

Application of a 3D Hydrogel-based model to replace use of animals for passaging patient-derived xenografts

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Abstract

Currently, *in vivo* mouse models are the gold standard for testing cancer treatments before progression to human trials, often using tissue derived directly from patient tumours (patient-derived xenografts, PDXs) propagated in mice to generate sufficient cells for an experiment. A 3D *in vitro* system for tumour passage would reduce animal use and provide an alternative means of expansion, with the additional benefit of being capable of maintaining a human signalling environment throughout.

This 3D *in vitro* cancer model for propagation of patient-derived cells, based on a synthetic self-assembling peptide gel and using human extracellular components, allows the formation of a fully characterised, tailorable tumour microenvironment. Unlike many existing 3D cancer models the peptide gel is inert apart from molecules and motifs deliberately added or produced by cells within the model.

Breast cancer PDXs were shown to be capable of expansion over several passages in the peptide gel, and contaminating mouse cells are rapidly removed by successive passages. The resulting human cells were shown to be compatible with a range of common assays useful for assessing survival, growth and maintenance of heterogeneity. Experiments were also performed to probe practical and effective approaches for inclusion of relevant human stromal cells to increase the model's resemblance to patient tumours.

With further development, this hydrogel model can be tailored by addition of tumour microenvironment-relevant molecules and cell types. Based on these initial findings, following further work to confirm maintenance of cell heterogeneity, genotypes and phenotypes across passage, the hydrogel has potential to provide an effective and practical breast cancer model for passage of PDXs which will have the added benefits of being relatively cheap, fully-defined and free of animal products, with potential for further development to cover other cancer types.

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List of Abbreviations

3D-TE – 3D Tumour Expansion

BME – Basement Membrane Extract

CIC – Cancer Initiating Cell

CSC – Cancer Stem Cell

DAPI - 4',6-diamidino-2-phenylindole

DE – Differentially Expressed

EHS – Engelbreth-Holm-Swarm (murine tumour)

ER – oestrogen receptor

FAP – Fibroblast Activation Protein

GSEA – Gene Set Enrichment Analysis

H&E – Haemotoxylin and Eosin staining

HER2 – Human Epidermal Growth Factor Receptor 2

HLA – human leukocyte antigen

HRM – High Resolution Melting

IL - Interleukin

ISX – Imagestream X

MMP – Matrix Metalloprotease

MSC – bone marrow-derived human mesenchymal stem cell (eGPF labelled unless otherwise specified)

NA - NetworkAnalyst

ORA – Over-Representation Analysis

PC – polycarbonate

PET – polyethylene terephthalate

PI – propidium iodide

PR – progesterone receptor

qPCR – quantitative PCR

RT – room temperature

TME – Tumour Microenvironment

List of Presentations

Poster Presentations:

European Association for Cancer Research's Goodbye Flat Biology Conference, 10 September 2018 (Berlin), "Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts"

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FRAME (Fund for the Replacement of Animals in Medical Experiments) Symposium, 17 July 2019 (Nottingham), "Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts"

Cancer Research Nottingham Poster Competition, 16 Sept 2019 (Nottingham), "Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts"

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Cancer Biology Seminar Series, 8 June 2018 (Nottingham), "Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts"

BACR Student Conference, 26 November 2018 (London), "Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts"

Cancer Biology Seminar Series, 22 March 2019 (Nottingham), "Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts"

Nottingham Breast Cancer Research Centre Seminar, 14 May 2019
(*Nottingham*), “Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts”

FRAME Symposium, 17 July 2019 (*Nottingham*), “Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts”

NC3Rs Midlands 3Rs Symposium, 10 September 2019 (*Leicester*), “Application of a 3D Hydrogel-Based Model to Replace Use of Animals for Passaging Patient-Derived Xenografts”

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1 Introduction

While impressive medical and scientific progress has been made thus far in diagnosing and treating cancer, with overall 10-year survival in the UK rising from around 25% to around 50% over the past few decades [1], there is still a great deal of room for improvement. Cancer drug development particularly is still associated with immense costs and worryingly low success rates even at the clinical trial stage.

Cancer is a particularly problematic condition to treat, as the tumour is derived from the patient's own cells and therefore unlike an exogenous pathogen presents relatively few targets which can be targeted without also harming healthy cells. Recurrence of apparently successfully-treated tumours either in the same site or in the form of metastases in other organs and tissues is also a significant problem, as well as the fact that some types of tumours particularly tend to give no symptoms until they have already metastasised and therefore have very poor prognoses. It is not hard to find compounds that will kill tumour cells growing in a flask, but developing treatments which can combat these issues is a much greater challenge as the relevance of cells grown in flasks to patient tumours is limited.

Low success rates owe a lot to unsuitable models used in early screening for potential treatments; while recent understanding of cancer cell behaviour and the complexity of real patient tumours is driving a push towards more complex models and particularly 3D ones, there are still many ways in which these models fall short. A core problem is the source of cancer cells typically used in high-throughput screening of potential anti-cancer compounds; having been grown sometimes for many years in 2D on plastic they no longer resemble their patient tumour origins. The patient tumour will have consisted of multiple genetically distinct clones which might have differing behaviours in terms of treatment responses and metastasis patterns, known as intra-tumour heterogeneity. Only those of these which happen to grow well in 2D on plastic, which bears minimal resemblance to the complexity of the actual patient tumour, will survive passage. A push towards use of tissues taken from patients and directly transplanted into mice (patient-derived xenografts, or PDXs) is improving matters in this regard by providing an environment in which patient tumour heterogeneity is better supported and maintained. However, these tissues are affected by the mouse cells they grow alongside, and ethical concerns

are raised by the large-scale use of mice in this way. This project aims to generate an alternative model to reduce mouse usage while still generating these patient-relevant cells for experiments such as drug screens, which may include potential for personalised medicine approaches.

The patient tumour consists not only of cancer cells but also of various healthy cell types, whose behaviours may have been altered by signalling from the tumour cells, as well as extracellular matrix consisting of both soluble and structural proteins secreted by both cancer and healthy cells. All of these may contribute to the expansion or loss of different sub-clones within a tumour, and subsequently to the response of the tumour to any given treatment in both the long and short term. Non-cancer cells and molecular components of the tumour also have direct effects on the functions of some potential treatments. By including components to recapitulate tumour features in methods for expansion of tissues or cells for experiments, one may support retention of more sub-clones. This will result in more heterogeneous tumour cells with which to seed experimental models, an important consideration for improving predictive capability of the resulting experiments.

The majority of researchers are now aware that the use of 2D-grown transformed cancer cell lines for novel drug screening is likely to be contributing to the low success rate of initially promising treatments by generating 'false positive' results which cost a great deal of money and time to bring forward into animal or pre-clinical trials where they are eventually dismissed. This project aims to generate a 3D model to be used as a more patient-like environment for expansion of tumour cells taken from patient-derived xenografts (PDXs) which have never been grown in 2D. This model would replace mice in passage of these PDXs, reducing the number of mice used in cancer research for this purpose. Additionally, human tumours grown in mice are infiltrated by murine stromal cells which produce murine signalling molecules; use of the peptide gel to dictate which stromal cells are present will generate a more human-relevant cancer cell supply for research. Breast cancer cells specifically were used as a starting point due to the supporting gel element of the proposed model already having been used as the basis for healthy breast tissue models.

There are many features of the tumour and its microenvironment with potential to affect tumour development and treatment responses, and it is important to have

an understanding of what these are and how they affect tumour development when approaching the problem of generating a relevant cancer model.

1.1 Commonly Used Cancer Models

Currently, a large proportion of cancer research is performed using models which neglect the heterogeneity of the tumour cells, the details of non-cancer-cell tumour components, or both.

1.1.1 *In Vitro* 2D Cell Line Models

Cell lines grown in 2D *in vitro* (or, for some cancer types, in suspension) account for the majority of cellular material in current and past cancer research. These may be human or animal-derived, but there is little reason not to use human cells in most cases and so generally the more patient-relevant option is chosen. While transformed cell lines grown for long time periods on plastic do not represent any intra-tumoural heterogeneity, inter-patient heterogeneity has been recognised and addressed by researchers using panels of cell lines in 2D. One example of this is breast cancer, which can be considered to consist of a number of subtypes defined by hormone receptor expression which affect treatment compatibility and outcomes; it is common for breast cancer research involving cell lines to use cell lines representing multiple different subtypes. Cell line panels such as NCI60 attempt to provide a consistent set of cell lines which can be used by researchers worldwide to account for a range of origin tissues and subtypes.

1.1.2 Subcutaneous *In Vivo* Cell Line Models

At the furthest end of the model complexity scale are the *in vivo* mouse models, which are not accessible to all research groups but very commonly used where possible. This is because they are considered a 'gold standard' for pre-clinical trials, the final experiments before treatments moved into clinical trials, in which treatments are given to patients for the first time to establish their actual safety and efficacy. *In vivo* models therefore function as a final test of treatment efficacy before extremely expensive human trials, making their relevance to patients critical.

These models feature a range of options when it comes to patient tumour similarity; the subject of mouse models of cancer is a vast one, including both mouse hosts of human tumours and mice with mouse tumours, the latter being covered in great detail by Cheon and Orsulic [2]. Often human cancer cell lines may be transplanted into mice as a tumour model for drug efficacy testing with the caveat that cell lines tend to adapt to growth in culture and become unlike their source tissue or tumour [3].

The use of human, non-cell-line tumour cells is ideal for modelling to maximally reflect patient tumours; however, when one considers the vast range of elements which affect tumour behaviour in patients it is no wonder that both a complex host setting such as an *in vivo* mode and a relevant source of tumour cells such as primary patient cells are required to provide informative results [4, 5].

1.2 Molecular Biology of the Tumour

Even before one considers the tumour microenvironment's (TME's) contributions towards tumour complexity, it has long been understood that the tumour cells themselves have a degree of diversity within a tumour. In order to become cancerous a cell must acquire certain cancer hallmarks, such as replicative immortality and evasion of growth suppression, as noted by Hanahan and Weinberg [6], essentially a set of molecular 'talents' resulting from mutation which allow the cell to escape normal cell behaviours. It stands to reason therefore, that genetic instability (leading to frequent mutation) and cancer would go hand-in-hand. Subsequently, within the many cells of a tumour one might find sub-clones that have acquired differing mutations, and over time the tumour would gather further mutations which might strengthen the cancer hallmark characteristics and complicate the study and use of treatments that target molecular pathways leading to these hallmarks. The tumour would evolve as clones which grow faster or survive better outcompete other clones, with the high mutation rate typical of cancer ensuring that there are many slightly different clones to potentially overcome any challenge; such time-dependent differences have been demonstrated [7].

While there is some disagreement as to the exact source and nature of this heterogeneity and which aspects are relevant, including whether the same tumour may consist of sub-clones which acquired their full set of cancer hallmarks through different mutations, many types of heterogeneity have been described even decades ago and at least some are undoubtedly very important to account for [8-10]. Heterogeneity affecting genes which are used to predict treatment outcomes or prognosis is particularly important to include in an ideal cancer model. For example HER2 expression has been found to be heterogeneous in some breast cancers, a status which may make treatment selection more challenging and uncertain as there is little value in targeting only a fraction of tumour cells (resistant clones will simply fill the niche they leave) but there is a limit to how many simultaneous treatments can be administered [11]. It is also important to consider that tumours change and develop over time, typically being diagnosed in later

forms, so a model for tumour initiation will typically need to differ from a model for tumour development [12].

Additional to the general idea that tumour cells may differ within a tumour is the understanding that some of these cells are more 'stemlike', and have tumourigenic properties which may aid recurrence of otherwise successfully treated tumours if they survive [13]. This has led to a search for treatments which can target these tumourigenic cells to avoid recurrence [14, 15], and it is important that models account for these cells where relevant.

The rest of the tumour mass, adding considerable heterogeneity in their own right, are non-tumour cell components including stromal cells and molecular features such as soluble or insoluble biological molecules with a range of origins including fibroblasts recruited from bone marrow and their products in addition to the cancer cells' own secretions [16]. Not only might different sub-clones be differently affected by treatments, but non-tumour-cell tumour microenvironment (TME) components may also have effects on outcomes; for example, extracellular matrix remodelling by the tumour might affect access of drugs to the tumour, or the types of immune cells present in the tumour might affect how well immune-system-mediated treatments are able to target the tumour.

Variation from patient to patient within broad tumour types is also becoming increasingly evident and in some cases very well documented and used to direct treatment. Breast cancer, for example, is typically sub-typed according to molecular markers which can determine which targeted treatments may work (Oestrogen receptor (ER), Progesterone receptor (PR), Human epidermal growth factor receptor 2 (HER2) and Ki-67). Four types are commonly described: HER2-enriched is HER2 positive but PR and ER negative; triple-negative is ER, PR and HER2 negative; luminal A is PR and ER positive and low in Ki-67 while luminal B is PR and/or ER positive and high in Ki-67 or HER2 (summarised recently in a review by Cho [17]). These subtypes can have remarkably differing progression in patients so it is preferable to avoid models which only apply to one subtype [18]. Treatments of hormone receptor positive breast cancers often target expressed hormone receptors, and so particularly from the perspective of treatment, and additionally with awareness that tumours may contain sub-populations of cell of different subtypes, it is important to avoid drawing conclusions purely from one tumour or cell line.

1.3 Tumour Microenvironment

1.3.1 Stromal Cells

A remarkable number of cell types have been found to interact with tumour cells and may affect the behaviour of the tumour cells or be 'educated' by the tumour into a tumour-supporting role. Any monoculture cancer model will inherently be missing the complex effects of these interactions on the growing tumour, though some compensation may be possible via molecular additions.

1.3.1.1 Vasculature

Tumour-educated lymphatic endothelial cells, such as those which would be found in a tumour which has been vascularised by lymph vessels, have been found to bi-directionally communicate with cancer cells. This includes both pro-tumourigenic effects such as increasing tumour characteristics such as metastasis and chemoresistance via Jagged1 in the Notch pathway [19, 20] or increasing metastasis efficiency by chaperoning circulating tumour cells [21] and anti-tumourigenic effects such as modulating chemokine CXCL12 levels [22] as well as, depending on the endothelial cells source, promoting or inhibiting tumour growth via high levels of EGF expression [23]. A more in-depth review from Maishi and Hida covers tumour endothelial cells in detail [24].

Through endothelial cells the tumour can influence its environment to an extent; lung endothelial cells have been found to respond to lung tumour cells, forming vessel-like structures around which the tumour cells gather, in a potential means of tumour vascularisation quite unlike the typical signalling of existing vessel networks to expand towards the tumour [25]. This would alter access of distant cell types and potentially change small molecule concentrations in and around the tumour. Both of these roles of endothelial cells in the TME underline the multi-faceted complexity of a 'perfect' model in their additional relevance to vascularisation.

1.3.1.2 Immune Cells

Like many aspects of the tumour stroma, immune cells have been approached as both an ally and an opponent in the search for a cure for cancer. While it makes sense that only tumours that have escaped the anti-tumour activities of the immune system become significant enough to cause disease, researchers are still investigating promising immune-focused leads for treatment [26, 27] and so relevant models are needed, in addition to models accounting for the range of effects of immune cells on other aspects of the tumour. The role of immune cells

in the tumour microenvironment is complex and two-sided; some immune cells and responses may act against a tumour, yet the tumour may also accumulate regulatory and protective immune cells in order to evade elimination and promote progression [28, 29].

Many immune cells affect tumour development and progression in a variety of ways. For example natural killer cells have been shown to be responsible for preventing tumour development, likely recognising ligands reflective of cellular stress [30]. Interactions between multiple immune cell types and signalling in both directions may also affect tumour progression; tumour associated macrophages recruit regulatory T cells to modulate interactions between the immune systems and the tumour via CCL20 [31] and tumour cells have been found to be able to polarise macrophages towards phenotypes with more pro-tumour activity, particularly via interleukins (ILs) including IL-1 β , IL-4, IL-10 and IL-13 [32]. These pro-tumour macrophages may initiate feedback loops (via monocyte colony stimulating factor, produced by cancer cells, and its receptor on macrophages, and conversely epidermal growth factor, produced by macrophages, and its receptor on cancer cells) which promote tumour proliferation [33]. The role of neutrophils in cancer is too broad a topic to cover here, even in brief; a recent review by Treffers *et.al.* covers both direct and indirect effects [34]. A variety of links with cancer development, and particularly metastasis, have been identified for platelets too. A link between platelet levels and metastatic capability was identified in neuraminidase treated cell lines as early as 1968 [35] while more recent work shows a great number of connections between platelets and tumours, including how platelets can reduce NK cell effectiveness [36] and that platelet depletion may improve cancer treatment delivery and effectiveness [37].

