Developing a PEG-based Hydrogel for the 3D culture of human colonic organoids

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Master of Science by Research

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School of Biological Sciences

September 2019

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Abstract

Human intestinal epithelial organoids are an emerging tool, both in research and in a clinical environment. Due to their structural and functional similarity to physical organs, human intestinal organoids (HIOs) are utilised to model the complexities of human disease pathology including colorectal cancer and inflammatory bowel disease, of which for both, the site of initiation is the colonic epithelium. Current culture methods for HIOs are dependent on growth in an ill-defined, tumour-derived extracellular matrix (ECM), that is of animal origin; which has confounded the use of organoids in a translational and regenerative medicine capacity. There is the therefore, growing appreciation for the use of chemically defined, synthetic hydrogels which have been optimised to support the growth and development of HIOs. In this study, we demonstrate the ability of a synthetic, fully defined hydrogel, based on the reaction between four-armed, thiol and maleimide-terminated poly(ethylene glycol) (PEG) macromers to support the three-dimensional growth and expansion of HIOs. We explore the effects of altered hydrogel parameters such as matrix stiffness and porosity on organoid growth and demonstrate the potential of functionalising inert PEG hydrogels, through assessing organoid growth and survival in response to the inclusion of degradable peptides and human recombinant ECM proteins in the matrix. Our research shows the potential of PEG-based hydrogels to overcome the limitations of current organoid culture systems and suggests a foundation by which PEG hydrogels can be further optimised to support the long-term growth and expansion of HIOs.

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Acknowledgements

I would like to extend my gratitude to both Dr. Mark Williams and Dr. Aram Saeed, who have been a continuous source of support, encouragement and knowledge throughout the duration of this research. I admire their dedication and commitment to their work, and hope to continue learning from them throughout my career.

I would also like to thank members of both the Williams and Saeed Laboratory, including Dr. Victoria Jones and PhD candidates Nicolas Pelaez Llaneza and Alvin Lee, who have been invaluable in their support. I would especially like to thank PhD candidate Noelia Dominguez Falcon who supported me enormously during the early stages of this work, and has continued to be a valued source of support throughout my research.

One final thank you to my brother, who is the source of my passion for science and research and the motivation in all things I achieve. I can never thank you enough.

1. Introduction

1.1 Colonic epithelial organisation

The large intestine, also known as colon, forms part of the human gastrointestinal tract and has key roles in nutrient absorption and digestion. To protect the systemic circulation of the host from the often-hostile luminal contents of the gut, such as exogenous bacteria, fungi and viruses; the intestine is lined with a monolayer of polarised columnar epithelial cells known as the intestinal epithelium (Okumura and Takeda, 2017). This colonic epithelium contains a number of specialised cell types as seen in Fig 1, such as enterocytes (colonocytes) necessary for water absorption, and goblet cells, which are responsible for secretion of mucus, and other proteins that are associated with the formation of the protective barrier (Allaire *et al.*, 2018). Both the protective and absorptive functions of the gut are dependent on maintaining an intact epithelium. Therefore the colonic epithelium is capable of rapid tissue renewal, with homeostasis being maintained through the shedding of differentiated epithelial cells, and the rapid, continuous proliferation and migration of undifferentiated cells to replenish lost cells, or repair tissue following injury (Humphries and Wright, 2008). The intestinal epithelium is highly dynamic, with the entire epithelial lining being replenished every 5 to 7 days (van der Flier and Clevers, 2009).

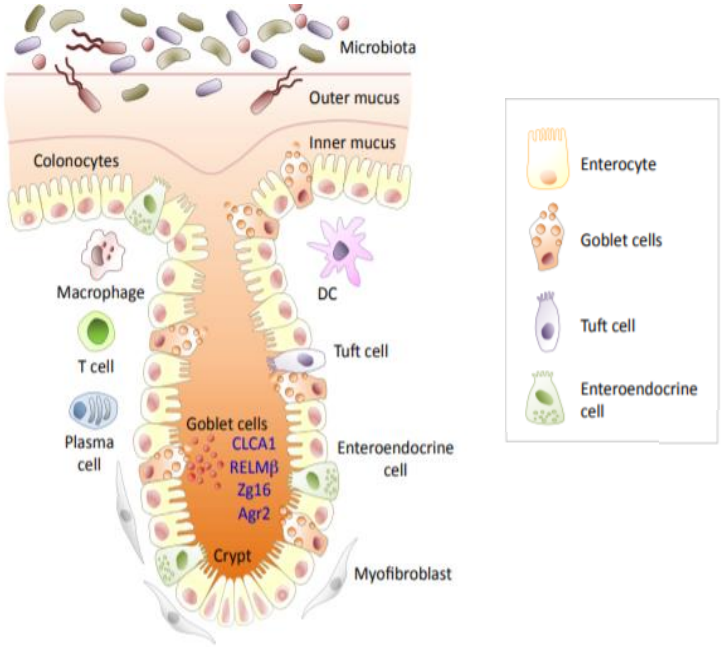


Figure 1. Schematic diagram showing the organisation and various specialised cell types of the large intestinal epithelium. (Allaire *et al.*, 2018)

1.2 The Intestinal Stem Cell Niche

The process of epithelial cell renewal is dependent on a subset of adult intestinal stem cells (ISC's) which give rise to progenitor cells, that are capable of differentiation into all cell types required for healthy gut function (Chee, Virshup and Madan, 2015). These stem cells are housed in specialised microenvironments which reside at the base of invaginations into the underlying mucosa, known as crypts of Lieberkühn. These crypts represent the single, unitary structure of self-renewal; where the base of each crypt serves as the intestinal stem cell niche, housing long-lived stem cells in a relatively sterile environment (Humphries and Wright, 2008). Here, ISC exposure to bacterial toxins and metabolites, dietary mutagens,

immunological cytokines and oxidative stress is reduced, minimising the risk of accumulating molecular damage (Chee, Virshup and Madan, 2015). ISCs at the crypt base fuel the constant replenishment of shed gut epithelial cells, whose brief residency in the gut also aid in reducing the probability that any singular epithelial cell will accumulate the complement of genetic mutations required for tumour initiation (Humphries and Wright, 2008).

1.3 Role of Wnt Signals Within Intestinal Crypts

Wnt signalling was found to be a driving force behind physiological crypt proliferation, through its dysregulation being the primary driver of colorectal cancer (Cernat *et al.*, 2014). Overall, around 80% of bowel cancer cases are fuelled by mutated APC genes (CRUK, 2012) which results in the failure of the APC destruction complex to function, and subsequent build-up of β-catenin, the key effector of Wnt signalling (van der Flier and Clevers, 2009) (Fig 2). Accumulation of β-catenin was found to induce transcriptional activation of Tcf4 target genes resulting in uncontrollable cell proliferation (van der Flier and Clevers, 2009). This, coupled with evidence that Tcf4 knockout mice lack proliferative crypts (Korinek *et al.,* 1998), highlights the importance of Wnt signals in regulating proliferation within the crypt and establishing the stem cell compartment.

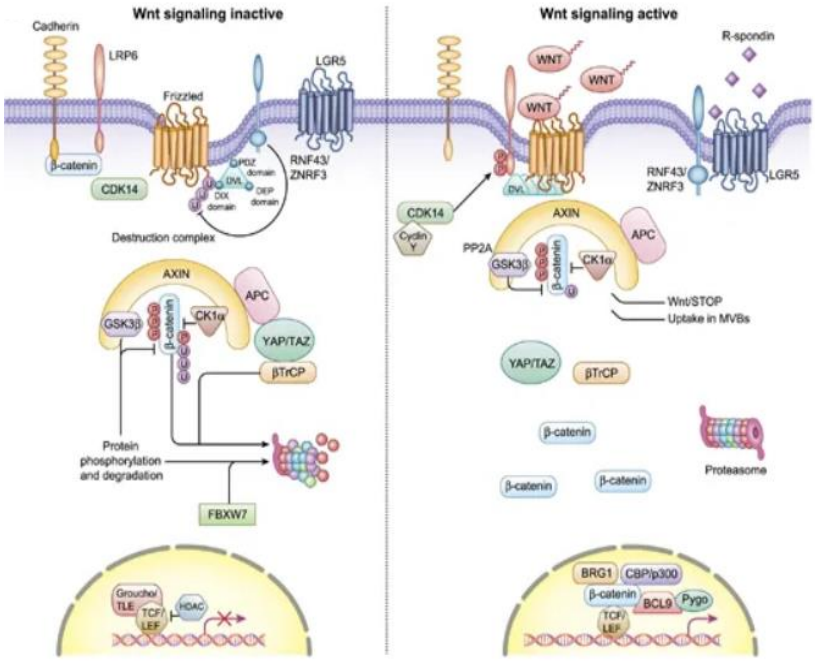


Figure 2. Schematic diagram of the Wnt signalling pathway in active (right) and inactive (Left) state (Zhan, Rindtorff and Boutros, 2017).

1.4 LGR5 – An Intestinal Stem Cell Marker

Early microarray experiments on the Wnt-pathway-controlled genes, expressed in both colon cancers and healthy crypts, unveiled the prominence of the Leucine-rich, repeat-containing, G-protein coupled receptor 5 (LGR5) gene. LGR5 encodes for a receptor of Wnt pathway agonists, R-spondins (Barker *et al.*, 2007). R-spondin signalling works to enhance Wnt signals in intestinal crypts by neutralising transmembrane ligases, that would otherwise remove Wnt receptors from the cell surface (de Lau *et al.*, 2014), and is therefore a prominent driver of crypt cell renewal. Using an inducible Cre knock in allele and the Rosa26 lacZ reporter strain, lineage tracing experiments were performed in mice and demonstrated exclusive LGR5 expression in cycling crypt base columnar cells (CBC), which were capable of generating all epithelial cell lineages over a 60 day period (Barker *et al.*, 2007). This data allowed the

identification of the intestinal stem cell and demonstrated the significance of LGR5 as an intestinal stem cell marker.

1.5 Human Intestinal Organoid Culture

It was later discovered that single LGR5^{+} intestinal stem cells could be expanded in culture to generate 3D gut epithelium cultures, otherwise known as organoids (Fig 3) (Sato *et al.*, 2009). These organoids maintain the LGR5~~(+)~~ stem cell hierarchy, are self-organising and do not require an epithelial cellular niche. Organoids can be cultured indefinitely in epithelial growth factor (EGF), Noggin and R-spondin enriched conditions, supported by a 3D matrix, and retain the hallmarks of the *in vivo* intestinal epithelium (Sato *et al.*, 2013), therefore stem-cell-derived organoids have therefore become promising models of disease development, potential drug screening platforms and a potential source of transplantable tissue (Rossi, Manfrin and Lutolf, 2018).

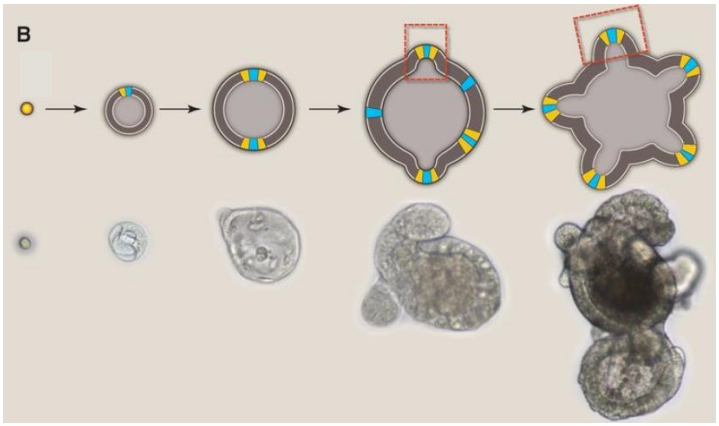


Figure 3. Schematic and brightfield images highlight the stages of organoid growth and development. Budding structures are highlighted (red rectangle) and demonstrate crypt-like morphology. CBC cells including LGR5+ ISCs are highlighted in blue (Sato *et al.*, 2013)

1.6 Organoids as Models of Development

Organoids have proven vital models of development, homeostasis and regeneration, in particular, mouse derived small intestinal epithelial organoids were used to demonstrate how LGR5+ mouse ISCs are dependent on Wnt signals from neighbouring Paneth cells in the small intestine (Sato *et al.*, 2011). Paneth cells were seen to express essential signals for stem cell maintenance including EGF, TGFα, Wnt3 and the Notch-ligand Dll4, and their co-culture alongside ISCs greatly improved organoid formation (Sato *et al.*, 2011). This work revolving around an organoid model, in addition to other observations, helped indicate a model by which the confinement of Paneth cells to the base of small intestinal crypts defines separation between ISC self-renewal and differentiation in the small intestine.

1.7 Organoids as Models of Disease

Organoids are also capable of mimicking disease pathology at an organ level, and therefore prove advantageous for disease modelling, drug discovery and personalised medicine, when compared to traditional single cell type cultures. (Rossi, Manfrin and Lutolf, 2018). Organoid-based disease models have already been developed for several disease types and have provided evidence that organoids are capable of recapitulating well known pathological features. An example of this is the development of human gastric organoids, that when injected with *Helicobacter pylori* bacteria, successfully reproduce the typical signs of the infection, including species-specific features of human gastric pathology, i.e. the progression to ulceration and cancer, which make animal models unsuitable for these studies (McCracken *et al.*, 2014; Bartfeld *et al.*, 2015). The usage of organoids in mimicking disease pathology has led to studies exploiting this for drug testing and screening applications (Fig 4). Patient derived intestinal organoids have laid the foundation for organoid research in personalised medicine by being successfully used to screen for drugs to treat cystic fibrosis (CF), a genetic disease

caused by mutations in the CFTR gene (Jane C Davies, 2007). Intestinal organoids were derived from a patient with a rare CFTR mutation, with no known treatment, and a standardised functional test by which cAMP-induced organoid swelling indicated restored CFTR functionality, was used to screen existing known CF drugs, allowing the patient to access treatment (Saini, 2016).

1.8 Intestinal Organoids Biobanks

An emerging application for organoids is the generation of organoid biobanks for different pathologies, that will be capable of acting as a powerful screening platform covering a large amount of genetic variance from the general population (Rossi, Manfrin and Lutolf, 2018) (Fig 4). Current work in the Williams laboratory has involved the generation of an intestinal organoid biobank, establishing organoid cultures from the healthy and diseased tissue of colorectal cancer patients as well as sufferers of IBD. This has allowed the disease process to be studied in native human tissue and will facilitate the discovery of both preventative and therapeutic drugs that are aligned to the genetic mutations associated with an individual patient.

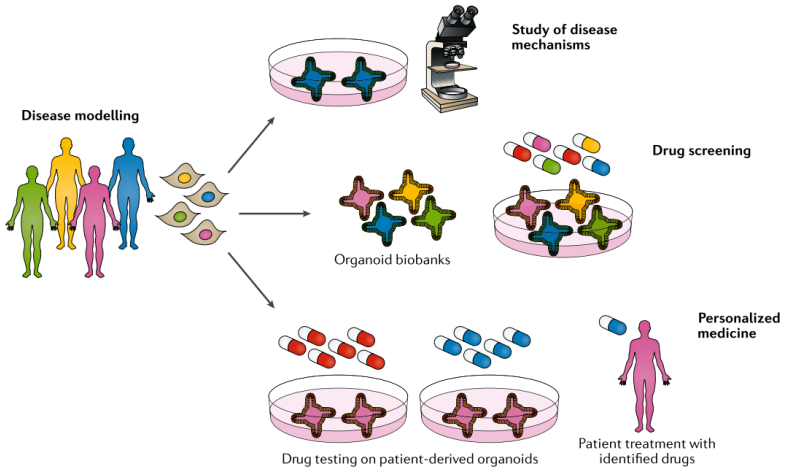


Figure 4. Schematic diagram showing key applications of organoids in disease modelling. The use of organoids in a research environment is indicated through the study of disease mechanisms and the development of drug screening platforms, whilst the clinical applications are highlighted in the form of personalised medicine (Rossi, Manfrin and Lutolf, 2018)

1.9 Current Organoid Culture Methods

Organoids have the potential to be a powerful tool for research and at a clinical level, it is of the utmost importance that their 3D culture conditions allow for highly reproducible generation of organoids, with a high level of functional and structural similarity to physical organs. ~~As laminin (α1 and α2) is enriched in the physiological crypt base~~ Currently, routine ~~,~~ 3D culture methods have relied on the commercially available, ~~laminin-rich~~ hydrogel matrixces ~~known such~~ as Matrigel, a basement membrane-like gel secreted by mouse sarcoma cells (Sato and Clevers, 2013). Proteomic analysis of Matrigel revealed its composition of a complex assortment of 1851 unique proteins, glycoproteins and proteoglycans, primarily consisting of ECM proteins such as laminin, collagen type IV and enactin (Hughes, Postovit and Lajoie, 2010). In addition several growth factors were detected in varying quantities including epidermal growth factor, fibroblast growth factor, transforming growth factor beta

and others (Hughes, Postovit and Lajoie, 2010). Although effective in promoting cell growth and self-organisation, Matrigel is limited in its ability to direct organoid morphogenesis and offers no control over the 3D self-organisation process (Hughes, Postovit and Lajoie, 2010). This is owing to its poorly defined chemical composition, containing largely variable concentrations of growth factors and proteins from batch-to-batch, as well as its inability to be chemically or physically modified, preventing specific tailoring of biophysical or biochemical matrix properties for intestinal organoids (Gjorevski *et al.*, 2016). As both chemical and mechanical/physical cues from the ECM drive organogenesis, it is important to be able to modulate matrix cues such as mechanical stiffness, adhesive ligand presentation and degradability in order to model the complex interplay between cell behaviour and the physical environment that is demonstrated in the intestinal epithelium (Raeber, Lutolf and Hubbell, 2005). Furthermore, Matrigel is an animal derived product which confounds its use in a clinical environment due to the risk of immunogen and pathogen transfer (Gjorevski *et al.*, 2016).

1.10 ECM and the Intestinal Stem Cell Niche

In the human gut epithelium, crypts are formed by epithelial invaginations into the extracellular matrix (ECM) which is supported by stromal cells (Meran, Baulies and Li, 2017). The intestinal stem cells, which are located at the crypt base, are maintained by their surrounding niche for regulation of both differentiation and self-renewal under homeostasis (van der Flier and Clevers, 2009). The ECM provides the physical niche, and is comprised of a network of fibrous structural proteins including proteoglycans (PGs) and glycoproteins which act as a physical scaffold, maintaining a 3D morphology (Meran, Baulies and Li, 2017). The ISC niche is also surrounded by other ECM components such as different laminin isoforms, collagens, glycosaminoglycans and fibronectins (Czerwinski and Spence, 2017) that provide

structural support, as well as influencing stem cell behaviour through mediation of instructive cell signalling (Vazin and Schaffer, 2010). For example 2 isoforms of the heterotrimeric ECM protein Laminin (α1 and α2) are enriched in the physiological crypt base (basal laminae) and have been seen to interact with ISCs via integrins – transmembrane heterodimeric receptors located on the cell surface that link the ECM with the intracellular cytoskeleton, mediating cell anchorage and mechanotransduction, as well as suggesting a potential role for integrin-mediated signalling in ISC regulation (Meran, Baulies and Li, 2017). In addition to the physical niche, cells embedded into the ECM form the cellular niche (Fig 5), secreting a range of matrix components and growth factors which are capable of altering ISC proliferation and differentiation (Chee, Virshup and Madan, 2015). The ECM is therefore vital to maintain the ISC niche, and both physical and cellular elements of the ECM are known to regulate both proliferation and differentiation through several pathways including the Wnt/β-catenin cascade, Notch signalling, TGF- β/BMP and Hedgehog pathways (Meran, Baulies and Li, 2017). In addition, the ECM is suggested to function as a reservoir for growth factors, through the ability of heparin sulfate proteoglycan, a common component of many ECM PGs, to bind growth factors such as FGF and VEGF allowing for the establishment of morphogen gradients – which plays a vital role in patterning, growth and development (Hynes, 2009). These growth factors may also be released during degradation of the ECM, highlighting how proteolytic remodelling of the surrounding ECM by secreted matrix metalloproteinases (MMPs) may enhance ISC proliferation (Yin *et al.*, 2016).

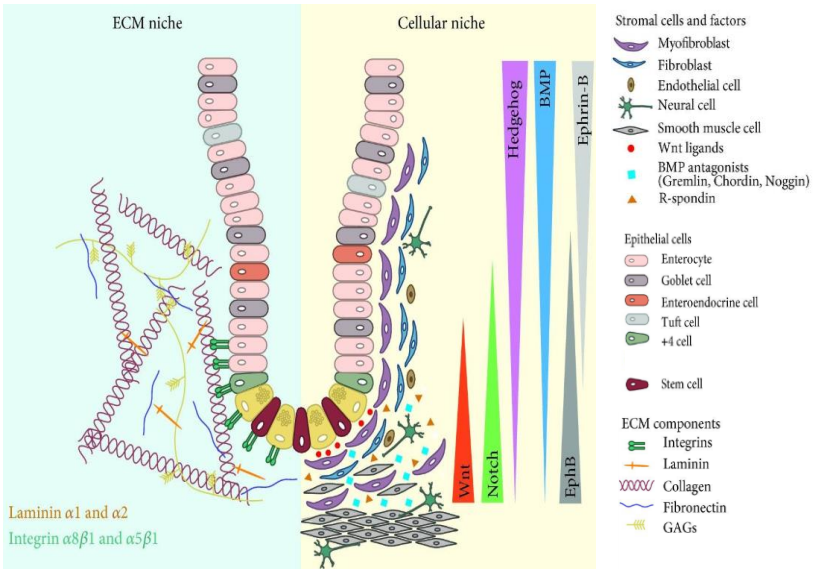


Figure 5. Schematic diagram of the interaction between intestinal epithelial crypts (the intestinal stem cell niche) and the surrounding microenvironment. Detailed on the left is the interaction between the highly structured ECM which is mediated through integrins, whilst the right-hand side details interaction of the crypt base with the cellular niche. Communication between the ECM and cellular niche is achieved via the highlighted signalling pathways (Meran, Baulies and Li, 2017).

1.11 ECM and Organoid development

Several studies have revealed the importance of choosing an appropriate ECM or ECM-mimic when developing a 3D matrix for organoid culture. Mouse-derived small intestinal organoids grown in conventional plastic adhered Matrigel droplets were seen to grow as singular

budding cysts, while in contrast, mouse small intestinal organoids grown in floating collagen gel rings formed a seemingly continuous macroscopic tube, with budding crypts along the tube axis, highlighting that an alteration of the ECM constituents has a drastic effect on organoid formation and organisation (Sachs *et al.*, 2017). Fibrin based hydrogels have also demonstrated that organoid formation is dependent on matrix properties such as mechanical stiffness and availability of an RGD motif, a tripeptide originally identified as the amino acid sequence within the ECM protein fibronectin that mediates cell attachment (Ruoslahti, 1996). This study using a fibrin-based gel identified that increasing matrix stiffness whilst decreasing RGD availability reduced organoid formation. In addition, supplementing the gel with laminin, a major component of Matrigel, led to organoid yields in the fibrin/laminin gel that were comparable to Matrigel (Broguiere *et al.*, 2018).

1.12 Chemically Defined, Synthetic Hydrogels and Organoid Culture

As the growth of LGR5+ stem cells into self-organising organoids is enhanced by a 3D extracellular matrix like-gel, there is a requirement for a synthetic, chemically defined hydrogel, whose chemical and physical properties are subject to manipulation in order to fully optimise the growth and maturation of organoids embedded into the gel. By controlling the distribution of signalling molecules and ECM proteins, as well as manipulating the gels' mechanical properties such as porosity and stiffness, there is the potential for organoid cultures within an optimised synthetic gel to reproducibly surpass the growth and self-organising potential achieved in Matrigel. A class of synthetic biomaterials that have been of great research interest are hydrogels. Hydrogels can be formed from synthetic, or naturally occurring polymers that closely mimic the natural environment of cells, through their susceptibility to physical and chemical manipulation (Caliari and Burdick, 2016). They possess a high-water content and can form 3D complexes around viable cells, allowing for three-

dimensional growth and signalling. Hydrogels have several characterisable properties including mechanics such as crosslinking density, swelling, mesh size and degradation, all of which influence the utility of the gel for cell culture applications (Caliari and Burdick, 2016). For example, matrix stiffness can influence stem cell differentiation through the activation of cellular mechanotransduction pathways, i.e. the activation of biochemical signalling pathways from the mechanical information of the microenvironment (Ingber, 2006). It is therefore necessary to consider the properties of each hydrogel system, in order to select a gel that is optimal for intestinal organoid expansion.

1.13 PEG Hydrogels and Organoid Culture

Polyethylene glycol (PEG) is a readily available hydrophilic polymer, that when crosslinked into hydrogel networks, possess a high-water content, making them suitable to emulate soft tissues (Leach, 2011). A major advantage of PEG based hydrogels is their relative inertness and cytocompatibility, as evidenced by their failure to initiate an immune response in the cells they encounter (Mäder, 2011). Often referred to as a 'blank slate material' PEGs are amenable to functionalisation with user-defined crosslinking groups, allowing for control over the crosslinking properties of the gel (Caliari and Burdick, 2016). This is due to the basic structure of PEG, which is capped with hydroxyl end groups, allowing the polymer to partake in a variety of chemical reactions (Zhu, 2010). Raeber, Lutolf and Hubbel pioneered the use of PEGs functionalised with vinyl sulfone (VS) in hydrogel preparations, as PEG-VS reacts specifically with free thiols, such as peptides terminated with cysteine residues (Raeber, Lutolf and Hubbell, 2005). Cells and proteins within the gel rarely possess exposed cysteine residues on their surface and thus peptides can be incorporated that render the gel with biological activity, such as adding cell adhesive domains from ECM proteins and peptide substrates for

matrix metalloproteinase degradation, allowing cells to mimic attachment and migration through the ECM (Leach, 2011).