The immune system's ability to target tumours is further complicated by the activity of cancer-associated fibroblasts, the more direct effects of which are discussed here later. As reviewed by Fearon [38], fibroblasts expressing Fibroblast Activation Protein (FAP) reduce the ability of would-be anti-tumour T cells to access the tumour cells, thereby promoting tumour survival.

1.3.1.3 Adipocytes

Cells of the microenvironment specific to the location of the tumour may also affect cancer aggressiveness and drug resistance; adipocytes, which are particularly relevant in breast cancer, have been found to produce an environment which favours cancer progression and survival.

Adipocytes in breast cancer stroma have been found to be linked with increased metastasis to the lymph nodes compared to more fibrous stroma [39] and the same study showed that indirect co-culture with adipocytes caused breast cancer cell lines to adopt more mesenchymal morphological, gene expression and migration patterns, predictive of cancer progression and metastasis. Adipocytes have also been shown to increase migratory and invasive behaviour in breast cancer cell line through expression of IGFBP-2, which is also linked with increased metastasis in patient breast cancer samples [40]. Similarly, adipocytes from the omentum, to which ovarian cancer often metastasises, increase migration in ovarian cancer cell lines [41]. Indirect effects via adipocytes have been proposed as a mechanism contributing to the anticancer effects of metformin against ovarian cancer [42]. Further underscoring the multi-directional interactions between the different cell types that may be found in the tumour stroma, incubation of the breast cancer cell line MDA-MB-231 with adipocyte conditioned medium increases production of immune signalling proteins, some of which are also associated with cancer migration and invasion [43].

1.3.1.4 Cancer-Associated Fibroblasts

Fibroblasts and related cells are widely acknowledged as important factors in the tumour microenvironment, though the details of their roles and even identities are subject to ongoing debate [44-47]. Cancer- (or carcinoma-) associated fibroblasts (CAFs) primarily from bone marrow [16], myofibroblasts, mesenchymal stem cells (as well as mesenchymal stromal cells, a broad population not specifically identified as being stem cells; both are referred to in the literature as MSCs however here MSCs will refer to Mesenchymal Stem Cells only) and pericytes are among (potentially overlapping) fibroblast-like cell types investigated for their effects on cancer progression and behaviour. Significantly, fibroblastic cells of nominally the same type originating from healthy versus cancerous breast tissue can have opposite effects on co-cultured normal and pre-neoplastic breast cell lines [48].

In healthy tissue, fibroblasts are primarily responsible for extracellular matrix (ECM) production. Myofibroblasts in the stroma are associated with a wound healing response, and in association with cancer's similarities to a 'never-healing wound' have been found to be predictive of poor outcome in some cancers [49, 50]. While myofibroblasts are commonly distinguished from other cell types by α -smooth muscle actin expression, their origin and relationship with other fibroblast types is inconsistently described throughout the literature. Yeung *et al.* [51] treats

myofibroblasts as a distinct cell type which becomes activated in cancer metastases, whereas others (e.g. Takahashi *et al.* [52] and Brennan *et al.* [53]) treat myofibroblasts as activated fibroblasts and Sinha *et al.* [54] present myofibroblasts as a possible source of CAFs. This difficult situation is reflective of the level of clarity and consensus regarding terminology, identity and origins of fibroblast-like cells in general, which complicates inclusion of potentially highly relevant and otherwise simple model features.

Mesenchymal stem cells have been implicated as having both inhibitory and supportive roles in cancer progression [55], depending at least in part on the cancer cell line investigated. A review by Torsvik and Bjerkvig provides a detailed overview and critical review of the roles and effects MSCs and their secreted paracrine factors in cancer [56]. They are also a proposed origin cell type for the activated fibroblasts found within tumours [16, 57].

1.3.2 Acellular Components of the TME

1.3.2.1 Adhesive Proteins, Glycoproteins and Glycosaminoglycans

Many important and common elements of the matrix are glycoproteins or proteoglycans [58]. For example, the presence of different syndecans, shed from cell surfaces, in breast carcinomas is linked to different prognoses [59]. Agrin and perlecan, at least at transcript levels, are upregulated in some cancers including oral squamous cell carcinoma. Knockdown of either of these proteins in different oral squamous cell carcinoma cell lines can reduce the ability of the cells to adhere to Matrigel and migrate [60]. Additionally, the matrix glycoprotein vitronectin has been shown to have a role in accelerating tumour growth [61].

To go into the details of every proteoglycan that has been linked to cancer would be impossible here; instead some examples of the effects of more common or better known components will be outlined. A more detailed analysis of one group of basement membrane glycoproteins, the heparan sulphate proteoglycans, specifically in cancer can be found in Blackhall *et al.*'s review [62].

Laminins, another group of basement membrane glycoproteins composed of three peptide chains, have likewise been scrutinised comparatively in cancer and healthy tissues, with expression levels of some laminins or individual laminin chains having been linked with tumours. This includes the γ 1 chain, whose increasing expression is linked with progression through stages of developing uterine cancer [63]. The laminin 332 trimer (or its γ 2 chain) is conversely lost from the basement membrane with bladder tumour progression, instead being retained inside cells, and *in vitro*

addition of laminin 332 to a rat carcinoma cell line increased motility and invasion [64].

Hyaluronan is an unusual case in that it is similar to the sugar components of proteoglycans and is found alongside them in the ECM but unlike these includes no protein component at the point of synthesis. Covalent and non-covalent attachments to ECM proteins may form after hyaluronan's synthesis. Like many ECM glycoproteins, hyaluronan's presence affects the progress and behaviour of tumours. Hyaluronidase can act as a tumour chemosensitiser and reduce the resistance cancer gains in the realistic 3D setting [65], while particularly metastatic examples of breast cancer cell lines have been found to contain cancer stem cell populations which overexpress Hyaluronan Synthase 2, which enhances invasion and growth of the tumour into bone [66].

Highlighting the complex network of changes and effects encompassing the tumour microenvironment, both tumour cells and normal cell types may, with or without one another's influence, produce matrix components which may affect cancer development and progression. Increased expression of fibronectin by mesothelial cells, stimulated by cancer cells, has been associated with metastasis of ovarian cancer [67]. Breast cancer stem cells produce a matrix of laminin 511, with multiple pro-tumourigenic effects including maintaining a feedback loop to maintain their stemness and interacting with integrins, a group of membrane-bound proteins, to allow tumour growth [68].

Integrins act to convey signals between proteins of the ECM and the inside of the cell, and subsequently may feature in interactions and pathways identified as contributing to cancer development and behaviour [69]. In addition to proteoglycans, integrins may convey signals to and from collagens, another group of ECM proteins.

1.3.2.2 Collagens

A major component of basement membrane extracts used in some 3D models [58], structural, or otherwise insoluble proteins in the matrix affect the biophysical properties of the tumour microenvironment. They may also contain important binding sites (for homotypic binding or interacting with other proteins or matrix glycans) or be cleaved to produce cryptic functional fragments as is the case for collagen XVIII, of which endostatin is a cleavage product [70].

There are many types of collagen, found in different tissues where they serve a wide variety of different functions, including exerting effects on cells through interaction of integrins with binding sequences within the collagen fibres. They therefore have a range of effects in cancer, depending on the cancer's location and type. A review by Fang *et al.* summarises the various collagens, covers some of their effects on cancer and outlines how these effects may be positive or negative [71]. In addition to the previously discussed case of collagen XVIII, whose cleavage product endostatin was proposed as a cancer treatment due to its anti-angiogenic properties, some illustrative examples include collagen III's protective effect against spontaneous tumour formation and metastasis in mouse breast cancer models [72], while collagen I in contrast promotes growth and invasive properties in cancer cell lines [73]. Collagen II is a critical component of a pro-migratory matrix produced by CAFs [74] and collagen IV has been found to promote growth and migration in a pancreatic cancer cell line via integrin engagement, as well as reducing apoptosis [75] while the absence of a collagen IV subunit $\alpha 5$ prevents lung tumour progression in mouse models and has similar effects on the A549 human lung cancer cell line in addition to reducing migration of the latter [76].

1.3.2.3 Diffusible Proteins

In addition to the insoluble proteins which contribute to the structure of the TME, many soluble proteins including growth factors and matrix remodelling proteins are found in the TME and may be dysregulated compared to healthy stroma. Many of these may originate from the tumour cells themselves or the stromal cells described above.

Matrix metalloproteinases degrade extracellular matrix and are subsequently of interest in the contexts of tumour growth and metastasis. Matrix metalloproteases 2 and 9 (MMP2 and MMP9) have been particularly scrutinised for their roles in cancer, including investigation of the details of how expression is regulated and how they interact with 3D patient-like models as opposed to 2D models [33, 77-79]. Differences in the promotor regions of MMP1 and MMP9 are associated altered likelihoods of cancer progression outcomes such as survival or metastasis to lymph nodes [80]. MMP7 for example is responsible for cleaving collagen XVIII to produce endostatin [81]; while endostatin specifically proved ineffective in treating human cancer the potential roles of cleaved matrix fragments in cancer are evident in the effects of endostatin treatment on mouse tumours [82]. Less

often considered but nonetheless likely to be important is the role of MMP inhibitors and proteins which can sequester or over-concentrate MMPs, altering their effective concentration further.

Similar to the role of MMPs, increased heparanase expression in a range of cancers degrades heparin sulfates in the extracellular matrix, releasing sequestered molecules and altering the heparin sulfate structure to induce a plethora of signalling changes [83]. The breakdown of matrix molecules by MMPs or heparanase may also rapidly release sequestered growth factors and cytokines from the stroma, allowing them to exert their effects on cells. Growth factors may affect tumour growth in ways which vary from tumour to tumour, as in the case of certain HER2 positive breast cancers [84]. These include IL-6 and IL-8 which may promote metastasis [41, 85], platelet derived growth factor which is involved in signalling between cancer stem cells and tumour-associated macrophages [66] and transforming growth factor- β (TGF- β) which appears to have differing effects at different stages of tumour progression [36, 86].

The result of all these intricately interacting TME features is that each patient tumour resides in a unique tumour microenvironment of proteins, glycans and non-tumour cells which influence and are influenced by the tumour cells and may affect the effectiveness of treatment. It is hardly surprising therefore that pre-clinical drug studies may therefore produce false negatives or false positives if relevant TME features are omitted from the models used. Careful model development and selection is therefore necessary, and some more complex models to begin to address these issues are coming into more frequent use.

1.4 3D *In Vitro* Cancer Models

The accuracy of a cancer model, and particularly of a cancer model which is to model drug responses, is tied to the extent to which it mimics the TME, however there is a limit to how realistic a model can be made while still being practically achievable. One approach that many groups take is to grow cells in 3D rather than in 2D.

Spheroids and similar spherical cancer models (for further context regarding the distinction between the many types of spherical cancer models see the 2015 review by Weiswald et al. [87]) are one approach that allows cells to be grown in 3D in a reasonably high-throughput-compatible format useful for experimental work [77, 88].

Basement membrane extracts (BMEs) are cell-free protein mixtures extracted from murine tumours grown *in vivo*. They are sold as products such as Matrigel™ and Cultrex™ and have a great strength in being as complex as a natural tumour's molecular environment. While they are expensive, basement membrane extracts are reasonably practical to work with and much more accessible than *in vivo* models. BME-based models are therefore often used to improve relevance where otherwise 2D culture might have been considered [89]. They do however suffer from batch-to-batch variation and particularly difficulty characterising lower-frequency components which appear to be present in some batches but not others [58], in addition to introducing murine molecular cues.

Single matrix protein gels such as collagen, fibrin and hyaluronic acid tend to be a less complex but more reproducible alternative to BMEs, and have the potential to use recombinant human proteins which will interact with cancer cells in a more patient-relevant manner [90-94].

Non-matrix-protein gels have the particular advantage in cancer modelling of being potentially highly consistent between batches, unlike murine tumour basement membrane extracts, of allowing cell binding sites to be adjusted independently of stiffness, which gives better flexibility for modelling of differing tissues or tumour stages, of being animal product-free, and of allowing encapsulated cells to define their own matrix [95, 96].

1.5 Models Incorporating Co-culture

One way to improve the complexity and subsequently relevance of cancer models in a wide range of formats is co-culture; the inclusion of non-tumour stromal cells which would typically be found in the tumour. Direct co-culture, in which the tumour and non-tumour cells are in the same compartment, allows bidirectional signalling as in a patient tumour and for non-tumour cells, where relevant, to lay down relevant matrix molecules. Indirect co-culture, in which a cell-impermeable membrane separates the cell types, allows the non-tumour cells to produce relevant soluble factors, allowing the effects of insoluble and soluble factors to be assessed separately and improving the relevance of the model without contaminating one cell type with the other. Likely due to these effects, cells grown in co-culture with fibroblasts in BME show highly patient-relevant treatment responses [97].

Many cancer models are amenable to direct co-culture by seeding the tumour cells in the same well as the additional cell types of interest (as in [25, 98]) or by growing

the tumour cells in medium in which the non-tumour cells of interest have been grown (conditioned medium) (for example in [43, 99]). For indirect co-culture, tissue culture well inserts can be used to investigate effects of signalling between cell types bi-directionally but without mixing of cells types or direct physical interaction of the different cell types, if soluble factors are of particular interest [100, 101]. An inherent complication of any co-culture approach, however, is that in many cases one cell type at least will have to be cultured in a different culture medium to its intended or accustomed medium and may subsequently grow or respond atypically. This medium mismatch problem demonstrates a further separation of current models from the reality of the patient; these are the media to which the cells have adapted during 2D growth. In an ideal model this problem would be self-correcting; patient-derived cells should not have experienced any medium adaptation and could be supported by including the needed medium components and any compartmentalising basement membrane as products of other included cell types.

Co-culture has been used in various formats to address cellular TME contributions. Paracrine signalling modulates many of the effects of fibroblast-like cells, therefore once the relevant fibroblastic cell type has been established, modelling the contribution of cells can be a fairly simple addition to 2D or 3D models. Direct co-culture, indirect co-culture and the use of medium 'conditioned' by the fibroblast-like cells may be used to model different types and degrees of cell-cell signalling, in addition to mixed microfluidics models which may allow greater control or automation over medium changes including conditioning [99]. Co-culture models have been used not only for combining tumour cells with another cell type but also for greater numbers of cells types [48]. Including co-culture may also function as a 'shortcut' to adding a range of physiologically relevant proteins, glycoproteins and metabolites to the model – rather than being added at times and concentrations determined by the researcher they are secreted by the different cell types as they would be in the patient as a result of the relevant cell-cell signalling.

Tumour modelling may be at first glance a simple case of co-culturing cancer cells alongside a relevant cell type. Those cells would behave differently if a third cell type were included however, and yet including that third cell type will add another cell type whose behaviour would be different and more relevant if a fourth were added. Each stromal cell type signals to many others in addition to the tumour cells, meaning any co-culture model must be limited by practicality rather than a patient-relevant stopping point regarding the numbers of cell types included. Co-culture

models have nonetheless proven informative on the subject of interactions between tumour and immune cells [28, 31] For many purposes additional cell types may need to be included in a manner that allows two-way signalling, as the immune cell type may be conditioned by the tumour to produce a pro-tumour effect or a feedback loop may occur, an effect which cannot be achieved by adding naïve-immune-cell conditioned medium to the tumour cell culture [33, 102].

From the often-neglected perspective of modelling for passage, in which the goal is to grow additional cells rather than immediately perform an experiment upon them, the ability of conditioned medium from fibroblast-like cells under some circumstances to increase proliferation as one of multiple effects on the tumour cells [86] presents both a convenient way to introduce a relevant TME feature into a model and potentially an improvement in the practical usefulness of the model for ensuring rapid production of material for testing of chemotherapeutics, for example. Similarly the importance of secreted factors means indirect co-culture could be used to improve model relevance while keeping the cancer or xenograft cells in their own easily-separated compartment. Potential points of particular difficulty in modelling stromal cell types, however, include signalling and travel through the bloodstream, which has been the subject of some disagreement [103, 104] and the role of pericytes which are particularly associated with blood vessels, both of which would require the impossible 'full model' to completely simulate.

At the simplest level, modelling of endothelial cells and adipocytes in the TME can and has been done by co-culturing relevant cell types alongside tumour cell lines in 2D [23] or 3D [25, 105]. The model becomes more complex, however, in cases where the roles of other factors such as matrix proteins and fibroblasts, which may influence how endothelial cells and adipocytes affect tumour behaviour, must be accounted for. Particularly in the case of endothelial cells the issue returns additionally to the challenge of modelling vascularisation, while the role of adipocytes, whose abundance varies within the body, highlights the necessity of considering the types and numbers of stromal cells likely to be found in the modelled tumour's native environment. Relevant adipocyte inclusion in models of cancer of the breast, ovary or other adipose-adjacent cancers such as colorectal cancers may therefore allow researchers to account for factors and problems in treating patients which are not typically seen *in vitro*.

1.6 *In Vivo* Cancer Models with Improved Relevance

Options are also available to improve the relevance of *in vivo* models, to ensure that the most relevant possible data is obtained from the animals used.

By introducing cancer cells into a mouse model into an orthotopic site, that is a site equivalent to where the tumour would have formed in the patient, rather a somewhat more convenient subcutaneous site, researchers can improve the relevance of the resulting tumours, often resulting in more patient-like metastasis patterns [106, 107]. This can be combined with use of patient-derived primary tissues rather than cell lines for maximal relevance (summarised in a review by Rashid and Takabe [108]).

Similarly, and particularly in experiments where the formation or treatment of metastases is of interest, cancer cells may be introduced into a mouse model via the bloodstream. In these models the expectation is that not all organs will be adequate hosts for the cells (in the same way as not all organs are commonly metastatic sites) and the cells will then survive and grow in organs likely to host metastases in equivalent patient tumours. These models however appear to require some caution regarding the location of injection to achieve relevant results [109, 110].

1.7 A Modelling Challenge

These options, which allow experiments to be performed in 3D or *in vivo* with greater resemblance to the patient tumour microenvironment, are a great improvement over 2D work, however a great number of realistic and important tumour elements may still be missing; human ECM molecules and the range of stromal cells that would be present in the patient tumour are often absent. In many cases the intra-tumour heterogeneity of primary patient cells in 3D *in vitro* models is lost in favour of easily-obtained but homogenous cell lines. Often it would be reasonably practical to incorporate some of these elements for a much more informative model.

At one end of the spectrum there are efficient, large-scale but 2D cell line screens, which increasingly fail to provide patient-relevant outcomes due to their highly simplified nature. On the other, there are *in vivo* pre-clinical trials which make use of the much greater complexity of an animal host and 3D environment, but are limited by their ethical, practical and financial burdens. An *in vitro* model which is relevant and complex, but not to such a point as to be too impractical to use, is needed.

A very large range of such models have been developed to fill this gap by providing a platform for smaller-scale, more accurate screening of potentially useful compounds identified by 2D screens Figure 1-1. These include models which may be highly complex to reflect the tumour microenvironment, easy to use, suitable for medium- to high-throughput experiments and/or compatible with a range of useful assays, and their use is therefore becoming widespread both for drug testing and for elucidating the underlying biochemistry of different cancers.

A key area in which these models are lacking, however, is applicability to passage; they have been designed to be a platform into which an existing stock of cancer cells can be seeded to perform drug screens or to understand specific mechanisms and behaviours in cancer. 3D *in vitro* models which can be used to expand cells in readiness for experiments are still a relative rarity, and particularly ones which can be applied to PDX-derived cells to address concerns regarding loss of heterogeneity that comes with the adaptation to 2D culture of cell lines.

1.8 The Problem of Expansion

Many modelling options for experiments involving cancer exist; while the sections above describe particular techniques in isolation, Figure 1-1 summarises the core elements of the most common and some emerging options which may be combined to form an overall model. However, in many cases these models are not suitable for prolonged expansion of cell populations and therefore are seeded with cells which have been expanded in 2D on plastic. It has become increasingly apparent that cells grown in two dimensions do not respond to treatments or manipulations as they do in the more realistic setting of three-dimensional culture [89, 111, 112]. While the immediate 3D environment of the experiment itself may improve accuracy of the model, homogenous cell lines are not an ideal starting point for the cellular element of the model. Changes in signalling pathways (including those important to cancer progression and resistance) occur during immortalisation or 2D culture and are not always restored to their original patient-matching state even when the cells are subsequently cultured *in vitro* in 3D [113]. Even the relatively simple 3D spheroid model can improve preservation of patient-like genome profiles [114].

The ability to take primary cells from patients or patient-derived xenografts and expand them in a 3D environment means that experiments performed in 3D

models using these expanded cells can be completely free of the unnatural selection and signalling effects of 2D culture and immortalisation procedures. In many cases there is the additional benefit of the potential for patient-matched tumour and healthy tissue samples handled in parallel. Some studies have found that while expansion in 2D conferred selection pressures causing gene expression or clone proliferation dissimilar to the patient tumour environment, *in vivo* expansion may also result in some changes from patient tissue to serially passaged cells [115, 116]. It is not a great stretch to imagine that the differences between the patient's human cells and the murine host cells' signalling molecules may contribute to these changes. This suggests a need for intermediate expansion models with human cells and molecules, greater complexity than 2D culture and greater optimisation capacity than *in vivo* passage. Additionally, there are ethical concerns associated with use of animals in research which are particularly pressing in the case of animals used purely to expand cell populations without direct benefit for human health.

While most experimental cancer models include an expansion stage between setup and assays or drug screening, many 3D models that expand a population of primary cancer cells but are ultimately intended for experimental purposes are not suitable for large-scale, long-term expansion. When creating a model for the purpose of primary cell expansion, there are several limitations that must be worked around which need not be considered for experimental models. These include the need to allow for a large change in cell mass and subsequently passage of the cells, the need to retain sample heterogeneity over potentially very long time periods, the need to eventually extract the cells in good condition to be seeded into subsequent experimental models or formats, and the need for maintenance of the cells during expansion to be something which can be achieved as a routine or background task. On the other hand, certain design pressures associated with experimental models are of reduced concern when considering models for expansion; there is no need for a setting that can compare dozens or hundreds of different variables, only for large bulk growing capacity, and immediate assay readiness is not a necessity.

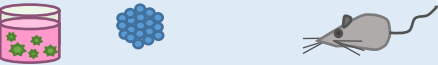
Tumour Cells	Scaffold, Shape	Added Features
 Established cell lines Immortalised and adapted to plastic – convenient but not highly representative	 2D on plastic Incorporates few features, but amenable to co-culture and some molecule additions	Co-culture Most models are amenable to inclusion of or inherently include other cell types such as fibroblasts and adipocytes – differing culture medium requirements and realistic cell number uncertainties may limit co-culture
 Primary cell lines Closer to patient samples, but few inherent features and a degree of adaptation to plastic	 3D matrix gels Range of synthetic through mixed to natural options, makes gradients possible and natural gels may provide realistic binding motifs	
 PDX Initially transplanted from patient into mouse, 3D and with other cell types and matrix molecules	 Spheroids May include other cell types and will mimic existence of molecular gradients (e.g. oxygen) in tumours	Natural Matrix Molecules Can be produced by tumour cells or co-cultured cells, or may be included in 3D matrix – full proteins or their motifs may also be added separately
 Induced or spontaneous animal tumours Typically purely murine models – effectively entirely natural but also entirely non-human	 Bioprinted scaffolds May mimic tissue boundaries or similar, made from a variety of materials overlapping with 3D matrix gel options	
	 Animal host Uniquely provides ‘full organism’ context. Hollow fibres can isolate tumour without losing all levels of complexity	Diffusible Molecules Signalling molecules and metabolites can be added or inherent to other aspects of the system, but realistic molecular gradients require 3D architecture

Figure 1-1: *Summary of commonly-used variations of the major components of a cancer model*

1.9 3D Expansion Models and Features

While immortalised cancer cell lines are resilient and easily expanded in many formats, primary cells, those which have not been immortalised and are more likely to retain some of the heterogeneity of the patient tumour, are less easy to expand. The few existing 3D models for expansion of primary patient-derived cancer cells come in various forms but certain themes can be identified, such as the particularly well-known organoid format, microfluidics and the gels sometimes used to suspend cells within them, and '2.5D' models in which cells are grown in a monolayer but with additional components beyond that of standard 2D.

The following sections summarise 3D model formats and features that can be used to expand primary cancer cells, with emphasis on their use for that purpose.

1.9.1 *In Vivo* Models

As well as functioning as experimental models, *in vivo* models can be used to passage patient-derived cells. PDXs, which use tumour samples from patients and do not harbour any risk of adaptation to plastic or loss of intra-tumour genetic and phenotypic diversity and heterogeneity following said adaptation, may be used as relatively close mimics of the tumours they originate from [117-119]. PDXs are not only strong general passage models but may offer insight into treatment routes for the specific patients from whom the tumour samples are taken [5]. Organisations like the Breast Cancer Now Breast Cancer Biobank give researchers who wish to improve the accuracy of their cancer models access to patient tissues which can be used to give highly patient-relevant research outcomes [120].

Orthotopic implantation of PDXs in mice allows researchers to account for site-relevant conditions and cell types in *in vivo* models, particularly producing patient-like metastasis outcomes [121, 122], albeit with the familiar problem that the mouse cells may not interact in the way that human ones would.

Implantation into a whole, living organism is required to truly recapitulate all levels of interplay between the tumour and other organs and cells via the vasculature, but species differences and subsequent necessity to use immunosuppressed hosts for xenograft models means important factors like immune cells cannot be accounted for.

The closest model of a human tumour currently available for whole-organism-scale effects of vascularisation is the use of immunodeficient mice which have been 'humanised' by a complex process ultimately engrafting human hematopoietic stem cells, producing a mouse with a human-like or human-mouse-chimeric immune system [123]. This underlines quite clearly the difficulties likely to be encountered when aiming to generate practical *in vitro* models of this particular feature of the TME.

1.9.2 Organoids

Organoids are derived from multi- or pluripotent stem cells and self-organise into structures which recapitulate their source tissue, including differentiated cell types. They are used particularly in gastrointestinal tissue modelling of healthy, cancer and other diseased states, in which the topological 'inside versus outside' distinction of the origin tissue is otherwise a challenge to model.

In 2011 Sato *et al.* developed a protocol, including a cocktail of required growth factors, to grow and passage organoids from primary human tissues including cancers from a range of gastrointestinal organs [124]. In this case the length of time human colon cancer could be expanded was unspecified, as was the percentage of samples for which successful organoid culture could be established, but nonetheless it was the beginning of the development of an important model for passage of primary human cancer tissues. Work since then has progressed the technology to applications in a much wider range of tissues, identifying and overcoming problem elements like overgrowth by normal cells along the way ([125-129]).

More recently the broad relevance of organoids to existing research approaches has been demonstrated; primary liver cancer organoids have been developed which recapitulate the patient disease in key criteria such as histology, biomarkers and drug response [130] and organoids have been used to probe Wnt signalling in transition between Barrett's Oesophagus and Oesophageal Adenocarcinoma [131]. A general protocol for organoid culture, including specifics conditions for certain tissues [132] has now been published, making organoids a fairly accessible model where patient tissue is available.

At the time of writing organoids are a flexible 3D model, suitable for a range of tissues and viable for significant expansion at reasonable speeds. Inclusion of Matrigel or an

alternative Engelbreth-Holm-Swarm (EHS) mouse sarcoma extract (basement membrane extract or BME) is ubiquitous in these cultures which may lead to a significant number of their potential drawbacks. It is perhaps worth considering that a vast number of proteins with signalling properties are retained (even in growth factor reduced BME [133]) and that these might provide means for non-cancer cells to outgrow cancer cells, along with (as indicated by Karthaus et al. [127]) slowed replication due to genomic instability in certain cancers. Use of BME also introduces considerable variation between labs and batches and may interact differently with different cancers, making reliance on BME a key weakness of the model. In light of some of these considerations, Broguiere *et al.* have developed a more defined organoid model using only a small percentage BME which might lead the way to an organoid model free of the key problems that limit their value currently [134]. While organoids derived from tumours have the benefit of maintaining some of the vital heterogeneity of patient tumours [135], organoids are not always ideal models as they lack many key features of the TME such as immune cells and human matrix composition.

1.9.3 Feeder Layers

While not strictly forming a 3D model, feeder layers of murine stromal cells can be used to encourage growth of primary cancer cells in tissue culture flasks and are sometimes referred to as '2.5D models'. This technique has been in use for more than a quarter of a century [136] initially using primary mouse cells grown on stromal cells from the same site. More recent examples grow human cells on an irradiated murine feeder layer, sometimes referred to as conditional reprogramming when used in conjunction with Rho kinase inhibition [137], and have demonstrated effectiveness for establishing primary breast [138, 139], prostate [139], B-cell lymphoma [140] and salivary gland [141] tumour cultures. Human stromal cell feeder layers have also been used in place of murine cells, for example to establish leukaemia primary cultures [142]. As with other methods used to establish cultures from primary tumour samples, overgrowth of non-tumour cell populations has been a persistent problem for some tumour types, particularly lung cancers [143, 144].

Ito *et al* found that CD40 ligand was the key signalling factor allowing stromal cells to support the B-cell lymphoma cells in their cultures [140], indicating the feeder layer itself is non-essential. Janik *et al* found that murine tumour basement membrane extract was able to support maintenance of primary breast and prostate cultures more effectively than a feeder layer or collagen alone [139]; while these experiments used a surface coating and therefore were effectively in 2D, gels such as these are commonly used to provide structural or signalling support to cells growing in 3D models.

1.9.4 Gels and Scaffolds

As well as being used as coatings for '2.5D' models and to encourage formation and survival of spheroids, gel matrices such as those derived from EHS mouse sarcomas may be used to form a 3D structure in which cells can grow, imitating to some extent the extracellular environment which supports a solid patient tumour. These may be complex tumour extracts (such as the EHS extracts used in typical organoid culture described above, reviewed by Benton *et al* [145]), but may also often be made from single (synthetic or purified) matrix proteins, simple mixtures of such proteins, and entirely synthetic/non-animal gels based on polypeptides, PEG-derived molecules or polysaccharides [146]. Natural polymers such as agarose [147] and single matrix components such as collagen [148] may be used as more consistent if somewhat less relevant supports for modelling tumours in three dimensions. Artificial polymers may be used with or without added natural matrix components such as hyaluronic acid [78, 149, 150]. The use of peptide hydrogels is also becoming more common, as such gels are practical and modifiable even when not matched to any particular protein [95]. Peptides would naturally be found in and around tumours and the peptide motifs of ECM proteins may provide the cells with survival-promoting or behaviour-altering binding sites in their surroundings [151, 152]. The availability of a variety of matrix polymers and the rise of 3D printing has translated into further advances in 3D tissue and tumour models. 3D printers using cell-laden bio-ink can generate tumour models with more structure (or structure on a larger scale) than is easily available in other models [153]. Specific cancers may call for highly tissue-specific scaffolds such as ossified structures in which to grow cells originating from cancers of the bone marrow [154].

In order to further introduce patient-mimicking complexity to 3D models, particularly those based on gels, microfluidic devices are sometimes employed to simulate blood flow, control cell cluster sizes in co-cultures, manage metabolite concentrations over time or form gradients [154-156]. These approaches are also used on non-cancer applications for primary cell expansion [157] and there may be lessons for cancer research to learn from these instances, and vice versa.