A pivotal study identified that a chemically defined, synthetic PEG based hydrogel could be used as a suitable Matrigel replacement to culture intestinal epithelial organoids (Gjorevski *et al.*, 2016). PEG as a biomaterial offers a highly amenable matrix that can be independently modulated to alter variables including matrix stiffness, degradability and adhesiveness, in order to assess their effects on ISC proliferation, organoid formation and cellular differentiation. This work observed that a mechanically dynamic matrix was required providing an initial stiffness that favoured stem cell expansion, but was hydrolytically active and therefore able to soften, alleviating mechanical forces and favouring cell differentiation (Gjorevski *et al.*, 2016). Hydrogel mechanical properties can be characterised using a technique known as rheology, in which a small amount of a sample is subject to rotational compressive forces, probing the stress-strain relationship of the gel and obtaining the samples equilibrium shear modulus (Zuidema *et al.*, 2014). This information can be used to reveal structural properties such as degree of crosslinking and mechanical stiffness. The hydrogels generated by Gjorevski et al., were optimised using a mouse intestinal epithelial organoid model, however. eExperiments have also demonstrated that PEG hydrogels could also include functionalisation with human-derived intestinal ECM components, as well as investigating how spatiotemporal control of mechanical cues and signalling gradients can enable hydrogels to better mimic the structure of the native human intestine (Wang *et al.*, 2017).

1.14 Objectives

In this study we explore the encapsulation, growth and functionality of intestinal organoids in synthetic PEG based hydrogels, based on the reaction between two functionalised PEGs,

PEG-thiol (SH) and PEG-Maleimide (MAL) (Fig 6). Maleimide, like VS, reacts readily with free thiols under physiological conditions allowing for the formation of a high-water content hydrogel, with a rapid gelation time and in conditions optimal for organoid survival (Jansen *et al.*, 2018). PEG-MAL was seen to exhibit faster reaction kinetics, and allow for the generation of hydrogels of lower polymer weight and across a larger range of Youngs moduli, than PEG-VS (Phelps *et al.*, 2012). Using a PEG hydrogel system as a foundation provides a platform~~allows~~ for further modification. The interaction between thiol and maleimide functional groups provide chemical cross-linking with tuneable reaction kinetics, whilst ~~through~~ varying the PEG molecular weight (PEG chain length) and overall % PEG content of the gel alters the physical interactions caused by entanglement of the PEG chains. PEG hydrogels ~~,~~ therefore provide tuneable~~altering~~ hydrogel characteristics including, stiffness, mesh size and porosity, as well as enabling the inclusion of adhesive ligands and degradable peptides.

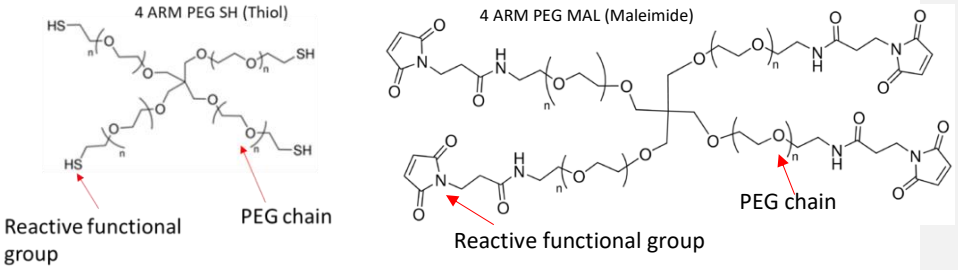


Figure 6. Chemical formula of PEG thiol and maleimide respectively (JenKem, 2019)

In this study we aim to

I. Engineer a synthetic, fully defined and optimised hydrogel, inspired by the colonic crypt ECM, that supports the growth of human intestinal organoids.

II. Characterise organoid growth, cell proliferation and stem cell biology of intestinal organoids embedded in a synthetic hydrogel to validate its usage

2. Materials and methods

2.1 Cell Culture

The Adult equine GFP+ tenocyte cell line was kindly donated by Dr. Deborah Guest (Animal Health Trust, Newmarket, UK). The cells were used in passage 8 for the experiments, and grown as monolayers in DMEM, GlutaMAX™ (catalogue number: 10566016, ThermoFisher Scientific), supplemented with Fetal Bovine Serum (FBS) and Penicillin-Streptomycin (P-strep). Both cell lines were expanded in 75 cm^2 Nunc™EasYFlasks™ incubated at 37^oC, 5% CO_2, until 80% cell confluence was achieved, refreshing 100% of the media every 4 to 5 days. For the cell seeding, cell monolayers were washed with PBS before detaching the cells using Trypsin-EDTA solution (CN: 25300054, ThermoFisher Scientific) for approximately 3 minutes at 37^oC. The trypsin was neutralised via the addition of fresh media and the cells were centrifuged before counting to remove residual trypsin.

2.2 Cell Encapsulation

Under sterile conditions degassed PBS 1X containing 4mM EA, 5mM EDTA was passed through a 0.22μM filter and 100 μL added independently to 4ARM PEG Thiol (JenKem tech. USA, SKU: A7008-1) and 4ARM PEG Maleimide (JenKem tech. USA, SKU: A7018-1) to make 4% w/v PEG solutions. A concentrated cell suspension of 5000 GFP+ tenocytes were added to the PEG-thiol solution and following gentle mixing, a 10 μL droplet was plated. An equivalent volume of PEG-Maleimide was then added to the cell-containing PEG-thiol and mixed rapidly with the pipette to ensure an even distribution of maleimide before gelation occurred. The droplets were left to polymerise at room temperature for 5 to 10 minutes before removing the gel using a sterile metal spatula and placing each droplet into individual wells of a 48 well plate

containing 1 mL of DMEM. The gels were incubated in a humidified atmosphere at 37 ^{o}C, 5% CO_2.

2.3 Fluorescent Imaging to visualise GFP+ Tenocytes

After 24 hours, 3 gels were removed from DMEM and placed into a fresh 48-well plate containing 1 mL of PBS for imaging. The remaining gels were maintained at 37 ^{o}C, 5% CO_2 for 7 days before imaging. Imaging was performed using a Zeiss Axiovert 200M microscope using AxioVision software. Images were obtained using a 20 X magnification Plan-Neofluar (0.5 NA) objective and a Zeiss AxioCam HRm CCD camera. Fluorescence was excited at 494-517 nm (GFP) and merged images were created using Image J software.

2.4 Live and Dead Viability Assay

Human colonic organoids were cultured in a 24-well culture plate (Nunc™ Surface, ThermoFisher scientific) in 10%, 10K 4arm PEG gels either unaltered or containing 10% LAM 111, 121, 511, 521, 511 and 521 together, or all 4 laminins in combination. After 7 days, the organoids were washed with HBS and freshly prepared Calcein AM-Ethidium homodimer-1 (ThermoFisher) was added to the organoids according to the manufacturer guidelines. Imaging was performed using an Inverted Nikon microscope and Hamatsu CCD Camera. Fluorescence was excited at 494-517 nm (calcein, green) or 528-617 nm (Ethidium homodimer-1, red). Merged images were created using Image J software

2.5 Tissue sample collection

Biopsy samples of healthy colonic epithelial tissue were obtained from patients at the Norfolk & Norwich University Hospital (NNUH) that had undergone exploratory colonoscopy procedures. The samples were collected by members of the Williams laboratory, with consent, and in compliance with the Human Tissue Act of 2004 and NHS research and development approval. Biopsy samples were kept cool within a sealed container and transported to the laboratory via a member of the Williams Lab.

2.6 Human Colonic Crypt isolation

Human colonic crypts were isolated from fresh biopsy samples obtained from the NNUH following a protocol outlined by Parris and Williams (Parris and Williams, 2015), whereby whole crypts are liberated from the mucosal tissue into fresh crypt isolation solution. A 100 µL sample of isolated crypt suspension is added to 400 µL of chilled Matrigel®, and mixed before dispensing 20 µL onto coverslips and placing at 37 ^{o}C for 15 minutes to allow polymerisation of the Matrigel. Once polymerised, the wells were flooded with 400 µL of human colonic crypt culture medium (hCCCM) and the plates returned to the incubator.

2.7 Human Colonic Organoids Culture

Human colonic organoids were obtained by members of the Williams lab after the passage of colonic crypts grown for 7 days. The samples were detached from the bottom of the plate by scratching the surface and mechanically dissociated into smaller fragments using a pipette. The suspension containing the crypt fragments was transferred into centrifuge tubes and pelleted at 4^{0}C. The supernatant containing cell debris and Matrigel leftovers was aspirated and then fresh media was added, and the crypt fragments resuspended. The final pellet was resuspended into a PEG-SH solution, before plating a 20 µL droplet. An equivalent volume of

PEG-MAL was then added, mixed rapidly and left to polymerise, before flooding the organoids with human colonic crypt culture media (hCCCM). Organoids were cultured at 37^0C and 5% CO_2, fed every 3 days and passaged every 5 to 7 days.

2.8 Hydrogel Preparation

Hydrogels were prepared using thiol-ene chemistry which has been utilised to generate PEG hydrogels for a range of applications (Toepke *et al.*, 2013). Thiol (SH) and Maleimide (MAL) functionalised 4-arm PEGs were purchased in various molecular weights (5K, 10K, 20K and 40K) from JenKem Tech. USA. Both SH and MAL functionalised PEGs of the desired molecular weight were independently dissolved in filtered PBS 1X containing 5 mM EDTA, degassed with N_2(g), to achieve precursor solutions of a desired w/v. Following a brief vortex, PEG precursor solutions were kept on ice, before a concentrated organoid suspension was added to the PEG-SH and after gentle mixing 20 µL of PEG-SH was plated. An equivalent volume of PEG-MAL solution was then added to the PEG-SH to generate hydrogel networks of a desired final PEG content, where the stochiometric ratio of SH:MAL was 1:1. PEG SH/MAL hydrogels polymerise rapidly under physiological conditions, after 1 minute each well was flooded with 400 µL of hCCCM. The final PEG w/v of the hydrogels used range from 5% to 10%.

2.9 Hydrogel Optimisation

Corning® Matrigel® Basement Membrane Matrix (CN: 356232), VPM peptide (GCRDVPMSMRGGDRCG) Lifetein (CN: LT255422) and human recombinant Laminins (LAM) 111, 121, 511 and 521 (BioLamina) were incorporated into the hydrogel backbone by mixing independently with the PEG-SH suspension at the relevant concentrations. Matrigel was incorporated at 2.5, 5 and 10% concentrations, VPM at 2:1 and 1:1 PEG: peptide ratios and recombinant laminins at 10% concentration.

2.10 Assessment of Organoid Growth

Organoid growth and morphology were monitored in real time by placing a multi-well plate into a climate-controlled chamber (5% CO_2, 37^{o}C) located on the stage of an inverted microscope. DIC illumination was established with a x10 objective and whole well images were obtained. Images were captured every other day over the period of 7 days. Organoid health and morphology were assessed visually and for living organoids, the size was determined using the Nikon, NIS-elements software to trace the circumference, obtaining a value of cross-sectional area (CSA). Healthy organoids were monitored over the 7-day duration and their CSA's were measured on alternate days. Organoids were stratified into two groups based on starting CSA and normalised growth was reported as organoid growth in relation to starting size.

2.11 Assessment of Cell proliferation

Human colonic organoids were cultured in 10% PEG or 10% PEG + LN511,LN521 on glass coverslips in hCCCM for 3 days before EdU was added to the media at a concentration on 10 μM, for a duration of 3 hours. Following labelling, organoids were fixed with 4% PFA for 1 hour at room temperature. Post fixation, organoids were washed with PBS before a 13-minute incubation at room temperature with NH_4Cl_2 and 2 subsequent PBS washes. The fixed organoids were then washed with 1% SDS, followed by several PBS washes. The organoids were then incubated, whilst covered, at room temperature for 30 minutes in the presence of 1% Triton X-100. The EdU reaction buffer was prepared according to The Click-iT EdU Cell Proliferation Kit (Invitrogen™) protocol, and following 2, 3% BSA washes, was added to the organoids, and incubated for 40 minutes, at room temperature in a reduced-light

environment. After 2 washes in 3% BSA the organoids were subjected to a standard immunocytochemistry protocol, including a 2-hour block of unspecific antibody binding via incubation in PBS containing 10% donkey serum, 1% BSA for 2 hours at room temperature. The primary antibodies E-Cadherin (goat) and Chromogranin A (mouse) were added at a concentration of 1:100 and stored at 4^{o}C overnight. Following 2 washes with PBS, the secondary antibodies Donkey Anti-Goat IgG H&L (Alexa Fluor® 488) and Donkey Anti-mouse IgG H&L (Alexa Fluor® 594) were added at a concentration of 1:200 before a 2-hour incubation at 4^{o}C. After 2 PBS washes, the coverslips were transferred to microscope slides using Vectasheild, Hoechst mounting media.

2.12 RNA isolation

RNA from freshly isolated colonic primary mucosa, freshly isolated crypts and human colonic organoids was isolated by the Williams ~~using~~lab using the ReliaPrep ™ RNA Miniprep System (Promega) according to the manufacturer's instructions. Total RNA concentration and purity was then measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.13 Generation of DNA and RNA biobank

The Williams lab ~~A biobank was~~ generated a Biobank containing the DNA and RNA from primary isolated colonic mucosa, crypts and colonic organoids of matching patients. Colonic crypt and isolated mucosa samples were obtained after a colonic crypt isolation. Colonic crypts were cultured and propagated into organoids and the DNA and RNA were isolated approximately after the 4th passage. The isolation of DNA and RNA was performed using the ReliaPrep ™ DNA Miniprep System (Promega) and ReliaPrep ™ RNA Miniprep System

(Promega) according to the manufacturers' instructions and the samples were stored at -20^{0}C and -80^{0}C respectively.

2.14 Data~~Statistical~~ analysis

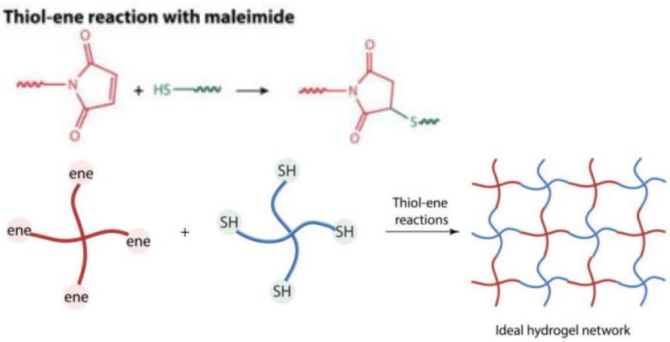
Organoid growth data is presented as overall mean organoid growth ratio ± SE for each gel where mean organoid growth <1 is indicative of cell death. The standard error of the mean is presented in order to predict how far the sample mean of the data is likely to be from the final organoid population mean. The variables of polymer ~~density~~concentration/PEG molecular weight combination, Matrigel concentration, VPM ratio and laminin type were each analysed using the one-way ANOVA statistical test to indicate significant differences in organoid growth. Where one-way ANOVA tests indicated significant differences, Tukey's multiple comparisons tests were performed allowing for selection of optimal organoid growth conditions. A comprehensive list of ANOVA p values and n numbers is compiled in supplementary tables 1-4.

3. Results

3.1 *Assessment of cell viability within a PEG-Hydrogel*

Encapsulating cells into a hydrogel matrix and maintaining their viability in culture post-encapsulation presents several challenges. Preliminary investigations involved generating a spectra of PEG-derived hydrogels ranging from low to high polymer ~~densities~~concentrations. A simplified schematic of the reaction between SH and MAL functionalised macromers is presented (Fig. 7A). Low ~~density~~concentration hydrogels failed to polymerise and would therefore not support three-dimensional growth, whilst high polymer ~~densities~~concentrations had an extremely short gelation time, preventing even distribution of PEG and the formation of homogenous hydrogel droplets. The un-workable nature of higher polymer ~~density~~concentration hydrogels also confounds their use for cell encapsulation. As organoids are large, multicellular structures, that are responsive to mechanics of their surrounding environment and PEG hydrogels differ mechanically and biochemically to the normal matrix that organoids are cultured in, we opted to first assess the viability of single cells encapsulated in PEG. A GFP+ tenocyte cell line was utilised due to their ease of visualisation, additionally, tenocytes are mechanically sensitive and have a clearly defined morphology making them suitable for assessing whether PEG hydrogels support viability. GFP + tenocytes were encapsulated in a 10K molecular weight~~, 8~~, 8% w/v PEG hydrogel and were seen to be viable through visual observation only at both 24 hours and 7 days post encapsulation (Fig 7). Although appearing visually viable, the tenocytes have adopted a rounded morphology, differing from their traditional, elongated, spindle-like morphology.

A

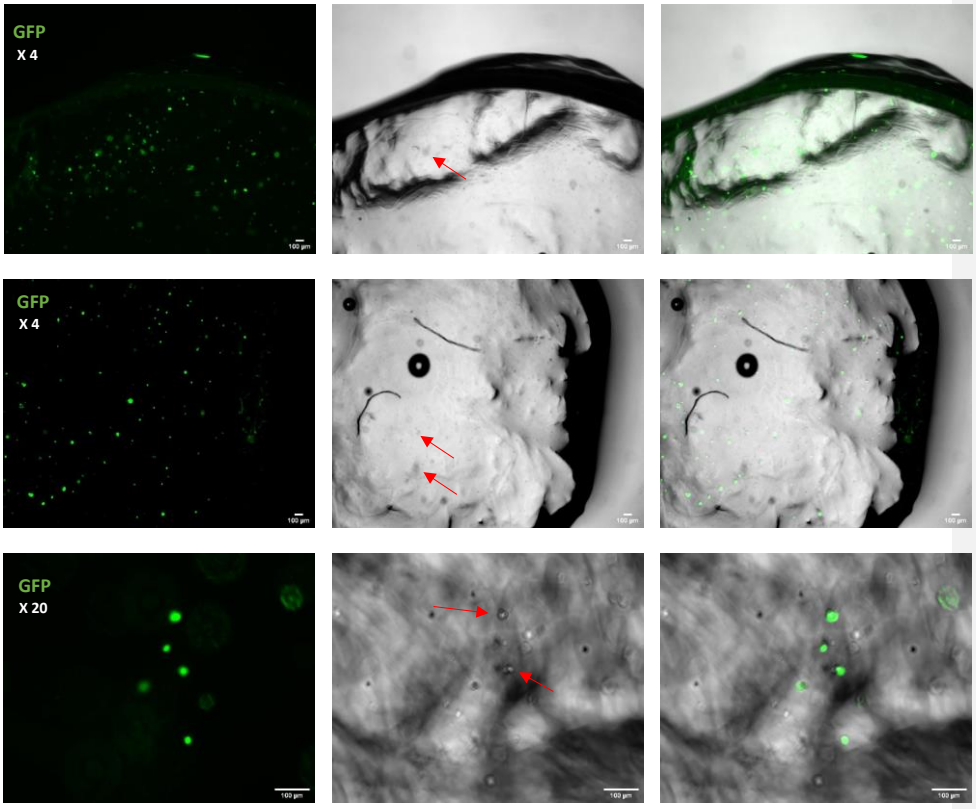


B

24 Hours

Cells | Gel | Composite

GFP+ Tenocytes



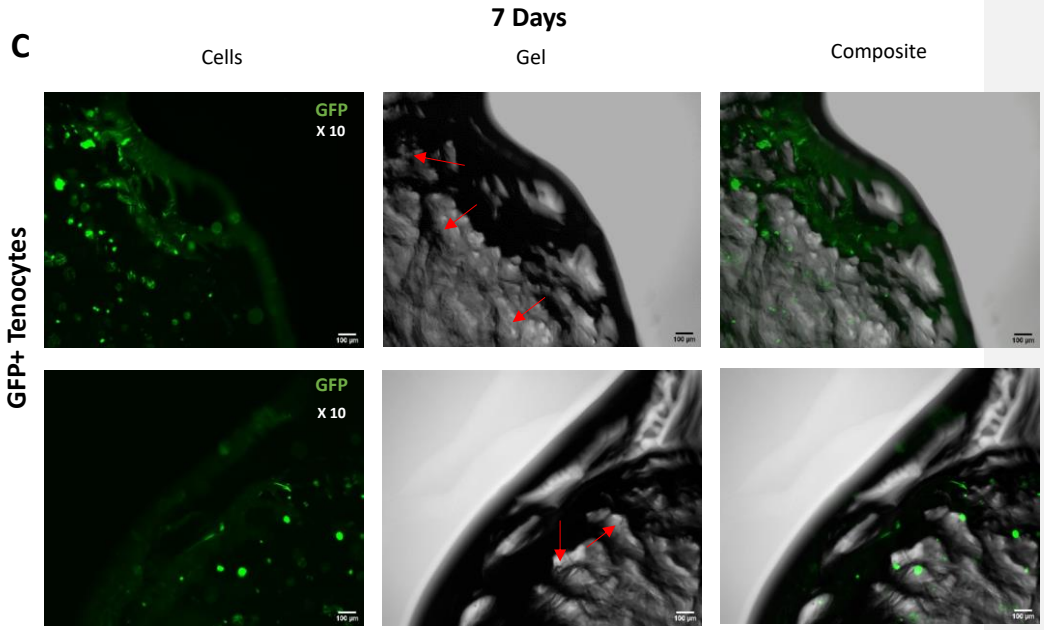
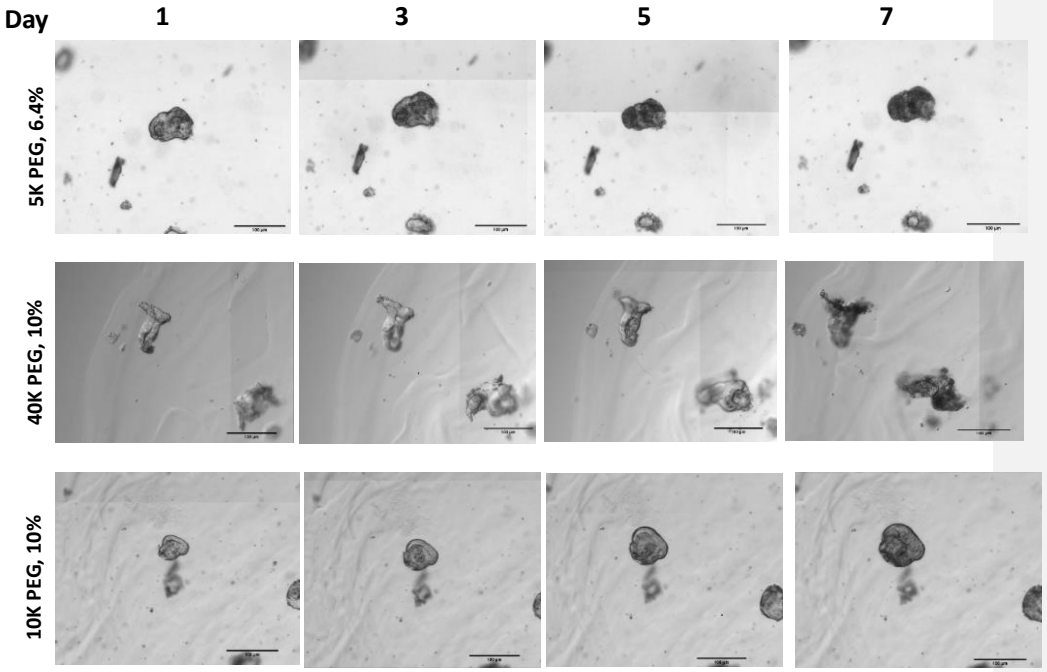


Figure 7. Encapsulation of GFP+ tenocytes in synthetic PEG hydrogel. GFP+ tenocytes were encapsulated in 4ARM, 10K PEG hydrogels of 8% total PEG content. (A) Schematic diagram highlighting the reaction mechanism between SH and MAL functionalised macromers (Jansen *et al.*, 2018). Representative images of encapsulated cells at 24 hours (B) and 7 days (C) are presented. Each image is presented as separate GFP, brightfield (BF) and composite images taken at the same magnification. Scale bar is 100 µM at the magnification displayed on the image.