1.9.5 Spheroids

Use of spheroids for expansion of primary tissue is not common, but has been performed by multiple labs for a range of purposes. Spheroid models typically consist of an approximately spherical collection of cells. Those established using cells taken from tumours in patients or animal models may contain other cell types from the tumour in addition to the tumour cells themselves. Likely as a result of more realistic cell-cell contacts and molecule gradients, even those consisting solely of tumour cells originating from established cell lines more closely resemble patient tumours than cells lines grown in 2D for a range of parameters including cell morphology, proliferation, histology and the activation of important signalling pathways [158-160], underlining the value of accounting for three dimensional factors in models. Key benefits of spheroids are their relative simplicity, enabling usage for wide applications and their resemblance in shape to many solid tumours, replicating the gradient of solute concentrations found in tumours.

Recently, Zhang *et al* were able to expand primary non-small cell lung cancer cells in a spheroid format for over 120 days [161]. With weekly passaging at a ratio of 1:2 this is a fairly slow expansion rate, which might limit its use for patient-specific testing, but not dramatically slower than some primary cells when grown in 2D. Importantly, the resulting spheroids after prolonged expansion still histologically resembled the patient tissues from which they originated.

Other research has focused on Cancer Stem Cells or Cancer Initiating Cells (CSCs or CICs) when expanding primary cultures in spheroid formats. A 2010 paper found that CD133+ (but not CD133-) fractions of patient colon cancer-derived cells, presumed to include CSCs, were able to retain spheroid-forming capacity of primary cultures to

allow expansion for 8 weeks [162]. Another group found in 2012 that while metastatic prostate tumour tissues could initiate prostatosphere formation in primary culture, 3D passage of localised prostate cancer in this manner was not possible over prolonged periods despite the apparent presence of cancer initiating cells [163]. This highlights a potential concern of using spheroids for passage if the intent is to reflect the patient tumour that may explain why it is a somewhat unusual approach; there is a clear selective pressure which limits which fractions of which tumours can be expanded. It is therefore only suitable as a passage method if the CSCs or CICs are the sole cell types of interest and the contributions of other tumour cell populations are to be discarded.

1.10 Hypothesis and Aims

This project tested the hypothesis that the FEFEFKFK peptide gel can be used to create a breast cancer model capable of replacing *in vivo* models for PDX expansion.

The FEFEFKFK peptide gel is already being used modelling of both cancer and healthy tissues; the gel's alternating polar (E, K) and non-polar (F) amino acids allow it to self-assemble [164] without processes such as ultraviolet light or addition of a second chemical which can be limiting when working with live cells. This peptide gel will be the supportive element of the model produced in this project, allowing excellent independent control over stiffness and composition [95] within relevant ranges to generate a tissue-relevant human-relevant model to expand patient-relevant PDX-derived cells.

In order to assess growth of differing breast cancer subtypes and identify any differences between PDXs established by different labs, several breast cancer PDXs were used within the project, including both luminal and triple negative tumours from two different labs.

As part of the overall testing of this hypothesis, the project worked towards the following aims:

- Establishing the basics of model elements (discussed in Chapter 3):
 - Exploring co-culture effects between Mesenchymal Stem Cells and cancer cells in 3D
 - Establishing production of the peptide gel in a new lab
 - Growing breast cancer PDX-derived cells in 3D

- Establishing assays and tests to characterise cells within the model (discussed in Chapter 4):
 - Identifying suitable metabolite-based proliferation and live/dead assays for 3D formats
 - Testing an assay to assess species of cells growing in peptide gel
 - Identifying assays to assess intra-tumoural heterogeneity in PDX-derived samples
 - Investigating stromal cell types in the context of co-culture with cancer cells
- Combining elements to form peptide gel model (discussed in Chapter 5):
 - Growing a range of PDXs in peptide gel
 - Establishing passage technique for PDXs in peptide gel
 - Passaging cells from multiple PDXs in the peptide gel for prolonged periods
 - Exploring freeze-thaw potential for peptide-gel passaged PDX-derived cells
 - Exploring the possibility of creating an animal serum-free model

2 Methods

2.1 Cells

2.1.1 Cell Lines

Cell cultures were incubated at 37°C, 5% CO₂.

MCF7 Fluc (constitutively expressing firefly luciferase) and MDA-MB-231 Fluc cells were cultured in phenol red free RPMI (Sigma #R7509) +10% FCS (Sigma, #F7524) + 1% L-glutamine (Sigma, #G7513). At passage the cells were washed with Hanks' Balanced Salt Solution (Sigma, #H8264) and lifted with trypsin (Sigma, #T3924).

Human bone marrow Mesenchymal stem cells (MSCS) constitutively expressing eGFP were cultured in MSC medium +5% FBS + MSC Growth Supplement (ScienCell, #7501, antibiotics omitted) and always used at passages lower than 14. At passage these cells were washed with Hanks' Balanced Salt Solution (Sigma, #H8264) and lifted with TrypLE™ Express Enzyme (Thermofisher, #12605028).

TERT-immortalised human bone-marrow MSCs (kindly provided by James Dixon, University of Nottingham School of Pharmacy) were grown in Dulbecco's Modified Eagle Media (Sigma D5671) 10% FCS (Sigma, #F7524) + 1% L-glutamine (Sigma, #G7513).

No antibiotics were used and (except where separately specified for primary cell lines) the cells were always used at passages lower than 50.

2.1.2 Patient-Derived Xenograft tissue

The following PDXs were used in this project:

*Table 2-1: **PDXs used in the project** ER=oestrogen receptor PR=progesterone receptor HER2=human EGF receptor 2 LN= Lymph node metastasis status. Mouse strain describes the strain used in Nottingham.*

PDX	Group	ER	PR	HER2	LN	Mouse Strain
BR8	A.M. Grabowska	-	-	-	+	Rag
BR15	A.M. Grabowska	+	-	+	+	Rag
BB6RC160*	R. Clarke	+	-	+	?	NSG
BB6RC87*	R. Clarke	+	-	+	+	NSG
BB6RC84*	R. Clarke	-	-	-	?	Rag

*[165], Data and metadata on PDX models are available in PDX Finder (<https://doi.org/10.1093/nar/gky984>, pdxfinder.org) and in the EurOPDX data portal (<http://dataportal.europdx.eu>)

Three PDXs derived by Rob Clarke's group at Manchester Cancer Research Centre were used in this project; BB6RC87, BB6RC160 and BB6RC84. These PDXs were derived by subcutaneous implantation of hormone receptor positive early breast cancer resection tissue (BB6RC87 and BB6RC160) or triple-negative metastatic breast cancer cells from pleural effusion (BB6RC84) into NSG mice following at most one freeze/thaw cycle. Matrigel was included with BB6RC84 on implantation, and BB6RC87- and BB6RC160-bearing mice were supplemented with oestrogen in their water in Manchester. BR-series PDXs were derived by implantation of tumour tissue fragments into the mammary fat pad of Rag2G mice along with MSCs.

Growth of PDXs Before *in vitro* work

Female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice (10-14wks), purchased from Charles River UK and allowed to acclimatise for a week prior to use, and female Rag2G (Rag2^{-/-} γ c^{-/-}) mice (8-10wks) bred in-house under PPL P375A76F, both immunodeficient strains, were used in this project. All mice used were of a known SPF (Specific Pathogen Free) health status tested following FELASA testing recommendations. Mice were maintained in Individually Ventilated Cages (IVCs) (Tecniplast UK) within a barriered unit illuminated by fluorescent lights set to give a 12 hour light-dark cycle (on 07.00, off 19.00), as recommended in the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. The room was air-conditioned by a system designed to maintain an air temperature range of $21 \pm 2^{\circ}\text{C}$ and a humidity of $55\% + 10\%$. Mice were housed in social groups during the procedure and provided with irradiated bedding and autoclaved nesting materials and environmental enrichment (Datesand, UK). Sterile irradiated 5V5R rodent diet (IPS Ltd, UK) and irradiated water (SLS, UK) was offered ad libitum. Tissue was generated by serial passage of tumour fragments using an implant trochar (VetTech Ltd. UK) into the mammary fat pad of the mice (BR8, BR15, BB6RC84) or subcutaneously (BB6RC160, BB6RC87) with MatrigelTM, by licenced competent *in vivo* technicians, under project licence PPL 3003444. Tumours were measured weekly using Vernier calipers and the volumes calculated using the formula $V = ab^2/6$, where a is the length and b is the

width. Mice were also weighed weekly and given a daily health check by an experienced technician. NCRI guidelines for the welfare and use of animals in cancer research, LASA good practice guidelines and FELASA working group on pain and distress guidelines were also followed, as were the ARRIVE guidelines on the reporting of *in vivo* experiments.

Disaggregation of PDXs

Samples were received minced in 12ml collagenase/dispase solution (collagenase type II (Invitrogen 17101-015) 100U/ml and dispase (Invitrogen 17105-041) 2.4U/ml in HBSS). After 80 minutes incubation at 37°C with rotation, undigested material was separated from cell suspension; 6ml RPMI medium was added and the sample washed through a 70µm nylon mesh. 6ml further RPMI was used to rinse any further loose cells through the nylon mesh, then the sample centrifuged again, with the supernatant discarded and the cell pellet re-suspended in 2 to 5ml RPMI.

2.2 General Growth/Survival Assays

2.2.1 3D Tumour Growth Assay

BME (Cultrex BME1 PathClear (amsbio #3432)) was defrosted overnight on ice at 4°C. Stripettes, multichannel pipette reservoirs and racked, sealed pipette tips were chilled in a freezer. BME was diluted on ice to twice the intended final concentration (standard 3mg/ml final) using chilled 3D-cTGA medium (phenol red free, serum free medium plus 1% L-glutamine.) directly from fridge and a chilled stripette. A plate heater was warmed to 37°C in or out of the tissue culture hood. Cells were harvested and counted, aliquotted into Eppendorfs according to seeding condition numbers and pelleted by centrifuging for 5 minutes at 3000rpm. On ice, cells were suspended in chilled 3D-cTGA medium (50µl per well plus 25-50% extra) and an equal volume of BME was added using chilled pipette tips, pipetting to mix avoiding bubbles. Either on ice or with the plate on 37°C heater in hood, 100µl of the mixture was added per well (leaving edge wells) using chilled pipette tips (96-well plate by Brand, #781671). When seeding on ice the plate was immediately moved to the 37° heater once filled, and completed plates were left on the heater for 10 minutes before transferring to an incubator. After 1-2 hours 200µl 3D-cTGA medium was added to each edge well. After 5-6 hours or the next morning 50µl 3D-cTGA medium was added carefully to each used well.

2.2.2 Growth Assays and Measurements

2.2.2.1 *Growth Assays in the 3D TGA Model*

All luciferase (approximation of proliferation of Fluc cell lines), AlamarBlue (approximate total proliferation) and eGFP readings were obtained using FluoStar Omega plate reader (excitation 485nm, emission 520nm and gain 1000). For luciferase assays 225µg (15µl of 15mg/ml aliquot) of D luciferin (PerkinElmer, #122799) was added, incubated at 37°C, 5% CO₂ and read after 15 minutes. For AlamarBlue assays reagent (Invitrogen, #DAL1100) was diluted 1:10 in 3D-cTGA medium, added at 100µl per well, incubated at 37°C, 5% CO₂ and read after 60 minutes using excitation wavelength: 544nm, emission wavelength: 590nm and gain 750. For Green Fluorescent Protein detection excitation wavelength used was 485nm and emission wavelength used was 520nm. Assays at different timepoints were performed using different wells.

2.2.2.2 *Growth Assays in the Peptide Gel model*

All readings were obtained using FluoStar Omega plate reader with two additional spacers relative to standard well plates to allow for additional height due to inserts. As much medium as possible was removed from each assayed insert before adding reagents. Luciferase and AlamarBlue were added directly to the top of the insert at the same concentration as for the 3D TGA model. Prestobblue (Thermofisher, A13261) was added at 20µl per insert. Inserts could be removed from wells to measure cell proliferation in the insert only for indirect cultures – in all cases 1ml (phenol red free) medium was present in the outer well in which the insert was assayed. Incubation times used were between 15 and 90 minutes. Inserts to be assayed again at later timepoints were rinsed three times with HBSS, returned to their co-culture plate where relevant and refed.

2.2.3 Imaging

2.2.3.1 *Routine Imaging*

Images were taken using Nikon Eclipse widefield microscope. Where separate wells were seeded for separate timepoints, either all wells were imaged or all images were taken from final-timepoint wells.

2.2.3.2 Confocal Imaging

Confocal images were taken using a Leica TCS SPE laser scanning system, with samples remaining in culture plates (plastic-bottomed) during imaging to prevent potential damage in moving to glass surfaces.

2.2.4 Live/Dead Staining

Live/Dead staining was performed using Live/Dead kit from Fisher (L3224). Gels were washed twice with PBS, cut from their inserts then submerged in stain solution (2µl ethidium homodimer and 0.5µl calcein AM per 1ml PBS) for 15 minutes in darkness at 37°C before imaging using a widefield microscope.

The following fluorescence channels on the Nikon widefield microscope were used during this project:

DAPI: Excitation: 350/50nm, Emission: 460/50nm

eGFP Excitation: 425/40nm, Emission: 505/40nm

TdTomato Excitation 540/40nm, Emission: 600/50nm

2.2.5 Antibody staining

Antibody staining was performed with care not to damage to gels at all stages. 1ml PBS was shared between the outer well and insert and removed. 4% PFA in PBS was added to each outer well and insert (1ml total each) and the plate incubated for 1 hour at RT. The gels were then washed again with PBS and stored in the plate wrapped in Parafilm at 4°C until staining.

PBS was removed and gels were cut from their inserts and placed in the outer wells their inserts sat in; a scalpel was used to cut the membrane from the insert. The gel was pushed through from the bottom and scooped into the outer well using the scalpel.

Each gel was covered with 0.5ml blocking buffer (0.5% BSA in PBS + 0.1% triton) for 45 minutes. Blocking buffer was then removed. 200µl of pre-conjugated cytokeratin 18 antibody (Thermofisher 53-9815-82) (1:100 dilution) was added to each gel. 20µl of 10x phalloidin (Life Technologies R415) was also added to each gel. The gels were refrigerated overnight wrapped in Parafilm and aluminium foil.

The gels were washed twice with PBS then incubated for 1 hour in DAPI (Invitrogen D3571) then imaged in place in their wells given their fragility.

2.3 Peptide Hydrogel

2.3.1 Gel Production

For each gel, 12.5mg FEFEFKFK peptide powder was added to 800µl sterile water in a 15ml falcon tube, vortexed for 3 minutes and centrifuged at 1000rpm for 3 minutes before baking at 80-90°C (oven set at 82°C) for at least 2 hours. 60µl 0.5M NaOH was then stirred into the peptide solution, vortexed to mix and briefly centrifuged. NaOH addition was repeated in 10µl increments until a self-supporting, completely clear gel was formed. 100µl 10x PBS solution was added followed by vortexing and centrifugation as for NaOH, resulting in a volume of approximately 1ml. This gel was then baked overnight at 80-90°C. Further NaOH could be added afterwards if the gel appeared cloudy following the overnight bake. Gels were then stored at 4°C.

2.3.2 Seeding of Cells in Peptide Hydrogel

Cells were disaggregated or dissociated according to the source and type, and resuspended in 250µl medium. Gels were heated at 80-90°C for at least two hours before seeding to ensure proper mixing, with a 37°C incubation period immediately afterwards to ensure gel temperature would not damage cells. The 250µl cell suspension was mixed into the 1ml gel (bringing the concentration to the final 10mg/ml and cell number to 5×10^5 cells per ml) by pipetting with care not to introduce bubbles. 200µl of the resulting cell-in-gel mixture was then pipetted into a 24-well-plate transwell insert (Greiner Bio-One 662610) or (less often) a 96-well-plate well. Typically 3 or 4 wells could be seeded per gel. Seeded gels were incubated at 37°C for 10 minutes before addition of 1ml of medium to each outer well and sufficient medium to fill the insert. This media was then changed twice more in the following hour, once the following day, and every 2 to 7 days afterwards (typically 2-3 days unless cells were exceptionally slow-growing).