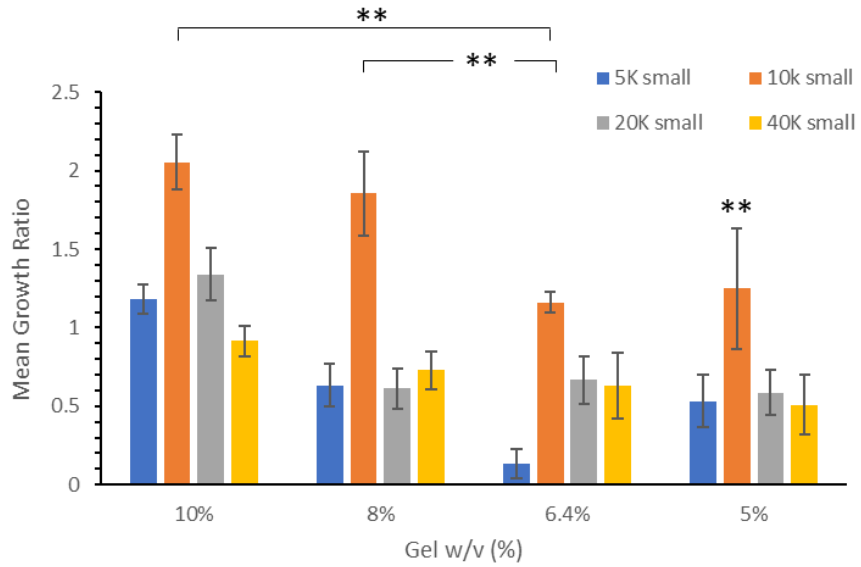
3.2 Selection of optimal PEG molecular weight

Matrix stiffness, crosslinking density, mesh size and porosity are key characteristics that affect organoid growth and can be controlled by altering the PEG molecular weight, and final polymer ~~density~~concentration of the hydrogel. As we observed prolonged viability of a mechanically sensitive cell type in a 10K PEG hydrogel of 8% w/v, we rationalised that PEG hydrogels, at the right mechanical stiffness and porosity, would support the viability of human intestinal organoids. In order to assess which molecular weight PEG best supported organoid growth, and at which % polymer ~~density~~concentration, organoids were embedded into a series of PEG hydrogels of increasing polymer ~~density~~concentration and increasing PEG molecular weight (Fig 8). Organoid growth was presented as mean ratio between final and starting cross sectional area, with values less than 1 indicating a loss of size and cell death. We observed that the 10K PEG provided the highest mean growth ratios, and among hydrogels produced with this MW PEG, the higher polymer ~~density~~concentration gels (10 and 8%) had significantly increased growth ratios for both small (P=0.001, P=0.003) and large (P=0.009, P=0.047) organoids, when compared to low polymer ~~density~~concentration hydrogels.

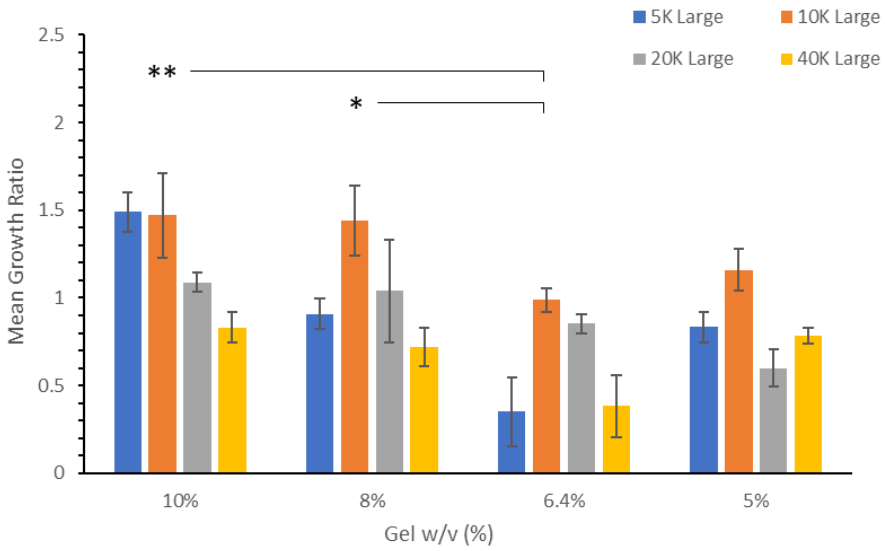
A



B


**
**
5K small
10k small
20K small
40K small
Mean Growth Ratio
2.5
2
1.5
1
0.5
0
**
10%
8%
6.4%
5%
Gel w/v (%)

C


5K Large
10K Large
20K Large
40K Large
**
*
Mean Growth Ratio
2.5
2
1.5
1
0.5
0
10%
8%
6.4%
5%
Gel w/v (%)

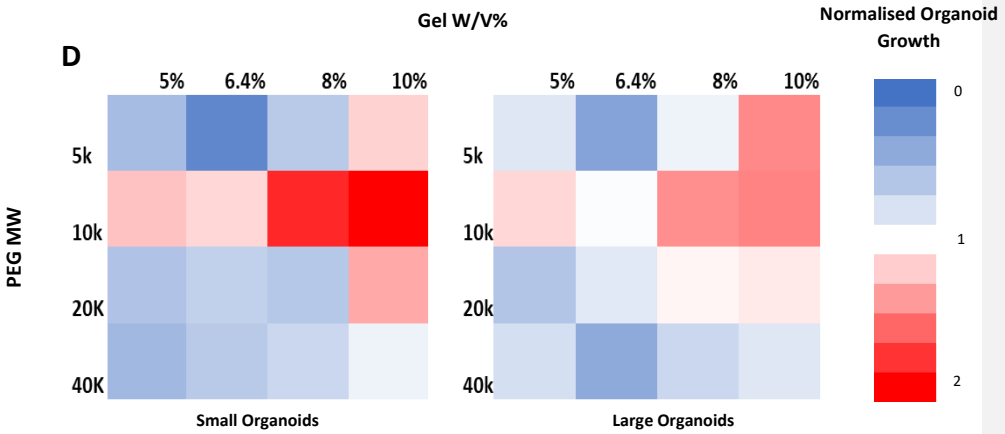
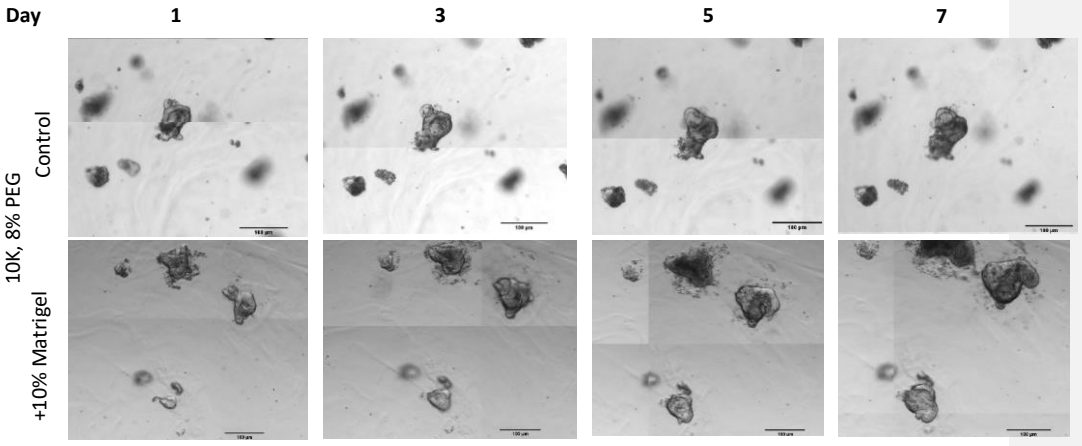


Figure 8. Assessment of human intestinal organoid growth in synthetic PEG hydrogels of varying PEG content (Gel w/v %) and differing PEG molecular weights (5K, 10K, 20K and 40K). (A) Brightfield images of human intestinal organoids grown over a 7-day period in hydrogels of the indicated PEG MW and final PEG content (%). Images were obtained at a 4X magnification, on alternate days. Scale bar is 100 µM. (B-C) Organoids were cultured in the relevant PEG hydrogel for 7 days and stratified into 2 groups based on starting cross sectional area ("small" organoids ≤ 15000 μm^2, B and "large organoids > 15000 μm^2, C). Data is expressed as the overall mean organoid growth ratio ± SE for each gel where mean organoid growth <1 is indicative of cell death. Statistical analysis one-way ANOVA with Tukey's multiple comparison test was applied for displaying significant differences between gel w/v and organoid growth ratio. (D) Heat map visualisation of organoid growth factors within PEG based hydrogels of increasing w/v% and PEG Molecular weight. Organoid starting cross sectional area Small (left) and Large (right)

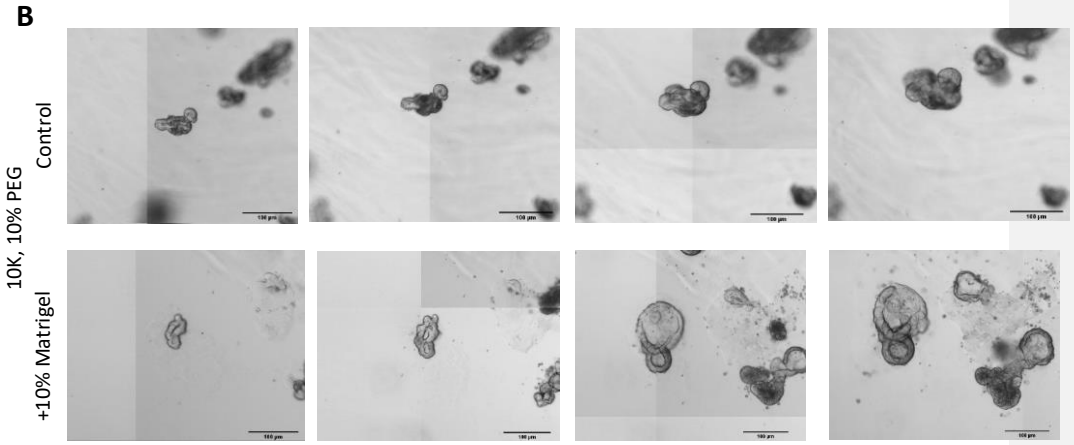
3.3 Organoid growth in synthetic PEG can be improved via the inclusion of natural ECM~~*M*~~

Although we ~~fter~~ established~~ing~~ organoid growth could be achieved in raw, unoptimised PEG, the overall growth achieved, in addition to the characteristic morphology of optimal organoid formation (lumen development and formation of new budding 'crypt-like' domains) was significantly reduced when compared with organoids routinely cultured in the Williams lab using Matrigel (data not shown). Organoids cultured in Matrigel alone have clear, pronounced lumens that expand greatly over the course of a 7-day culture, which is accompanied by the formation of new budding structures. ~~-l~~It was therefore important to assess whether organoid cultures in PEG could be improved by the inclusion of natural ECM components. Hybrid hydrogels were generated using the molecular weight PEG and final PEG concentration that best supported organoid growth in an unoptimized state. Low concentrations of natural ECM proteins in the form of Matrigel were incorporated into the PEG based hydrogels, and their growth over a 7-day period was monitored (Fig 9). For organoids grown in 10K 8% w/v PEG hybrid gels, the mean growth factor after 7 days was significantly higher in gels that contained 10% Matrigel, than in the control (P= 0.017), this trend was mirrored in the 10% gel (P= 0.032), although the mean organoid growth ratio in 10% PEG +10% Matrigel was seen to be greater than that achieved with the 8% gel. The improved growth characteristics in 10% 10K PEG + 10% Matrigel can be observed in the brightfield images, where there is a large increase in lumen size over the 7-day period.

A


Day
1
3
5
7
10K, 8% PEG
Control
+10% Matrigel

B


10K, 10% PEG
Control
+10% Matrigel

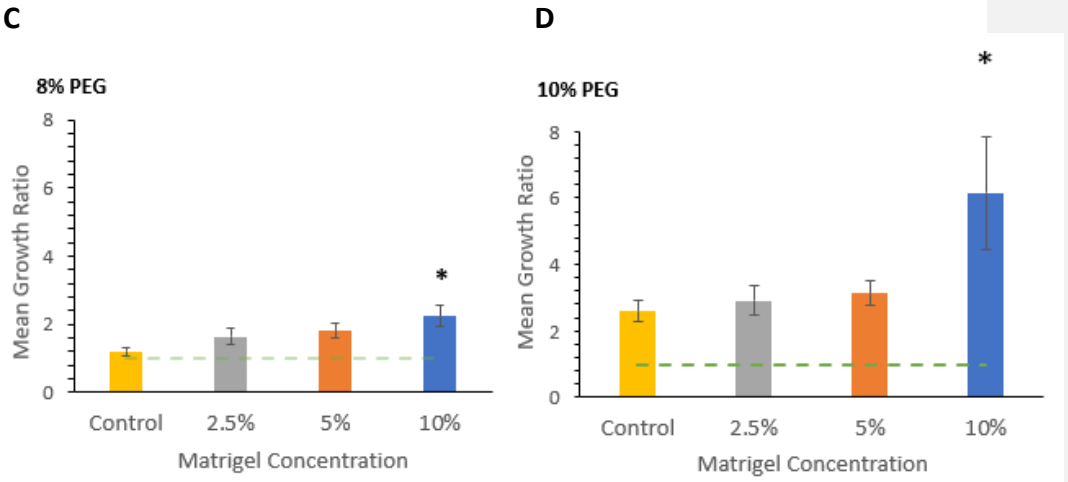
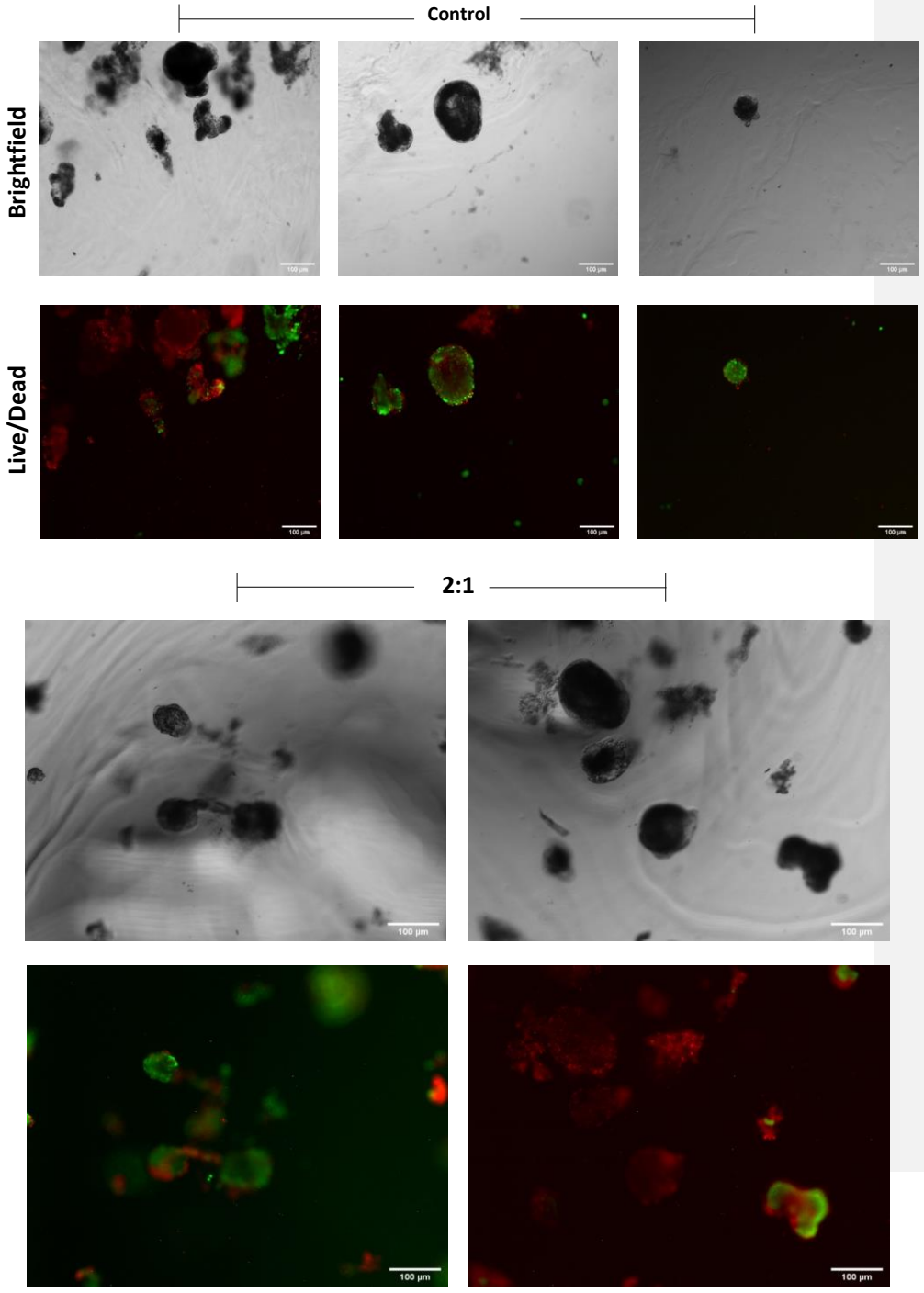


Figure 9. Effect of increasing Matrigel concentration on mean organoid growth in synthetic PEG hydrogel. (A-B) Brightfield whole well images of human intestinal organoid growth in synthetic PEG hydrogels of 8% (A) and 10% (B) gels with, and without (Control) Matrigel, over a 7-day duration at 4X magnification. Scale bar indicates 100 µM. Organoids were cultured in 4-ARM, 10K PEG hydrogels of 8% (C) and 10% (D) w/v. Dashed line represents a baseline growth ratio of 1. Data is expressed as the overall mean organoid growth ratio ± SE for each gel. Statistical analysis one-way ANOVA with Tukey's multiple comparison test was applied for displaying significant differences between Matrigel concentration and mean organoid growth ratio. *$p<0.05$

As matrix remodelling is important in regulating cell differentiation and maintaining stem cell niches, we aimed to assess the effect of rendering synthetic PEG hydrogels proteolytically degradable, on organoid growth. Organoids were encapsulated in 10K 10% PEG hydrogels, that included the matrix metalloproteinase sensitive peptide VPM (Fig 11). There were no significant differences in the mean growth factors of organoids with a large starting CSA in the control gels, and gels containing VPM at either ratio (P=0.391). For organoids with a smaller starting CSA, the mean growth factor after 7 days was seen to be significantly reduced in gels containing 1:1 ratios of VPM (1.33 ± 0.11), than in control gels (2.36 ± 0.20) or gels containing 2:1 ratios of PEG:VPM (1.95 ± 0.11) $P < 0.001$ and $P = 0.004$ respectively. Live/Dead staining revealed viable cells were present in all gel types after 9 days of culture.

B

C



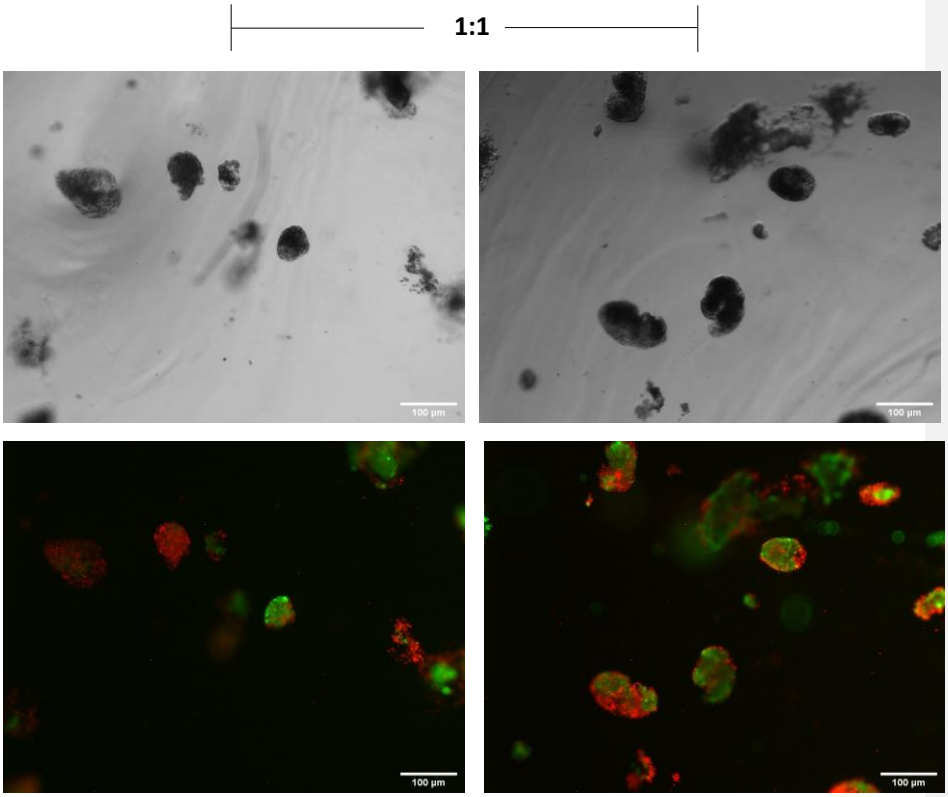


Figure 10. Effect of optimizing gel degradability on mean organoid growth. Human intestinal organoids were embedded in 4ARM, 10K PEG hydrogels with a total PEG content of 10%, containing either control, 2:1 or 1:1 ratios of VPM. The organoids were maintained in culture and (A) whole well brightfield images were obtained on alternating days at 4X magnification. (B) The mean growth factor over 7 days for organoids embedded in each gel was calculated

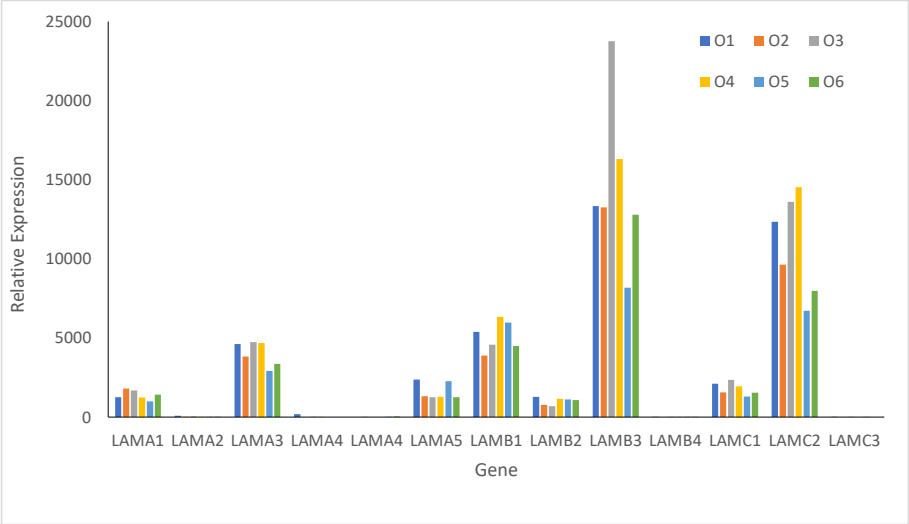
for organoids of small (solid colour) and large (striped colour) starting cross sectional areas. Data is expressed as the overall mean organoid growth ratio ± SE for each gel. Statistical analysis one way ANOVA with Tukey's multiple comparison test was applied for displaying significant differences between VPM concentration and mean organoid growth ratio. **P<0.01, ***P<0.001. (C) Live and dead cells were stained with Live/dead cytotoxicity kit on day 9 of organoid culture in optimised hydrogels. Live cells were stained with calcein AM (green), and dead cells were stained with ethidium homodimer-1 (red). Images were obtained at 20 X magnification. Scale bar is 100 µM.

3.5 Optimising synthetic PEG hydrogels with human recombinant laminin

As optimising hydrogel degradability in isolation offered no improvement to mean organoid growth (Fig 11), we investigated whether synthetic PEG hydrogels could be optimized to include human recombinant laminin proteins (a major constituent of Matrigel), rendering the hydrogel more physiological and improving mean organoid growth. RNA sequencing of human intestinal organoids –revealed organoids possess transcripts for a several laminin chains indicating the expression of laminin 111, 511 and 521. Using this data in combination with literature that identifies high expression of laminin 111, 121, 511 and 521 in Matrigel - (Hughes, Postovit and Lajoie, 2010), organoids were embedded in 4ARM 10K, 10% PEG hydrogels optimized with a single, or combination of biologically relevant laminin(s) (Fig 12). After 7 days the mean organoid growth in PEG gels optimized with a combination of laminin 511 and 521, were seen to display increased mean organoid growth when compared to the control (P=0.006). There were also significant differences in mean organoid growth between gels containing individual laminins, including an increased organoid growth ratio in organoids encapsulated in PEG optimized with laminin 111, however these were not seen to differ

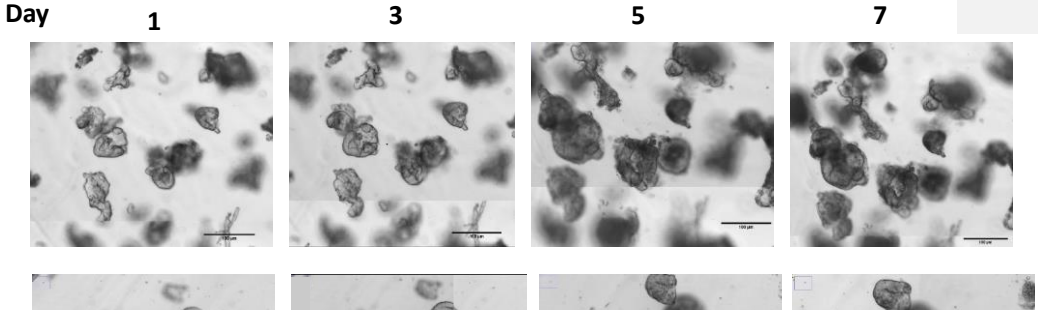
significantly from the control. Interestingly, in gels optimized with all 4 recombinant laminin proteins, mean organoid growth was seen to be significantly reduced when compared to un-optimized, control gels (P<0.001) as well as all gels that received either a single, or