2.3.3 Passage (Optimised Technique)

Medium was removed from insert and outer well. 100µl of disaggregation fluid was mixed into each gel by pipetting. Plates were incubated for 20 minutes at 37°C, 5% CO₂, mixing thoroughly halfway by pipetting. Insert contents was added to 1ml medium. For 1:20 passage 250µl of this mixture was seeded into a new gel; for 1:4 passage the medium/disaggregation fluid was centrifuged off the pellet (5 minutes at 300g) and the pellet resuspended in 250µl to seed into a new gel.

2.4 Characterisation and Tests of Heterogeneity

2.4.1 Species Testing by qPCR

Species testing was performed in collaboration with Catherine Probert.

DNA was extracted using QIAmp DNA mini kit (QIAGEN, 51304) (with one duplicate BR8 P0 sample additionally extracted using GenElute™ genomic DNA miniprep kit (Sigma, G1N350-1KT) for comparison) from cell pellets of excess PDX cells at passage between gels or immediately after disaggregation from the PDX itself. Extracted DNA concentration was determined using nanodrop, and 50ng DNA per well was amplified using PowerUp™ SYBR™ Green Master Mix (ThermoFisher, A25742). In a total 20µl reaction, forward and reverse primers were included at 500nM each. Primers were as follows (differing bases bolded):

Human Forward: CTGATCATCCGCAAGCCTGTGAC**G**

Human Reverse: G**CC**GGAAGGGCAGGCACAT

Mouse Forward: CTGATCATCCGCAAGCCTGTGACT

Mouse Reverse: G**AC**GGAAGGGCAGGCATAT

These primers are based on those used in Ealba and Schneider [166].

Amplification was performed using ViiA 7 Real Time PCR System with the following cycle:

1. 95°C 3m
2. 95°C 10s
3. 60°C 30s
4. Repeat 2.-3. 39x more
5. 95°C 10s

Followed by a melt curve from 60°C through to 90°C.

2.4.2 High Resolution Melting

HRM was performed using the 'inner' primers described by Ebili *et al* [167] for PIK3CA exons 9 (referred to within this thesis as exon 10) and 20 (referred to within this thesis as exon 21), with the analysis stage performed as described by Fadhil *et al* [168].

The following reagent mixture was used for the initial HRM experiment:

Master Mix:

20µl 10x Buffer

20µl 400µM dNTPs

20µl EvaGreen

22.5µl 3mM MgCl

8µl 250nM forward primer

8µl 250nM reverse primer

81.5µl Water

Plus 1µl (20ng) template per well

The following reagent mixture was then used for exon 21:

100µl 2x fast cycling Master

10µl EvaGreen

8µl 250nM forward primer

8µl 250nM reverse primer

34µl water

Plus 20ng template per well

1. 95°C 5m
2. 95°C 10s
3. 55°C 10s
4. 68°C 10s
5. Back to step 2, 39x

2.4.3 Imaging Flow Cytometry

Imagestream X imaging flow cytometer was used to assess cell cycle, cell death, PDX disaggregation and human fraction of BR8 PDX. BR8 PDX was disaggregated as described above (2.1.2) and the resulting cell suspension separated into two equal-sized pellets.

One aliquot was resuspended in 50 µl PBA (1% BSA in PBS). 2 µl HLA antibody was added to stain all human cells. After 30 minutes 2 ml PBA was added and the mixture was centrifuged. The pellet was resuspended in 50 µl PBA and 2.5 µl DAPI dilution

(1mg DAPI in 50µl water and diluted 1:100 in PBS) was added. DAPI was observed using channel seven and PE/Cy7 (anti human HLA) using channel 6.

One aliquot was resuspended in 500 µl PBS then mixed with 4.5 ml 70% ethanol and stored at 4°C for 13 days. The 70% ethanol was centrifuged off and the pellet was washed in 5ml PBS. PBS was replaced with propidium iodide (PI) staining solution (0.1% Triton-X-100, 10 mg/ml PI, 100 µg/ml and DNase-free RNase) and incubated for 10 minutes at 37° C. Channel 4 was used to observe PI staining.

2.4.4 DNA Extraction

DNA extractions were performed using QIAmp DNA mini kit (QIAGEN) or GenElute genomic DNA miniprep (SigmaAldrich) DNA extraction kits, according to kit directions except that exact cell counts could not be established and so cell pellets were used as-is, typically originating from a single well.

2.4.5 RNA Extraction, Sequencing and Analysis

RNA extraction was performed by Catherine Probert using a trizol method:

Cells were mixed with 500µl Trizol, incubated at room temperature for 5 minutes then mixed with 100µl chloroform. Samples were vortexed for 15 seconds then incubated for 10 minutes at room temperature. Samples were centrifuged at 12.000g at 4C for 15 minutes and the top aqueous phase transferred to a new Eppendorf tube. 500uL/mL of Isopropanol was added and incubated at room temperature for 10 minutes. The samples were centrifuged at 12.000g at 4C for 10 minutes, the supernatant discarded and the pellets washed with 500µl ethanol before vortexing for 15 seconds. Samples were centrifuged again at 12.000g at 4C for 5 minutes, supernatant discarded and ethanol permitted to evaporate from the pellet at room temperature. Pellets were resuspended in 20µl RNase free water.

Data Analysis

Used featureCOUNTS with HG19 reference within Galaxy. Counts files from Galaxy were combined in Excel and given headers denoting experimental conditioned to generate a counts file for analysis in NetworkAnalyst (NA).

Within NA submit .txt generated from Counts file. Gene Expression Table (organism: human, data type: bulk RNA-seq data, ID type: Official Gene Symbol, Gene-level summarisation: Mean).

NA identifies 17381 total features of which 15292 (88%) are matched genes. Abundances less than 4 and variances less than 15% were filtered out, as well as unannotated genes. Normalisation was to Log2-counts per million. EdgeR was used where not otherwise specified, with Mesenchymal Cell Type treated as a primary factor, specifically comparing MSCs with TE67s. An adjusted P value of 0.05 and log2 fold change of 1.0 were used for significance thresholding.

A count heatmap for genes specifically identified by Friedman *et al* was generated using Microsoft Excel – the VLookup function was used to pick out the specific gene set from the total counts data. Conditional formatting was then applied on a gene-by-gene basis to highlight differences in expression of that gene regardless of its counts relative to other genes in the set. The file was then exported as a webpage/html file and re-opened within Excel to allow preservation formatting without cell contents.

2.4.6 Embedding, Sectioning and Histology

Using a histological embedding mould as an outer mould and clean transwell inserts as inner moulds well shapes were formed in 2% w/v agarose. A peptide gel (fixed for 1 hour in NBF) was transferred to each and a layer of the same agarose solution pipetted to cover the top. This block was allowed to set fully, fixed overnight in NBF then stored in 70% alcohol before embedding in paraffin. 4um sections of the resulting blocks could then be cut onto slides.

Slides were bathed 3x for 5m in 100% Xylene then 2x for 1m in 100% Methanol to dewax and rehydrate, then rinsed for 1m in water before incubating for 3m in haematoxylin according to Mayer. A further 1m rinse in water was performed before incubating for 3m in Eosin, followed by another 1m water rinse, then baths of 3x 1m in 100% Methanol then 2x 3m in 100% Xylene to dehydrate. Slides were coverslipped using DPX mountant and dried overnight before imaging.

3 Core Elements of an Ideal Model for Passage

The overall aim of the study was to develop a peptide-gel based 3D culture model to allow passage of PDX-derived cells in the place of existing *in vivo* models. These PDX-derived cells had previously been grown in a mouse tumour ECM, Cultrex, for short term assays incorporating fibroblasts which reflect the presence of cancer-associated fibroblasts in patient tumours. The peptide gel which forms the basis of the study's model has been previously used to grow cancer cell lines as well as non-cancer cell types, but had not previously been used in the EVPC or for PDX-derived cells. Before combining these features, the following elements of the model needed to be investigated:

- Whether the well-known growth-supporting effects of fibroblast-like cell types on cancer cells in co-culture in 2D translate consistently to an established 3D model
- How the PDX-derived cells used in this project grow in an established 3D model
- Optimisation of production of the peptide gel within a laboratory setting where it had not previously been used.

By characterising cell growth in the peptide gel, the PDX in 3D *in vitro* culture, and the effect of stromal cell support in 3D, basic conditions for the eventual model where these are combined could be developed.

3.1 The Effect of Co-culture with Stromal Cells on Cancer Cell Growth

Past work from many groups has shown that stromal cells such as mesenchymal cells and fibroblasts can affect the rate of tumour proliferation [48, 55]. Including such cells in a model for passage could therefore simultaneously improve its practical value (by increasing the rate at which cancer cells grow within it) and biological relevance (as patient tumours also include stromal cells of similar lineages which may affect survival of particular clones within the tumour during growth or treatment and therefore how challenging the tumour is to treat).

3.1.1 Confirmation of Growth-Promoting Effect from Co-culture in 2D Assays

While the proposed eventual model is to be in 3D, initial experiments assessed the monoculture and co-culture growth of these cells in 2D. This made it possible to establish baseline behaviours for the cells involved to compare with later experiments

in 3D, as well as providing a format to judge the effectiveness of metabolic assays and imaging strategies for the cell types involved, together or separately. Three cell types were used in this experiment:

- Bone marrow-derived Mesenchymal stem cells (MSCs) from which CAFs have been hypothesised to develop in the presence of tumour cells
- MCF7 fluc, a relatively non-invasive breast cancer cell line
- MDA-MB-231 fluc, an invasive, triple-negative breast cancer cell line

The two cancer cell lines were labelled with firefly luciferase ('fluc') to allow cell-type-specific proliferation monitoring. An experiment was performed to test the hypothesis that inclusion of MSCs in 2D culture of tumour cell lines MCF7 fluc or MDA-MB-231 fluc cells would increase proliferation of the tumour cells, and to determine whether altering the ratio of tumour to mesenchymal cells changes this effect. A range of techniques for detection of labelled or unlabelled cells were considered, with the co-culture format being a significant factor in deciding which to use:

- Constitutive fluorescence label expression, such as eGFP or tdTomato, can distinguish multiple cell types in co-culture by imaging and allows plate-read assays without added reagents. Dead cells will continue to fluoresce however, which is not ideal for proliferation detection.
- Constitutive expression of luciferase, which metabolises the luciferin reagent using metabolic products of living cells, effectively reflects cell metabolism. It is therefore not subject to uncertainty from cells which have recently died. Luciferase expression is suitable to detect proliferation in plate-read assays, and by labelling only one cell line can detect that cell line's growth independently of other cells grown in co-culture, but is not useful for labelling for fluorescent imaging as there is no fluorescence without reagent addition.
- AlamarBlue assay, and similar assays such as PrestoBlue, give a signal based on reduction of an added reagent without the need for an exogenous enzyme. They therefore do not require any labelling of cells and will not give high signals from dead cells, but cannot give separate information about multiple cell lines in a well, and instead effectively show total metabolic activity in the well.

Luciferase-expressing cancer cell lines were used to ensure readings were reflective of live cancer cells only, while eGFP-labelled MSCs allowed the cell types to be distinguished in microscopy.

Cell numbers were selected on the basis of a seeding density experiment (not shown) which showed that seeding 2k cells per well gave as much growth as much higher cell numbers (suggesting no anoikis at the lower 2k seeding density) and resulted in slightly sub-confluent wells after 6 days unlike higher cell densities. Previous work within the group suggested primary MSCs have very limited growth in co-culture and therefore at the end-point the vast majority of cells are from the cancer cell line. For this reason the number of cancer cells was kept consistent, rather than maintaining a consistent total starting cell number. Other authors have observed and used ratios of epithelial to mesenchymal cells on the same order of magnitude [169], and so these numbers prompted the selection of the ratios used in this experiment.

MCF7 Fluc and MDA-MB-231 Fluc breast cancer cell lines were seeded at 2k cancer cells per well mixed with 0, 2k or 4k eGFP-expressing MSCs (1:0, 1:1 or 1:2 tumour: MSC ratio) in clear-bottomed black-walled tissue culture treated 96 well plates. Fluc expression allows growth of cancer cells to be monitored while the MSCs' constitutive eGFP expression allows survival of the eGFP cells to be assessed independently of the cancer cells with which they were co-cultured – wild-type cells' growth could be assessed by AlamarBlue, but not independently for multiple cell types in co-culture. Four types of data were collected at 24, 96 and 144 hour time points – microscope images, luciferase (reflecting tumour cell count), AlamarBlue (reflecting total cell count) and eGFP fluorescence (reflecting MSC count). 6 wells were seeded per assay – 3 for Luciferase and 3 for AlamarBlue. A seventh well per condition was seeded to provide a reagent-free control during luciferase assays.

A luciferase assay was used to judge the proliferation of the tumour cell lines – in all cases co-culture produced an initial boost in proliferation which does not appear to be affected by whether 2k or 4k MSCs are used (Figure 3-1). At 144 hours it appeared that either the pro-proliferative effect of MSCs was lost or the cells had reached confluence or exhausted the medium of nutrients as at this time point the difference between the three ratios was minimal – microscope images show that all cultures are fairly confluent, with only the 1:0 MCF7 cultures having large areas of room available to grow into (Figure 3-2), so confluence and medium exhaustion are likely reasons.

AlamarBlue readings were in agreement with the luciferase readings, showing clearly greater proliferation in the first 96 hours for co-culture conditions than monoculture, followed by less conclusive differences at 144 hours. At 144 hours AlamarBlue readings show similar proliferation between the two co-culture ratios for each cell line, in each case higher than the equivalent monoculture (Figure 3-1). This is not surprising as AlamarBlue will also account for the metabolism of the additional cell type present in the co-cultures, and the overall shapes of the graphs suggest a plateau in proliferation towards the end of the experiment.

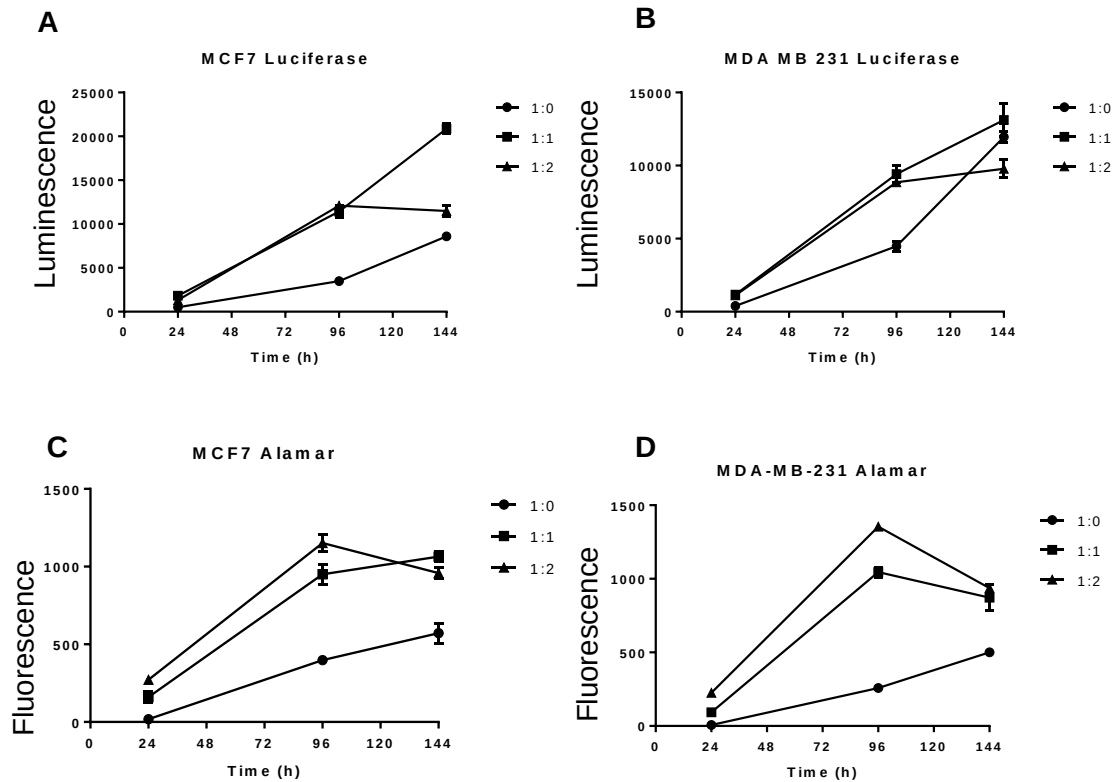


Figure 3-1: In 2D culture luciferase and AlamarBlue assays detect faster growth of cancer cell lines when co-cultured with MSCs Luciferase assay readings from MCF7 (A) and MDA-MB-231 (B) tumour cell lines grown in 2D in phenol red free RPMI, with differing ratios of tumour cell to MSC numbers. AlamarBlue readings from MCF7 (C) and MDA-MB-231 (D) tumour cell lines, grown under the same conditions. Error bars show standard deviation based on 3 technical replicates.