A



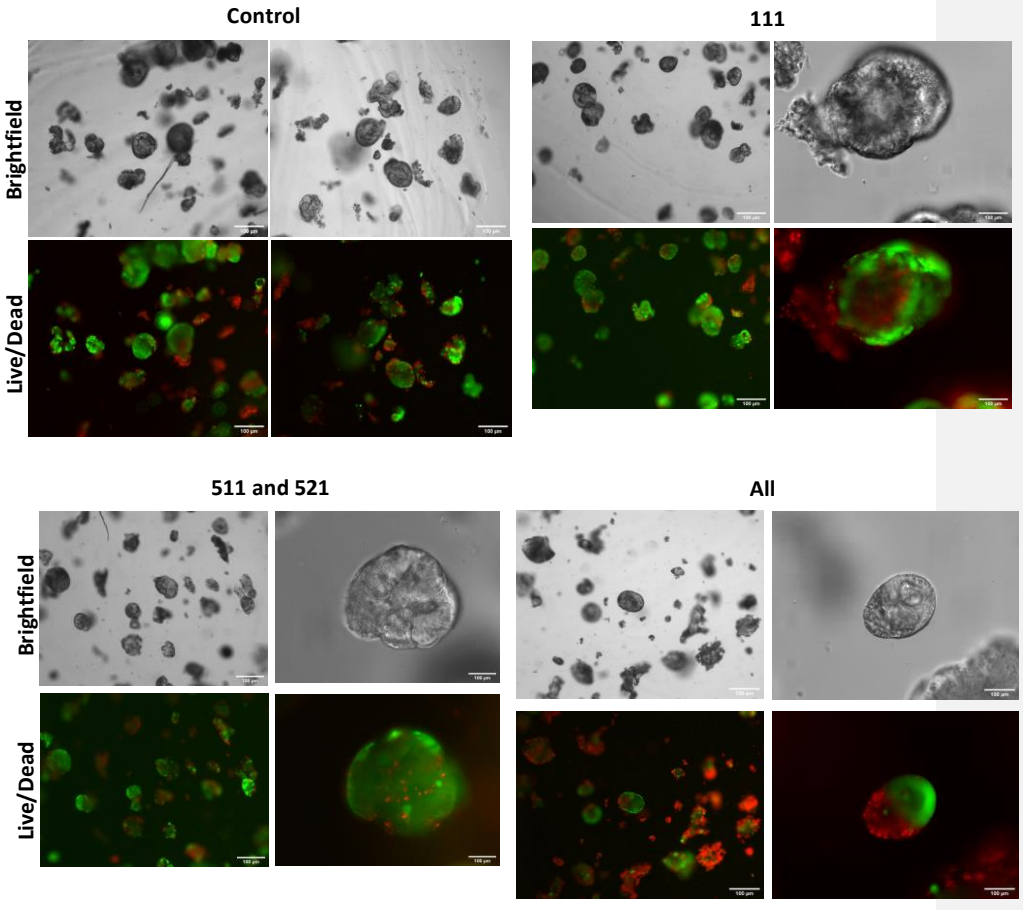
B

combination of laminin(s) (P<0.001). An assessment of cell proliferation in organoids encapsulated in control PEG gels and PEG hydrogels optimised with laminin 511 and 521 revealed cell proliferation occurs in both conditions



~~5.~~

D


Control
111
Brightfield
Live/Dead
511 and 521
All
Brightfield
Live/Dead

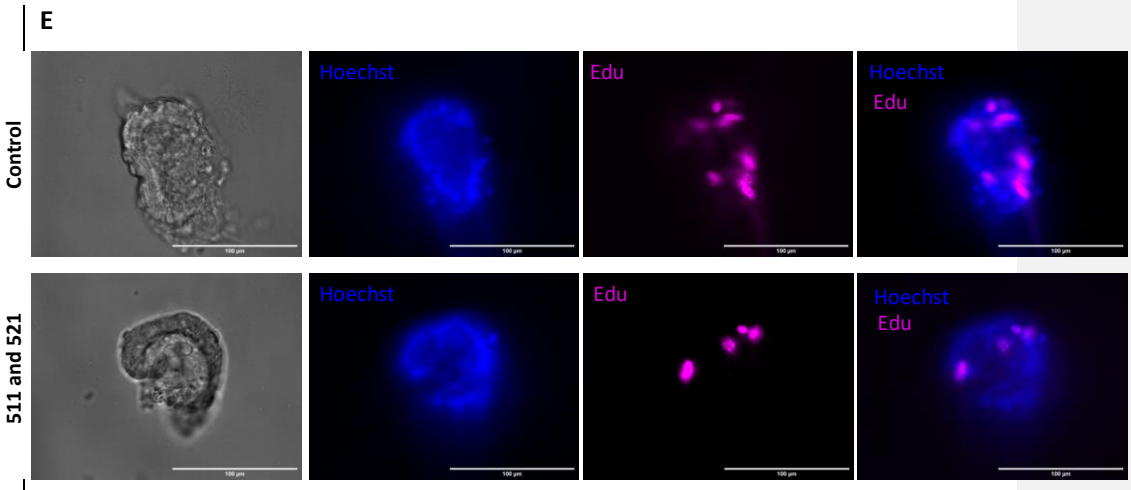


Figure 11. Effect of synthetic PEG optimisation with human recombinant laminin on mean organoid growth. (A) RNA sequencing was performed on RNA isolated from organoids after approximately 4 passages and the relative expression of laminin genes from 6 representative organoids is shown. (B) Brightfield whole well images of human intestinal organoid growth in synthetic PEG hydrogels optimised with 10% human recombinant laminin(s) over a 7 day duration at 4X magnification. Scale bar indicates 100 µM. Red dashed box indicates region of image that has been superimposed by an identical section at a different focus –(C) Human

intestinal organoids were embedded in 4ARM, 10K PEG hydrogels of 10% w/v that a contained a single, or combination, of human recombinant laminins at a 10% concentration. Data is expressed as the overall mean organoid growth ratio ± SE for each gel. Statistical analysis by one way ANOVA with Tukey's multiple comparison test was applied for displaying significant differences between laminin present and mean organoid growth ratio. *p<0.05 **P<0.01, ***P<0.001. (D) Live and dead cells were stained with Live/dead cytotoxicity kit on day 7 of organoid culture in optimised hydrogels. Live cells were stained with calcein AM (green), and dead cells were stained with ethidium homodimer-1 (red). Images were obtained at 10 and 20 X magnification. Scale bar is 100 µM. (E) Separately, HIOs embedded in PEG hydrogels optimised with a combination of human recombinant laminins or unoptimized 'control' PEG gels, were incubated independently for 3 hours with~~with~~ Edu to test for proliferation. Hoechst nuclear stain was ~~utilised~~utilised, and fluorescent images obtained at 40 X magnification. Scale bar is 100 µM.

4. Discussion

4.1 PEG based hydrogels support organoid growth and Viability

In accordance with literature (Gjorevski *et al.*, 2016b; Cruz-Acuña *et al.*, 2017; Broguiere *et al.*, 2018), this study demonstrates that synthetic PEG-based hydrogels can be used to successfully encapsulate different cell types, including more complex structures such as organoids. In addition, cell viability can be maintained within the gel, under normal culture conditions for extended periods of time. We do however, observe a distinct change in morphology for both tenocytes and human intestinal organoids (HIO) when encapsulated in PEG hydrogels. Tenocytes, which are highly mechanosensitive, do not maintain their elongated spindle-like structure when encapsulated in a 4 ARM, 10K PEG gel of 8% w/v. Instead, we observed smaller, rounded cell morphologies that were viable at both 24 hours and after 7 days. Similarly, for organoids encapsulated in PEG hydrogels, we do not regularly observe the same expansion of overall organoid size and lumen diameter, or the formation of budding, crypt-like domains, that are demonstrated by organoids embedded in Matrigel. As mechanical forces and interactions between the ECM influence cell shape and function, it is reasonable to assume that in an ~~unoptimized~~unoptimised PEG based hydrogel, that does not contain any ECM proteins and is not biologically active, cells will not interact with their surrounding matrix, and therefore be unable to achieve their desired morphology. However, as the base mechanical properties of a PEG based hydrogel supports cell and organoid viability for extended durations, they serve as a suitable inert substrate for optimisation through alteration of mechanical properties and the incorporation of ECM elements.

4.2 Tuneable mechanical properties of PEG hydrogels

PEG is an ideal encapsulation material due to its tuneable mechanical properties, that can therefore be tailored, in this circumstance, to best support human intestinal organoid growth and development. By altering the PEG molecular weight (MW) we can alter physical properties of the gel, where an increase in PEG MW reduces the cross-linking density and therefore increases the mesh size, resulting in decreased matrix stiffness. The mesh size is the average distance between crosslinks within the polymer network and therefore affects the overall porosity of the gel. Furthermore, we can further alter the overall cross-linking density and gelation time by varying the final polymer content (w/v %) of the gel. We assessed organoid growth in a range of hydrogels constructed from PEGs of 5K-40K molecular weight in the w/v polymer ~~density~~concentration range of 5-10%. We observe a large variation in mean organoid growth when altering the mechanical properties of the hydrogel. Markedly, a higher final polymer ~~density~~concentration of 10% was seen to favour organoid growth for PEG gels of all MW's. Hydrogels constructed from 10K PEG consistently and significantly outperformed other PEG MW gels in relation to mean organoid growth ratio at all polymer ~~densities~~concentrations, with the highest organoid growth identified in the 8 and 10% categories. This data suggests that human intestinal organoid growth within synthetic PEG hydrogels reflects the Goldilocks principle. This is to say that the overall porosity, which affects perfusion of media through the gel and removal of waste material, and matrix stiffness is at an optimal level within 10K/ 10% gels to support organoid growth. Increasing or decreasing the mechanical stiffness by altering the PEG MW and reducing the polymer content of the gel are only seen to reduce the growth potential of organoids encapsulated within the gel. Final polymer contents of >10% were not investigated due to the increased reaction rate that reduced the efficiency of organoid encapsulation.

Matrix stiffness of synthetic PEG hydrogels is a clear factor in regulating the growth potential of encapsulated HIO's. This is due to a cells' ability to perceive their environment through physical and mechanical cues such as ECM stiffness, and their ability to translate this stimuli into biochemical signals through mechanotransduction pathways, which then influence cell behaviour including growth and differentiation. How stiffness mechanosensing directly influences nuclear transcription activity is not wholly understood. Recent studies have found that matrix stiffness influences intestinal stem cell expansion by influencing YAP (Yes-associated protein) activity and nuclear localization, with high matrix stiffness enhancing ISC survival and proliferation through a YAP-dependant mechanism (Gjorevski *et al.*, 2016b). The same study also identified that in stiff matrices, the proportion of cells with nuclear YAP activity rapidly decrease over time, whilst organoids cultured in Matrigel maintain intermediate levels of nuclear YAP activity. This indicated that whilst organoid culture within a stiff matrix initially increases YAP activity and supports organoid growth, continued exposure to a stiff matrix results in compression and YAP inactivation, preventing further growth (Gjorevski *et al.*, 2016b). The data produced in this study is concurrent with this published data, as we identified that 10K PEG, which offers increased matrix stiffness and therefore, predicted higher YAP activity, over 20 and 40K PEGs, provided the highest mean growth ratio. Increasing the stiffness further with a 5K PEG offered no growth benefit for the organoids and this may be due to the reduced mesh size and porosity, effecting the delivery of fresh nutrients to the organoids, further enforcing the Goldilocks principle.

Recent research has also demonstrated that mechanical signals from the surrounding matrix can be 'overridden' through the inhibition of the mechanoresponsive ion channel PIEZO1, in order to increase proliferation and differentiation of oligodendrocyte progenitor cells in the ageing central nervous system, whose stem cell niche stiffens with age (Segel *et al.*, 2019).

PIEZO1 is a non-selective cationic ion channel that are directly influenced by mechanical forces, and is expressed in mechanically active tissues, such as the gastro-intestinal epithelium (Alcaino, Farrugia and Beyder, 2017). The exact signalling mechanism of PIEZO1 in the colonic epithelium is still unknown, however, it is has been demonstrated that the piezo channel opens upon direct, physical deformations of the lipid bilayer, such as increased membrane tension and remains closed in the absence of external mechanical force (Botello-Smith *et al.*, 2019). It is therefore conceivable that the stiffening of the stem cell niche may alter piezo 1 sensitivity and through the inhibition/activation of PIEZO1 channels expressed in intestinal epithelial organoids, organoid growth and proliferation can be enhanced even in stiffer matrices, and future research will explore this. In sum, the growth potential in the optimised relatively stiff PEG matrix is still reduced in relation to organoid growth in Matrigel, which through its susceptibility to degradation, softens over time, allowing maintenance of nuclear YAP activity and conceivable optimal activation of other mechanosensitive pathways such as PIEZO1.

4.3 Matrix degradability and organoid growth

We successfully demonstrate that intestinal organoid growth can be achieved in biologically inactive PEG, however the observable differences between organoids cultured in sub-optimal PEG and organoids in Matrigel, include the failure of the organoids encapsulated in PEG to form budding structures, and reduced expansion of organoid luminal diameter. As Matrigel is proteolytically degradable, and there is matrix turnover over time, we rationalised that organoids in Matrigel, unlike in PEG, degrade their surrounding matrix, creating sufficient space required to facilitate their expansion. Our data however shows that incorporating the proteolytically degradable VPM peptide into the synthetic PEG hydrogel backbone offered no benefit to the mean organoid growth ratio and failed to encourage bud formation. This was

despite organoids appearing more mobile within the VPM optimised PEG gels, as the overall position of individual organoids within the gels were seen to fluctuate more over the culture period (data not shown). As ECM remodelling around the physiologically intestinal crypt would also release sequestered growth factors such as Wnt signalling molecules and FGF (Chee, Virshup and Madan, 2015), we reasoned that optimising PEG hydrogel degradability alone in order to provide organoids with the space to grow, was not sufficient to improve mean organoid growth.