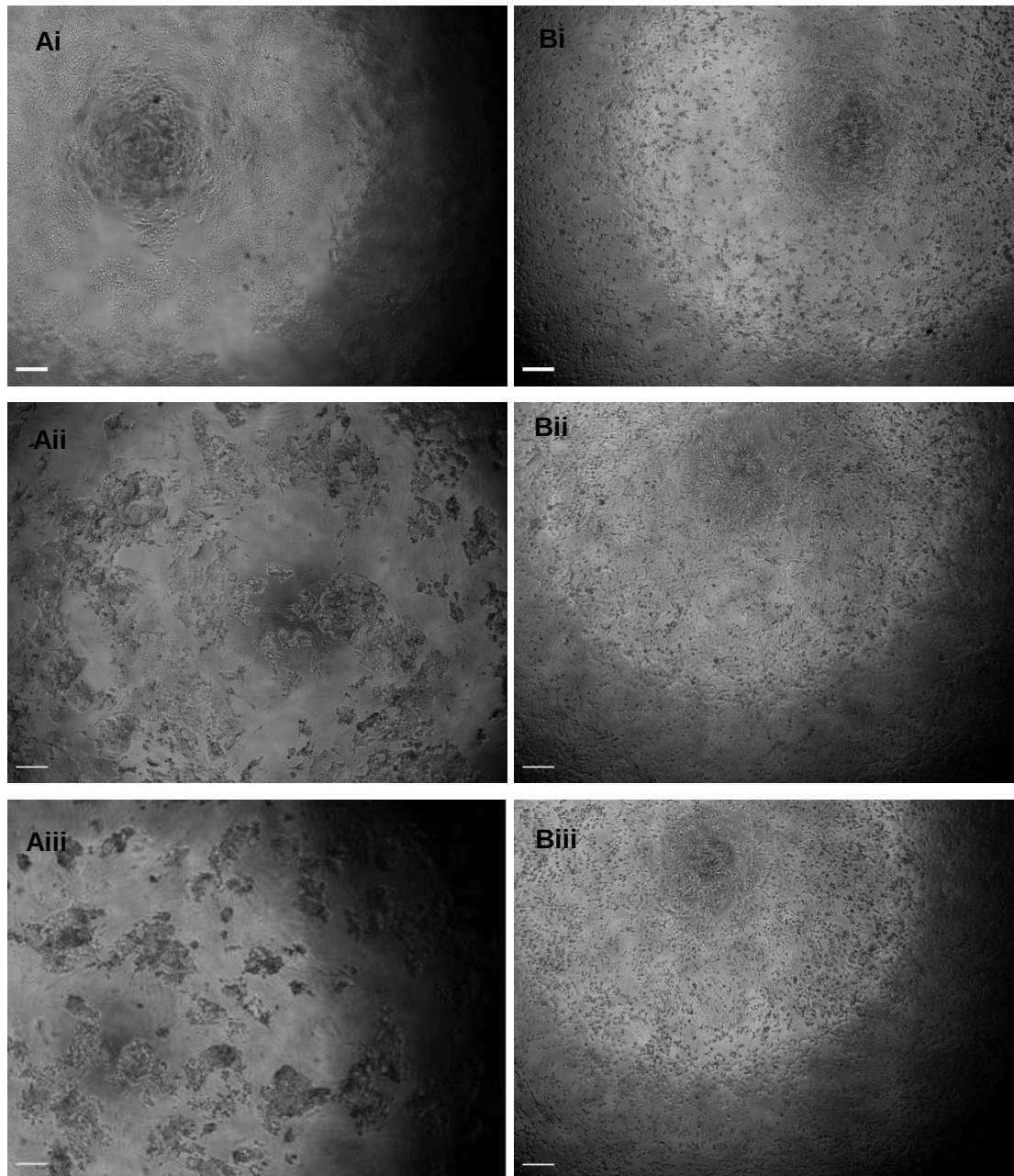


Figure 3-2: Effect of co-culture ratio on visual phenotype and growth of cancer cells differs between MDA-MB-231 and MCF7 cells Representative images from 6 replicate wells, taken at 4x using Nikon Eclipse widefield microscope. MCF7 (**A**) and MDA-MB-231 (**B**) in 3D-TE at 144 hours at 1:0 (**i**), 1:1 (**ii**) and 1:2 (**iii**) ratios tumour cells to MSCs. Scale bars are 200 μ m. Cells were grown in phenol red-free RPMI.

Consistent with previous observations from the group, MSC proliferation as determined by eGFP detection was minimal, with most co-cultures simply surviving and showing no meaningful change over the 144 hour period (Figure 3-3a). Only the

1:2 MCF7 co-culture's MSCs proliferated continually throughout the experiment. These readings combined with luciferase readings are in agreement with the general trends shown in the AlamarBlue readings. Microscope images are in agreement that, particularly after the first 96 hours during which some growth was detectable, differences between conditions were more significant than growth over time for most conditions (Figure 3-3Bi, ii and iii). Growth in the first 96 hours did, however, appear more significant in the microscope images than suggested by fluorescence readings.

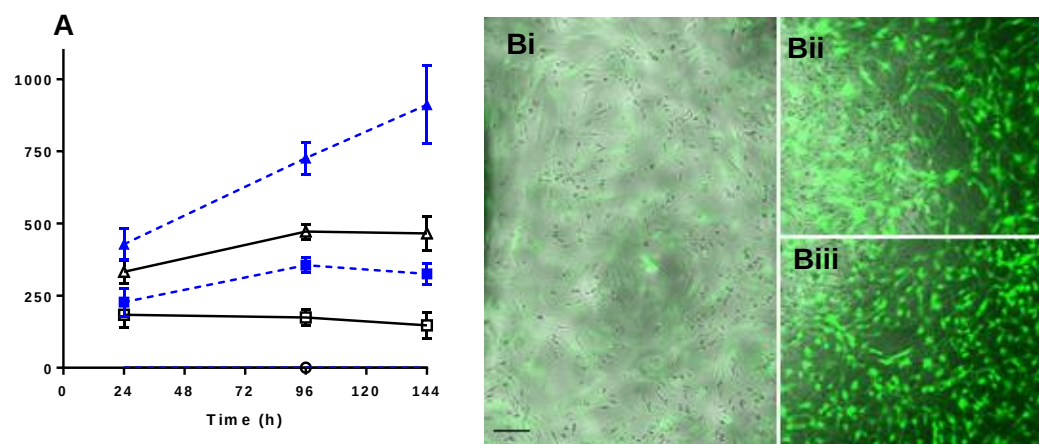


Figure 3-3: Fluorescence readings and microscope images both reflect differing MSC growth between co-culture conditions (A) eGFP fluorescence readings from co-cultures with MCF7 (dashed blue lines) or MDA-MB-231 (solid black lines) co-cultured with MSCs at 1:0, 1:1 or 1:2 ratio (circular, square and triangular point respectively) in phenol red free RPMI, calculated using values from 1:0 tumour cell:MSC wells as zero values. Error bars show standard deviation, based on seven technical replicates. **(B)** Representative images from 7 replicates; MDA-MB-231 cells grown in 1:2 ratio with MSCs at 24 **(i)**, 96 **(ii)** and 144 **(iii)** hours, reflecting initial growth then plateau as observable from fluorescence readings in **(A)**. Scale bar is 200µm.

From this experiment it can be concluded that at least on the scale of 96 hours in this arrangement, and likely in the longer term if, as suspected, competition between cells for space and nutrients in the limited size of the well was responsible for the levelling-off of proliferation at 144 hours, inclusion of MSCs in co-culture with both MCF7s and MDA-MB-231s increases proliferation. At the tested ratios of 1:1 and 1:2 there did not seem to be a significant difference resulting from adding differing amounts of MSCs in the initial conditions. Confirming that this effect persists in three dimensions is an important next step.

3.1.2 Initial Optimisation of 3D Tumour Expansion Format

A Cultrex-based assay was initially used for 3D experiments; this assay is an established 3D model for drug screening of non-breast-cancer PDX-derived cells [97, 170]. It was therefore chosen as a setting to probe MSC co-culture effects in a format

that can be directly related to existing literature before moving into the peptide gel, but required some optimisation to ensure reliable behaviour of the cells involved and compatibility of their specific growth media.

In this assay cells are grown in murine basement membrane extract, allowing assays of growth or survival (for example by AlamarBlue or luciferin) in a 3D setting. Typically this would be referred to as '3D-TGA' (3D tumour growth assay); given that many assays of tumour cell growth in 3D settings will be discussed here, and that the actual goal within this project is expansion rather than a growth assay '3D-TE' (3D tumour expansion) will be used to specifically refer to this Cultrex-based model, as opposed to the peptide gel model used more often in the project, or other 3D settings in which tumour cell growth can be assayed.

Prior to testing co-culture effects in the 3D-TE it was necessary to optimise seeding densities and protocol technique details for 3D Tumour Growth Assays with MCF7 and MDA-MB-231 cells, two cell types for which the assay had not frequently been used. In the 3D-TE format cells are suspended in a gel consisting of basement membrane extract from a murine tumour. This extract is very different to 2D assays because of the 3D arrangement, because it includes a large number of extracellular molecules which may signal to the cells, and because it must be seeded as an ice-cold liquid before warming to gel at 37°C. This format, despite more closely resembling a tumour's environment than 2D assays, has much less commonly been used with the MCF7 and MDA-MB-231 cell lines – first it was necessary to optimise cell numbers and seeding technique for these monocultures.

Three optimisation experiments were performed.

1. **Optimisation for ideal starting cell number** Initially MDA-MB-231 Fluc and MCF7 Fluc cells were seeded at 0, 15k, 20k and 25k cells per well; too low a number might induce anoikis as cells are much more sparsely distributed in 3D, while too high a number might result in available nutrients being depleted before the end of the experiment, as the 3D-TE does not allow media to be changed, only added. This seeding was performed according to the 3D-TE protocol with the exception that each plate was heated for 2 minutes after each new seeding condition was added to it, rather than for 10 minutes once fully seeded. Four replicate wells were seeded for each timepoint (24h, 72h, and

144h), for both AlamarBlue and luciferase assays for each cell density, and six cell-free wells were seeded per timepoint per assay.

In this initial experiment AlamarBlue and luciferase assay results suggested that within the range tested (15k to 25k cell per well) the cell count for MCF7s made no significant difference to proliferation. For MDA-MB-231s seeding with 15k cells resulted in significantly lower proliferation throughout the experiment than seeding with 20k or 25k, though at any density MDA-MB-231 proliferation was much lower than MCF7 proliferation. While the metabolic assays used indicated normal growth, the majority of cells sank to the bottom of the well as determined by microscopy.

2. **Optimisation of seeding technique to avoid sinking of cells** As the intent of the previous experiment was to perform a growth assay in a 3D model, it was a considerable concern that microscopy revealed the majority of cells to in fact be in 2D; while they were in contact with the BME on all but the bottom surface, they were nonetheless forming the monolayer arrangement which 3D culture aims to avoid. This was likely a result of the way the cells were seeded; each condition was seeded in several separate plates so that all assays could be performed in one plate per day. All three plates were therefore seeded simultaneously rather than finishing and heating the first before moving onto the next, and the BME was therefore not heated to prevent cells from sinking until many already had. In a further experiment to identify how to correct this sinking, five replicate wells each of no cells or 25k MCF7 Fluc cells were seeded per time point (24h, 72h, and 144h) for both AlamarBlue and luciferase assays according to the 3D-TE protocol, seeding on ice and rapidly transferring the plate to the plate heater after seeding for ten minutes. The results of this experiment confirmed that suspension was much improved when the plate was seeded all at once and immediately placed on the heating block for the full ten minutes (results not shown).
3. **Optimisation for ideal starting cell number without sinking** In a final optimisation experiment MDA-MB-231 Fluc and MCF7 Fluc cells were seeded at 0, 20k, 25k and 30k cells per well, according to the 3D-TE protocol, with the plates seeded while directly on the plate heater. Five replicates were seeded for every experimental time point.

AlamarBlue and luciferase assays suggested that within the cell density range tested (20k to 30k cells per well) there was limited difference in proliferation effects (Figure 3-4). MCF7s appear to proliferate more around the 72 hour time point, whereas MDA-MB-231s do not appear to proliferate before 96 hours if at all; however, it is possible that the actual growth patterns are similar as MCF7s were observed at 72 hours but not 96 hours and MDA-MB-231s were observed at 96 but not 72 hours. Suspension of cells in this experiment was much improved (Figure 3-5) particularly in the case of MCF7 cells.

Following this experiment AlamarBlue assay was not used to measure growth in direct co-culture, as Luciferase and eGFP readings were more informative due to their ability to distinguish cell types and overall in agreement.

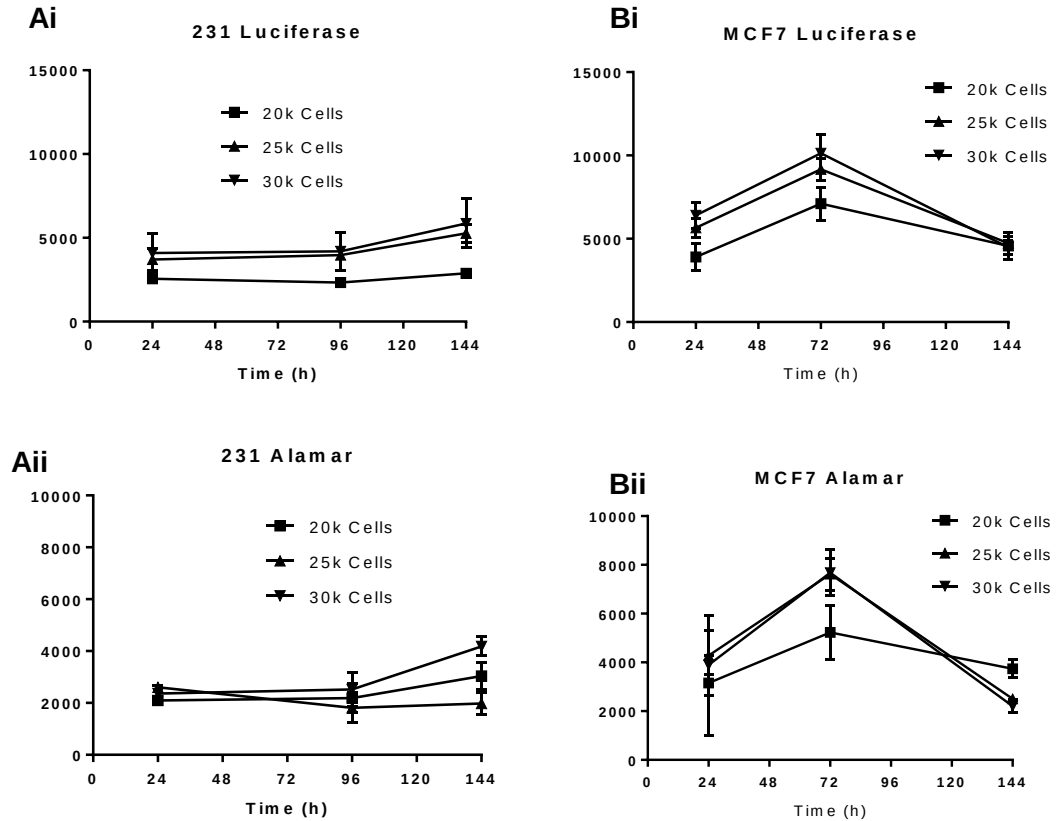


Figure 3-4: MDA-MB-231 and MCF7 show different growth patterns in optimised 3D-cTGA, while initial seeding number has a limited effect. Luciferase (i) and AlamarBlue (ii) assay results from both MDA-MB-231 (A) and MCF7 (B) cell lines in the final 3D-TE monoculture optimisation experiment. Error bars show standard deviation based on 5 technical replicates. Y-axes have been matched by assay to illustrate differing proliferation between cell lines.

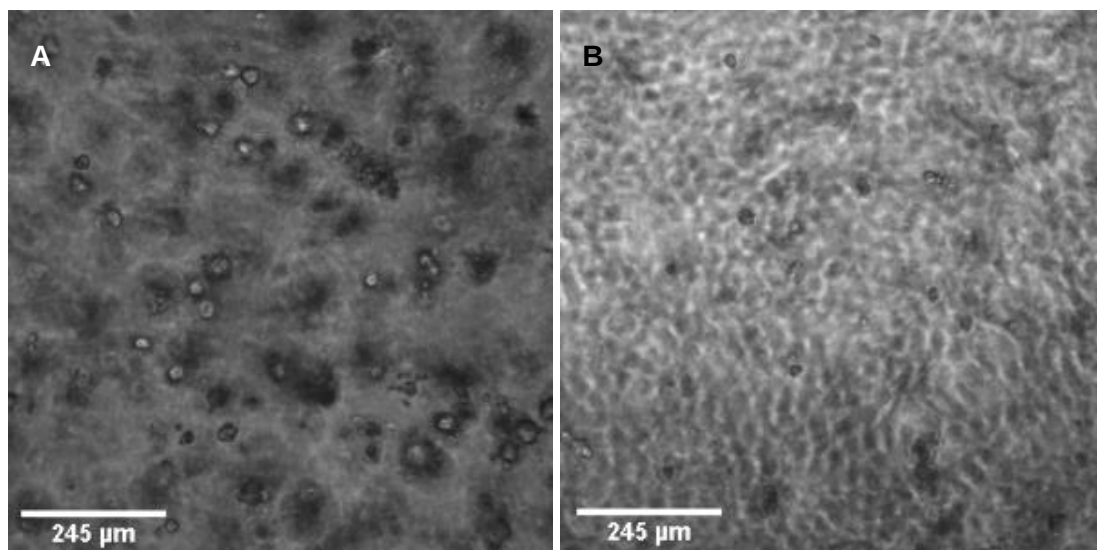


Figure 3-5: Rapidly warming 3D-cTGA plate to 37°C after seeding improves suspension of cells
 Representative images from 5 replicates taken at 4x using Nikon Eclipse widefield microscope of 3D-TE assays above the bottom of the well at 144 hours in wells containing 25k MCF7 (**A**) or MDA-MB-231 (**B**) cells. In both cases some cells can be seen to be approximately in focus while the cells which appear as dark shadows are suspended at different heights out of focus or on the bottom of the well.

As growth seemed similar at the four seeding densities observed throughout this series of experiments, the 25k per well density suggested in the protocol based on experience with other cell lines and types was selected to be used for further experiments where no specific changes to allow for the nature of the experiment were required.

3.1.3 Co-culture with Stromal Cells in the 3D-TE Model

Based on Sasser *et al's* work [171] and results in 2D assays it was hypothesised that inclusion of MSCs would increase the growth rate of MCF7 cells in a 3D-TE, and stimulate growth of the MDA-MB-231 cells which does not proliferate in monoculture under such conditions.

It was necessary to ensure that the 3D nature of the peptide gel model would not inherently prevent this effect from occurring regardless of the other features specific to the peptide gel, such as the absence of both murine and human matrix components and signalling elements or the range of stiffnesses that can be achieved, any of which might positively or negatively affect cancer cell growth with or without stromal cells. Additionally, while the specific MSCs planned for use in this project are of the same

type and origin previously used in some previous work demonstrating this effect, there is value in establishing consistency between cells grown in different laboratories.

Sasser *et al* have previously demonstrated a supporting effect from MSC co-culture in the 3D-TE model for some cell lines [171], however a key difference in the following experiment is that the following experiment uses metabolite-based assays, ensuring that readouts are a product of current cellular activity and will not be contributed to by non-metabolising cells such as those which have recently died.

A 3D-TE was performed in using MDA-MB-231 Fluc and MCF7 Fluc cell lines seeded at 25k cells per well, with or without MSCs seeded at 12.5k cells per well in 3mg/ml Cultrex BME, with 5 technical replicates per condition. Luciferase assay was used to assay tumour cell line growth, and eGFP fluorescence to monitor MSC growth.

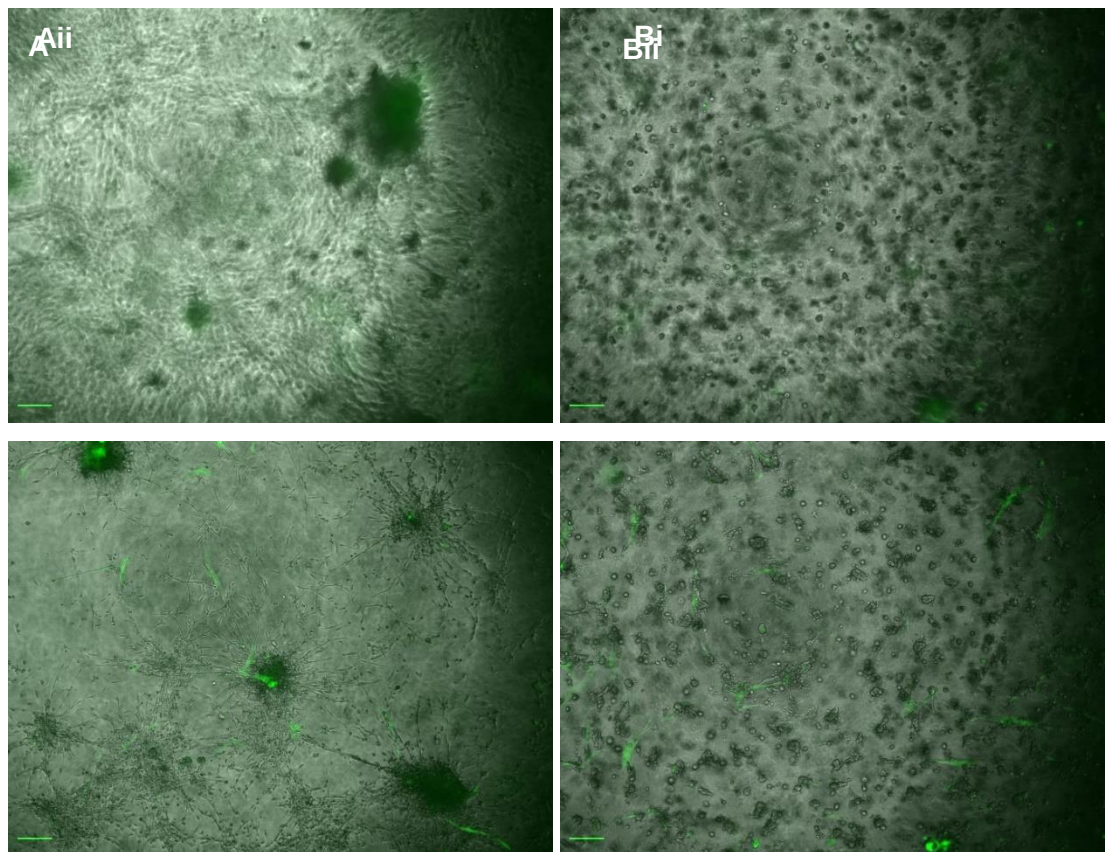


Figure 3-6: MCF7 and particularly MDA-MB-231 cancer cells lines appear to sink to the bottom of the well in 3mg/ml 3D-TE Representative images from 5 technical replicates taken at 4x using Nikon Eclipse widefield microscope. MDA-MB-231 (**A**) and MCF7 (**B**) cells in 3D-TE at the well bottom (**ii**) or further up in matrix (**i**) at 144h. Green cells are eGFP-labelled MSCs. Scale bars are 200µm

Each tumour cell line was grown in monoculture and in co-culture with MSCs. Additionally, a set of wells without cells (but with BME) was assayed for comparison, to confirm that what the plate reader detects originates from the growth of cells rather than the BME or plate plastic.

Microscope images confirm the presence of MSCs in +MSC wells. However, particularly in the case of MDA-MB-231 cells, many of the tumour cells sank to the bottom of the well rather than remaining suspended in the BME matrix (Figure 3-6). The MDA-MB-231s seemed to grow relatively well at the bottom whereas MCF7 cells looked considerably less healthy on the bottom of the well – given that the wells are non-treated it is the response of the MDA-MB-231s which is more surprising. This sinking affect limits the extent to which any other results can be interpreted.

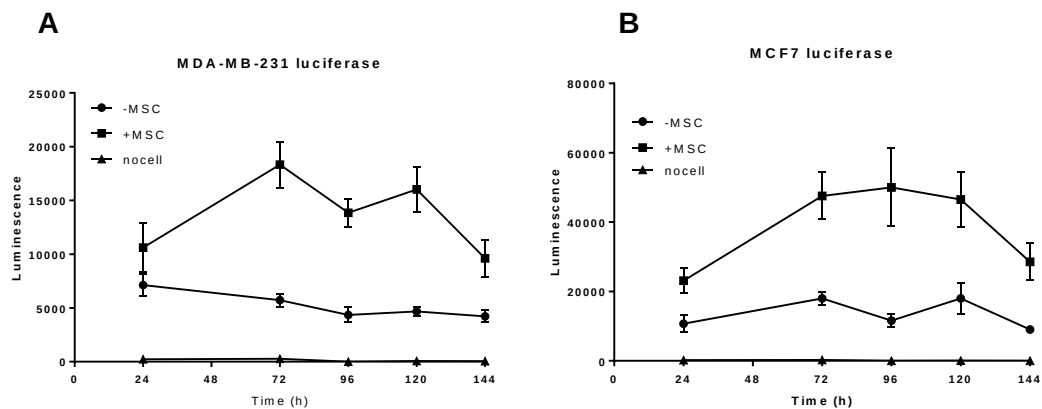


Figure 3-7: Co-culture with MSCs in 3D-TE increases growth of cancer cell lines Growth of (A) MDA-MB-231 and (B) MCF7 cell lines with versus without MSC co-culture. Error bars show standard deviation based on 5 technical replicates.

Growth was, however, consistent with previous experiments in that MDA-MB-231 cells failed to proliferate in monoculture in the 3D-TE and MCF7 growth was observed though just barely greater than standard deviation (Figure 3-7). For both cell lines, when co-cultured with MSCs, there were significantly increased luminescence readings in the first 72 hours compared with monoculture. This indicates a positive effect of the MSCs upon tumour cell line proliferation. After 72 hours luminescence readings reached a plateau for both cell lines and then particularly in the case of MDA-MB-231 had dropped again by 144 hours. This may be due to nutrient depletion or approaching confluence limiting further cell growth; although the luciferase readings

do not reflect MSC numbers, MSCs will nonetheless contribute to these potential limiting factors.

These results suggest a proliferation-enhancing effect on both the highly invasive MDA-MB-231 cell lines and the less invasive MCF7 cell lines by MSCs. Future work must establish whether eGFP readings can be used to enable tracking of both cell types in co-culture to give a fuller picture. Additionally, optimisation of BME concentration may reduce the number of cells which sink to the bottom and are subsequently not wholly in a 3D environment. While the majority of conclusions drawn from this experiment are consistent with Sasser *et al*'s work, here it was found that MSCs increase MDA-MB-231 proliferation, while Sasser *et al* found no such increase. More replicates would be needed to confirm the results presented here, but this difference may be real, due to biological factors such as batch variation in basement membrane extract, differing behaviour in true 3D versus mostly-sunk cells, or differences between the MSCs used in the two groups.

To establish whether the second of these possibilities is the case, the next step was to repeat this experiment using a higher concentration of BME to identify whether this reduces the number of cells sinking to the bottom of each well.

3.1.4 Further Optimisation of 3D-TE to Improve Cell Suspension

It was hypothesised that the normal basement membrane extract (BME) concentration of 3mg/ml used in the 3D-TE allowed MDA-MB-231 cells and to a lesser extent MCF7 cells to drop to the bottom of the well, and that a higher BME concentration of 6mg/ml might retain the cells better in 3D. As this series of experiments served primarily to bridge between probing the effects of co-culture in 2D and in the peptide gel model by providing an established 3D model, retention of cells in 3D was vital.

A 3D-TE was performed using MDA-MB-231 Fluc and MCF7 Fluc seeded at 25k cells per well in 3mg/ml or 6mg/ml Cultrex BME. Luciferase assay was used to assay tumour cell line growth. Every well was photographed at the bottom of the well at each timepoint and wells containing cells were additionally photographed at another vertical coordinate within the matrix – where possible the horizontal coordinates of the images for each well were the same. An eGFP plate read prior to luciferin addition confirmed that the 3D-TE assay itself has only a small background fluorescence in eGFP's wavelengths, suggesting it was the luciferin interfering with these results in past experiments.

Each cell line was grown in 6mg/ml BME and in 3mg/ml BME with five technical replicates per condition. Additional wells of each BME concentration were seeded without cells as controls.

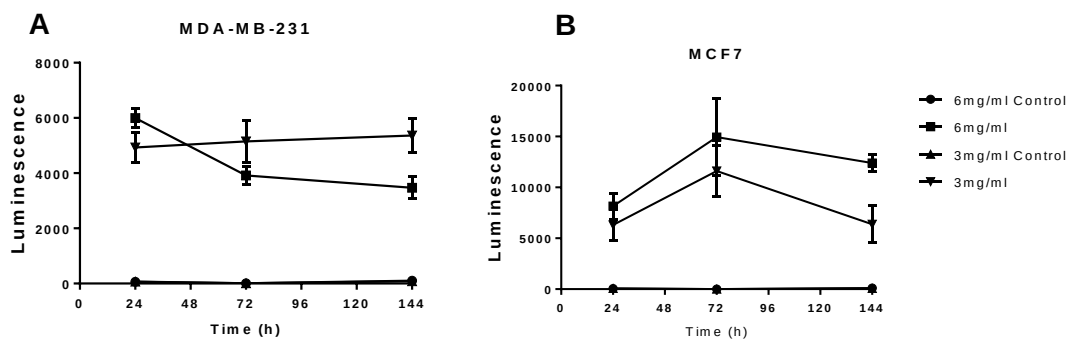


Figure 3-8: Altering BME concentration in 3D-TE minimally affects growth pattern of cancer cells in monoculture Growth of MCF7 (A) and MDA-MB-231 (B) cells in different BME concentrations in 3D-TE as measured by Luciferase assay. Error bars show standard deviation based on 5 technical replicates.

There was little difference between the two BME concentrations in terms of growth as determined by luciferase assay – MCF7s appeared to grow slightly faster in the higher concentration while MDA-MB-231s failed to proliferate in either concentration, as is typically observed (Figure 3-8). Possibly, MDA-MB-231s proliferated less in the higher BME concentration, which may be a result of their apparent preference for proliferating on the bottom of the well.

The increased BME concentration did appear to both reduce the number of MDA-MB-231 cells sinking to the bottom of the well and increase their numbers in the matrix (Figure 3-9).