4.4 ECM composition and organoid growth

Proteomic analysis of Matrigel reveals the presence of an abundance of proteins related to the binding and signalling of growth factors, as well as various structural proteins (Hughes, Postovit and Lajoie, 2010). Although ill-defined, a prominent component of Matrigel is the ECM protein Laminin, including laminins 111, 121, 511 and 521 (Hughes, Postovit and Lajoie, 2010). Considering the abundance of laminin isoforms within the ECM of the intestinal crypt, and the use of Matrigel as the current gold standard for intestinal organoid culture, we expected, as demonstrated, mean intestinal organoid growth in PEG hydrogels would be significantly improved by the inclusion of diluted Matrigel (5%). This result served to validate the approach of optimising inert synthetic PEG hydrogels and informed our decision to pursue the incorporation of human recombinant laminin proteins into the PEG hydrogel matrix.

Through RNA sequencing of cultured intestinal epithelial organoids, we learned that organoids possess several transcripts for laminin chains including expression of LAMA5, LAMB1, LAMC1 (Laminin 511) and LAMA5, LAMB2, LAMC1 (Laminin 521). This would suggest that organoids are capable of synthesising some elements of their own ECM, in the form of laminin, and provides a sound rationale as to why organoids remain viable in a synthetic PEG

hydrogel, which is biologically inert. Furthermore, the presence of RNA transcripts for laminins 511 and 521 suggest a potentially vital role for these specific laminin isoforms, in the survival and proliferation of intestinal organoids. Interestingly, the same combination of laminins (511 and 521) are also expressed at significantly high levels in Matrigel (Hughes, Postovit and Lajoie, 2010) furthering the notion that these specific isoforms are uniquely responsible for organoid development. Not surprisingly, organoids cultured in PEG hydrogels that were supplemented with a combination of laminin 511 and 521, possessed a significantly increased mean organoid growth ratio when compared to control. This increase is also significantly different from hydrogels that possessed laminin 521 in isolation, suggesting a potentially additive effect in the mechanism of action by which these laminins confer improved cell proliferation.

4.5 Potential of PEG hydrogels for further optimisation

Although intestinal organoids embedded in PEG hydrogels optimised with human laminin 511 and 521 display improved growth characteristics, there remains a distinct and regular absence of budding, crypt-like domains, that is characteristic of organoid development under optimal conditions. This would suggest that although laminins are an important ECM element whose inclusion enhances organoid growth, they are not the sole contribution to optimal organoid architecture. An aforementioned study suggests the importance of a mechanically dynamic matrix that begins with high stiffness, but softens over time (Gjorevski *et al.*, 2016b). This highlights the necessity to incorporate multiple areas of optimisation, namely both biological matrix composition, and degradability, into a single hydrogel. By rendering a PEG based hydrogel mechanically dynamic through VPM incorporation, as well as incorporating the adhesive properties of biologically relevant laminins, it is conceivable that organoids will

create the space they need to expand whilst interacting with the surrounding matrix, prompting the formation of budding structures.

Furthermore, as our data suggests that laminins are not capable of solely replicating the organoid growth that is demonstrated in Matrigel, it is necessary to explore other elements of optimisation. Heparan sulfate (HS) proteoglycans are complex molecules consisting of one or more HS chain bound covalently to a protein backbone, and are distributed throughout the ECM as well as being present on many cell surfaces (Vicente *et al.*, 2018). Membrane bound HS proteoglycans have been known to co-operate with cell adhesion receptors such as integrins, and help facilitate cell-ECM interactions (Kim, Turnbull and Guimond, 2011). HS proteoglycans also serve as a ligand for CD44, a family of cell adhesion and signalling molecules that have effects on proliferation and survival, and in the intestinal mucosa, serves as an intestinal stem cell marker and direct target of Wnt signalling (Zeilstra *et al.*, 2013). We would hypothesise that the inclusion of HS proteoglycans into the synthetic PEG matrix would further aid in organoid growth and the formation of budding structures, and can be used in combination with laminins and the degradable peptide to create a highly optimised and defined matrix that specifically supports the growth of human intestinal organoids.

4.6 Limitations of study and Future Research

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In progressing with this research, it would be necessary to combine the functional data of organoid growth from altering the PEG molecular weight and polymer ~~density~~concentration, with rheological data for each gel, as there appears to be a narrow physiological window at which mechanical stiffness best supports organoid formation and development. It is necessary to quantify this stiffness, allowing for better comparisons to be drawn between individual optimised PEG gels, and other matrices such as Matrigel. Furthermore, as we wish

to explore the effect of interfering with mechanostransduction pathways and mechano-sensitive ion channels has in relation to organoid growth in matrices of varying stiffness, it is important to fully quantify hydrogel properties at a rheological level.

In addition, it would be necessary to expand the methodology of obtaining organoid growth data. Taking measurements of cross sectional area in one plane of focus across multiple time points, although providing a consistent point of comparison, does not account for the complexity of 3D organoid structures, or organoid growth that may be occurring across different dimensions. It would perhaps be more beneficial to use more advanced, high resolution, confocal live cell-microscopy in order to more accurately assess the growth of 3D organoids. This can be coupled with fluorescent labelling to visualise different cellular processes as well as overall tissue architecture.

Another potential area for further analysis is the swelling characteristics of PEG hydrogels. Hydrogel swelling is the amount of water or buffer taken up by the hydrogel and can indicate the hydrophilicity of the polymer network, as well as crosslinking density, where stiffer networks exhibit less swelling (Caliari and Burdick, 2016). Hydrogel swelling, which is likely to occur when organoids within the hydrogel are flooded with media, will have an effect on the gels microstructure and mechanical properties such as mesh size. Concentrations of PEG can be expressed as a molarity and this can be used to compare swelling ratios of hydrogels comprised of different molecular weight PEGs. This will allow for a more comprehensive comparison between hydrogels and can provide rational behind the performance of different PEG hydrogels.

4.~~7~~6 Concluding remarks

In this study, summarised in figure 12, we demonstrate the ability of synthetic, ~~unoptimized~~unoptimised PEG based hydrogels to support human intestinal organoid growth and viability under normal culture conditions. To further this, we begin to explore the versatility in the optimising potential that is offered by PEG hydrogels, through the alteration of matrix stiffness by using PEGs of increasing molecular weight as well as altering the , polymer ~~density~~concentration within hydrogels. Furthermore we assess how , hydrogel degradability influences organoid growth and morphology through the inclusion of a degradable peptide, and we explore how ~~and~~ adhesive ligand presentation through the incorporation of low concentrations of Matrigel and human derived laminin influence organoid growth, morphology and survival. These data in combination, highlight the possibility by which the animal derived natural matrix of Matrigel, can be replaced by fully optimised, synthetic, PEG based hydrogels that are tailored specifically to the growth of human intestinal organoids and compatible with clinical applications.

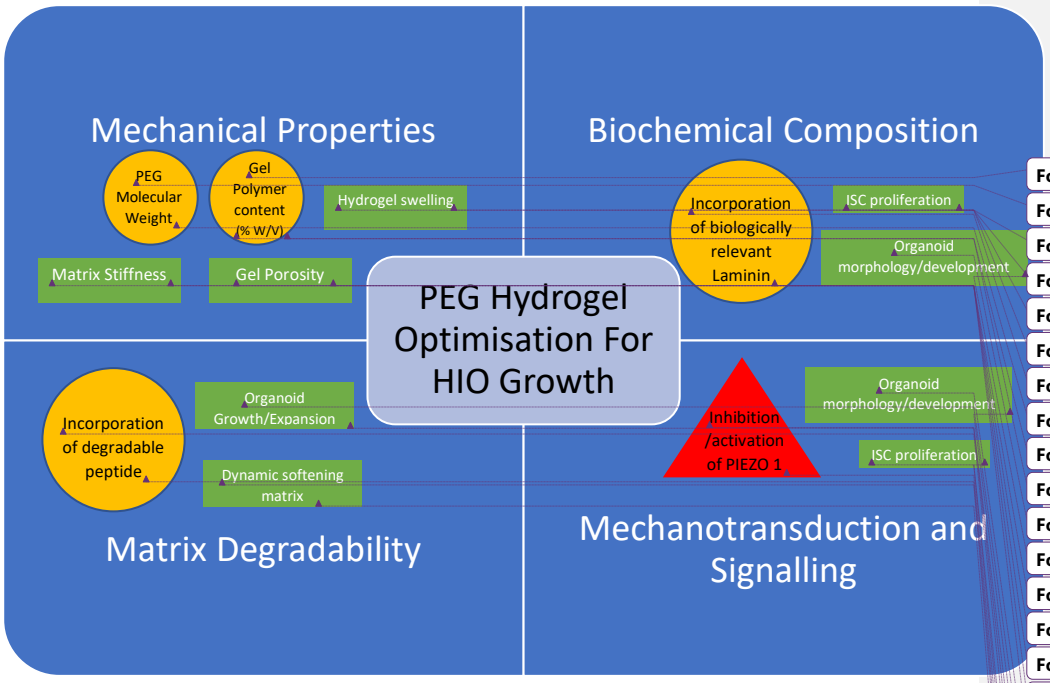

Figure 12. Summary schematic highlighting the different tuneable properties of PEG hydrogels that can be relevant for human intestinal organoid growth (blue squares). Each category contains the properties altered and tested in this body of work (orange circle) and the effects or potential effects that altering these properties will have are also highlighted (green rectangle). Properties not directly tested, but of potential biological relevance that future work will hope to explore is also depicted (red triangle)

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Supplementary Table 1. Statistical data for one-way ANOVA applied for the variables of organoid growth in response to polymer ~~density~~concentration/PEG molecular weight combination. N = the number of individual culture replicates, n = number of organoids analysed.

Gel	N	n				ANOVA
		10%	8%	6.40%	5%	P
5K small	2	8	11	8	9	<0.001
5K large	2	8	6	6	5	<0.001
10K small	2	13	15	12	15	<0.001
10K large	2	7	9	4	5	0.008
20K small	2	11	11	8	9	0.004
20K large	2	11	8	11	11	0.022
40K small	1	9	11	5	5	0.864
40K large	1	10	7	4	4	0.685

Supplementary Table 2. Statistical data for one-way ANOVA applied for the variables of organoid growth in response to Matrigel concentration. N = the number of individual culture replicates, n = number of organoids analysed.

Gel	N	n				ANOVA
		10%	5%	2.50%	control	P
8% PEG + Matrigel small	2	9	9	8	9	0.029
8% PEG + Matrigel large	2	6	7	8	6	0.004
10% PEG + Matrigel small	2	18	16	17	17	0.024
10% PEG + Matrigel large	2	7	7	11	14	0.74

Supplementary Table 3. Statistical data for one-way ANOVA applied for the variables of organoid growth in response to VPM:PEG ratio. N = the number of individual culture replicates, n = number of organoids analysed.

Gel	N	n			ANOVA
		Control	02:01	01:01	P
PEG + VPM small	2	18	22	24	<0.001
PEG + VPM large	2	16	22	13	0.391

Supplementary Table 4. Statistical data for one-way ANOVA applied for the variables of organoid growth in response to laminin type. N = the number of individual culture replicates, n = number of organoids analysed.

Gel	N	n							ANOVA
		Control	111	121	511	521	511+521	All	P
Laminin	2	18	21	23	25	21	26	25	<0.001

6. List of Abbreviations

ISC	Intestinal stem cell
CBC	Crypt base columnar
EGF	Epidermal growth factor
TGF alpha	Transforming growth factor alpha
CF	Cystic fibrosis
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
APC	Adenomatous polyposis coli
Tcf4	Transcription factor 4
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
IBD	Inflammatory bowel disease
3D	Three-dimensional
ECM	Extracellular matrix
PG	Proteoglycan
FGF	Fibroblast growth factors
VEGF	Vascular endothelial growth factor
MMP	Matrix metalloprotease
PEG	Polyethylene glycol
VS	Vinyl sulfone
SH	Thiol
MAL	Maleimide
GFP	Green fluorescent protein
DMEM	Dulbecco's Modified Eagle Media
P-strep	Penecillin-streptomycin
PBS	Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
HBS	Hepes buffered saline
NNUH	Norfolk and Norwich University Hospital
NHS	National Health Service
hCCCM	Human colonic crypt culture media
w/v	Weight by volume
DIC	Differential interface contrast
CSA	Cross sectional area
EdU	5-ethynyl-2'-deoxyuridine
SDS	Sodium dodecyl sulfate
BSA	Bovine serum albumin
IgG	Immunoglobulin G
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
HIO	Human intestinal organoid
MW	Molecular weight
YAP	Yes-associated protein
HS	Heparin sulfate

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