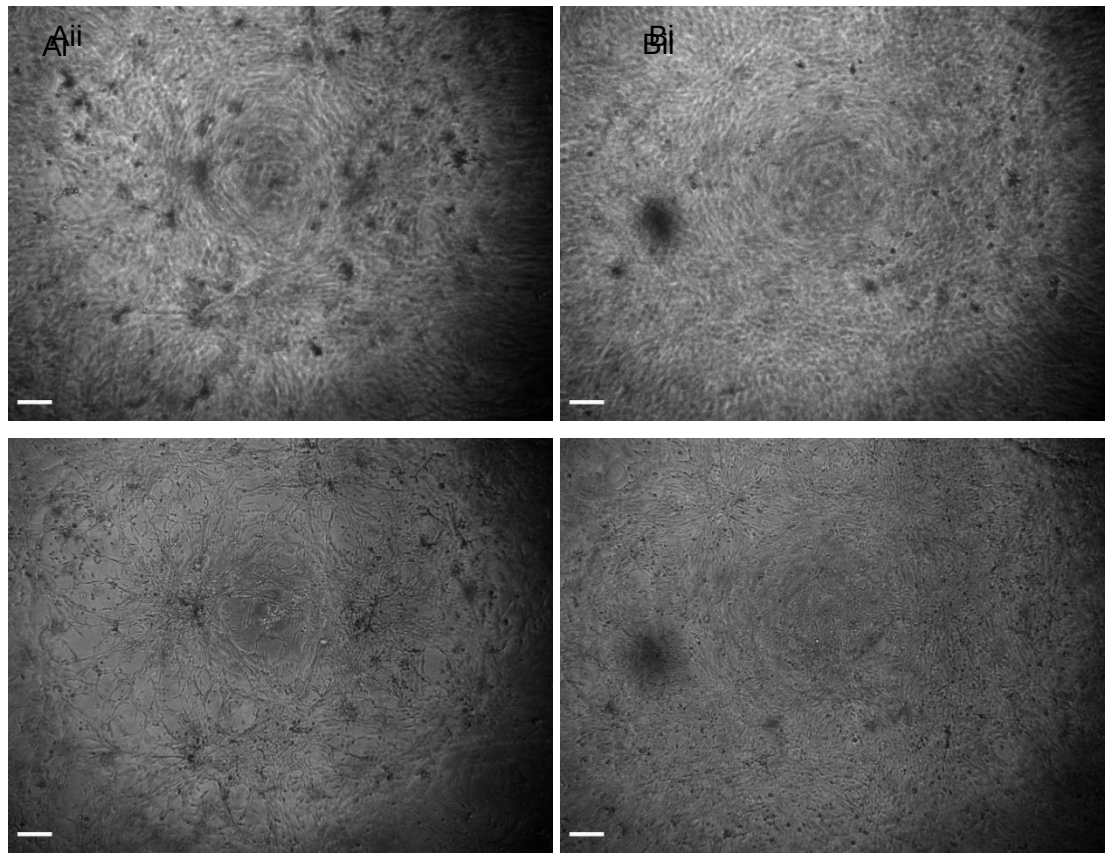


Figure 3-9: MDA-MB-231 cells are better suspended in 3D in 6mg/ml than 3mg/ml BME
Representative images from 5 replicates taken at 4x using Nikon Eclipse widefield microscope. MDA-MB-231 cells growing in 6mg/ml **(A)** and 3 mg/ml **(B)** BME 3D-TE after 144 hours. **(i)** shows cells suspended in the BME while **(ii)** shows the cells which sank to and grew on the bottoms of the same wells at the same time. Scale bars are 200 μ m

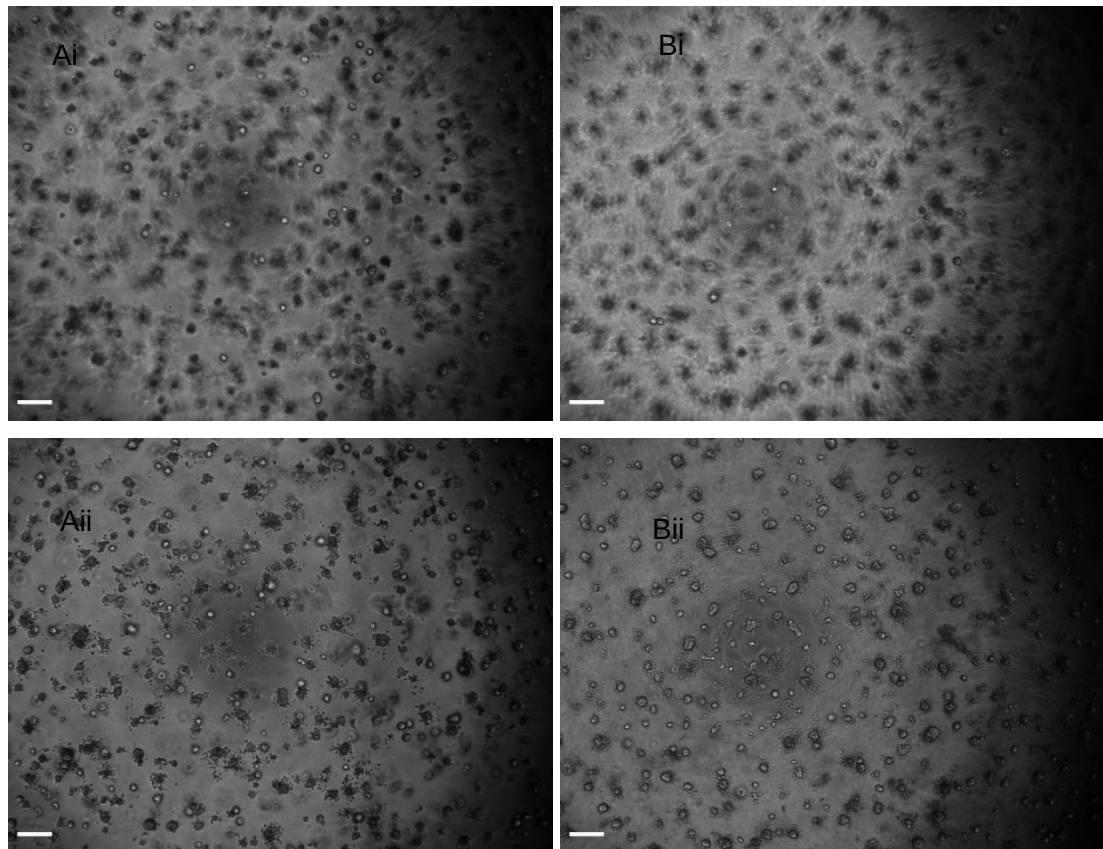


*Figure 3-10: **MCF7 cells are better suspended in 3D in 6mg/ml than 3mg/ml BME** Representative images from 5 replicates taken at 4x using Nikon Eclipse widefield microscope. MCF7 cells growing in 6mg/ml **(A)** and 3 mg/ml **(B)** 3D-TE in suspension **(i)** or sunk to the bottom **(ii)** after 144 hours. Scale bars are 200 μ m.*

A similar effect is seen with the MCF7s (Figure 3-10) – the differing growth assay patterns may be a result of MCF7 cells growing better when properly suspended in the BME whereas MDA-MB-231s appear to proliferate better on the bottom of the well despite the well's lack of tissue-culture coating. Unexpectedly, while there are clearly more MCF7 cells visible in the matrix at 6 mg/ml than at 3 mg/ml, the cells growing on the bottom of the well in 6 mg/ml conditions, appear to have an unusual morphology (likely a result of apoptotic cell death) compared with those at 3 mg/ml, although cell numbers are similar in the two conditions.

Increasing the BME concentration does generally improve suspension of the cell in the BME and reduce the numbers which fall through and grow on the bottom of the well.

3.1.5 Confirmation of Co-culture Effect on MDA-MB-231 in Optimised 3D-TE

Having established that MDA-MB-231 cells are much more effectively suspended in the BME of a 3D-TE if 6 mg/ml BME rather than the suggested 3 mg/ml BME is used, a repeat of the 3D-TE co-culture experiment was performed using 6 mg/ml BME to ensure that the results obtained reflect a truly 3D environment. This experiment served to establish whether the pro-proliferative effect of the MSCs observed at 3mg/ml BME is retained in the higher concentration assay in which cells are truly growing in 3D.

A 3D-TE was performed in using MDA-MB-231 Fluc cells seeded at 25k cells per well. MSCs constitutively expressing eGFP were seeded at 0 or 12.5k cells per well. All wells were seeded with 100µl of 6mg/ml Cultrex BME. Luciferase assay was used to assay tumour cell line growth at 24, 96 and 144 hours after seeding, with eGFP readings taken before luciferin addition each time. This experiment was also performed using MCF7s but it later became clear from luciferase readings and passaged cell behaviour that the MCF7s were unhealthy and results have therefore been omitted.

Luciferase readings from MDA-MB-231 in the 96-well plate format showed survival without growth in monoculture and strong growth in co-culture, consistent with 3mg/ml experiments (Figure 3-11A). MSCs survived in co-culture with MDA-MB-231 (Figure 3-11B) – the dip in readings at 96 hours were present in empty well and MSC-free wells and therefore not likely reflective of an actual change in cell number.

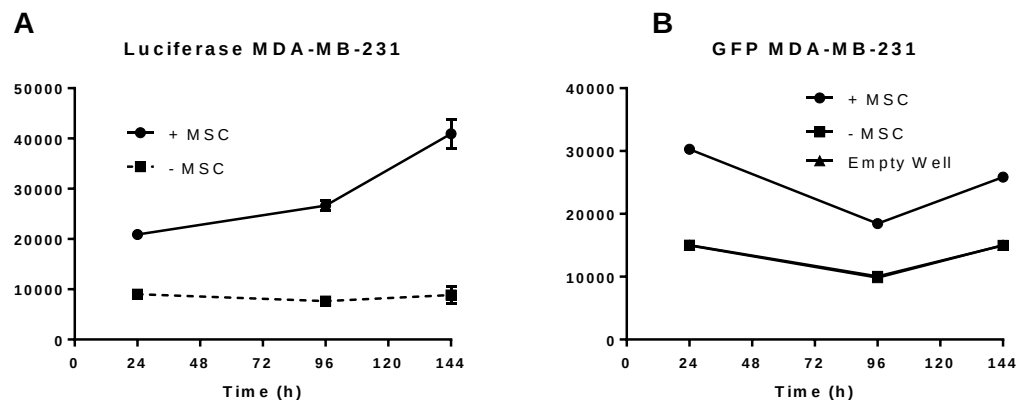


Figure 3-11: Co-culture with MSCs increases MDA-MB-231 growth compared to monoculture in 6mg/ml BME Luciferase readings showing growth of MDA-MB-231 cells in mono- and co-culture over time in 6mg/ml 3D-TE (**A**) while eGFP readings show survival without growth. Error bars show standard deviation between 4 technical replicates.

Microscope images (Figure 3-12) were consistent with luciferase readings; at 144h both monocultures show a relatively small number of cells which appear to be alive. In co-culture MDA-MB-231s have proliferated considerably, and form exceptionally large clusters of cells. All show a reasonable number of in-focus cells in images taken well above the membrane, suggesting suspension has remained improved by the increase in BME concentration.

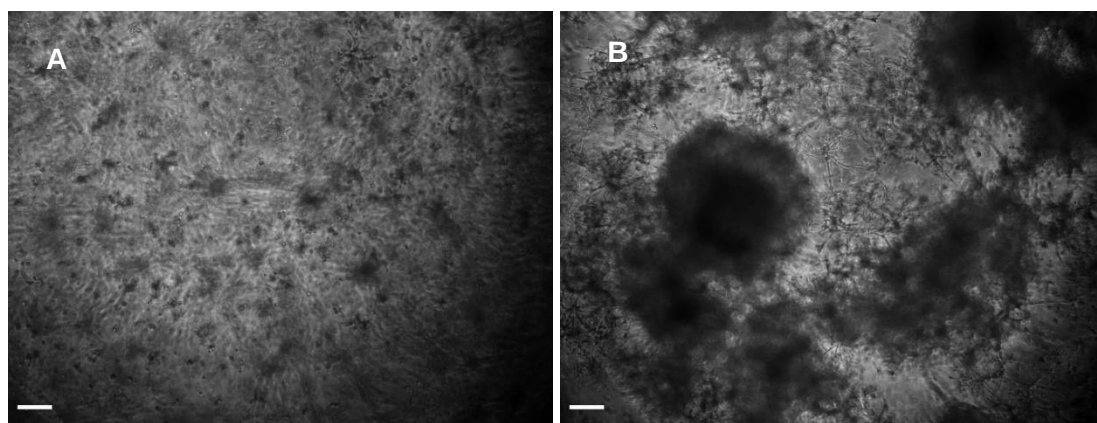


Figure 3-12: MDA-MB-231 cells grow with differing distribution in monoculture compared to co-culture with MSCs in 6mg/ml 3D-cTGA. Representative images from 4 replicates taken at 4x using Nikon Eclipse widefield microscope. Brightfield images of MDA-MB-231 in monoculture (A) or direct co-culture with MSCs (B) after 144h growth in 6mg/ml 3D-TE. Scale bar is 200µm

Overall these results suggest that the increase in BME concentration does not noticeably alter the proliferation-supporting effect of co-culture in the 96-well format compared to monoculture. In both 3mg/ml and 6mg/ml 3D-TE co-culture increases proliferation over monoculture.

3.1.6 Further Confirmation of Co-culture Effect in Fully Optimised 3D-TE

Having established that using 6mg/ml BME improved suspension of cells in the 3D-TE, an experiment was performed to assess the behaviour of MCF7s under these conditions and to assess whether the increased concentration affected growth of cancer cells in co-cultures.

Fresh, infection-free MCF7s were used, alongside the same MDA-MB-231s and MSCs as previously used. All were additionally confirmed free of mycoplasma and continued to grow normally in passage after the experiment concluded. A 3D-TE was

performed in 96-well plate according to protocol. MCF7 Fluc cells and MDA-MB-231 Fluc cells were seeded in co-culture with MSCs as before, in 3mg/ml BME or 6mg/ml BME, and luciferase activity was assayed at 24, 96 and 144h. Brightfield images were also captured at these timepoints.

Both cell lines experienced overall apparent proliferation during the experiment, with the differing BME concentrations making no significant difference to luciferase readings at any point (Figure 3-13A). MCF7 growth was more pronounced than MDA-MB-231 proliferation and appeared to plateau.

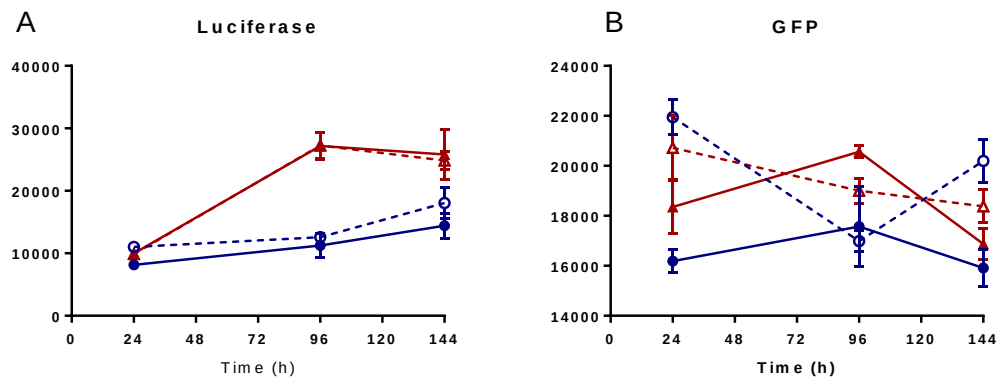


Figure 3-13: Increased BME concentration in 3D-TE does not affect growth/survival of cancer cell lines or MSCs in co-culture. Results of luciferase assay (A), reflecting cancer cell line growth, and eGFP fluorescence read (B), reflecting MSC growth, on simultaneous 3D-TE co-cultures. Solid lines show 3mg/ml BME, dashed lines show 6mg/ml BME. Red lines with triangular points show cultures including MCF7 cancer cell line, blue lines with circular points show cultures with MDA-MB-231 cancer cell line. All conditions include MSC co-culture. Error bars show standard deviation based on 4 technical replicates.

The previously-observed effect of increased sinking of cells in 3mg/ml BME compared to 6mg/ml BME was again evident. For MCF7s cell numbers on the bottom appear lower in 6mg/ml BME throughout the experiment (Figure 3-14) while for MDA-MB-231s the reduced number of sunk cells in 6mg/ml BME effect is evident at 24h but mostly obscured by later sinking or rapid cell growth on the well bottoms by 144h (Figure 3-15). 6mg/ml produced consistently high numbers of cells in suspension while in 3mg/ml wells were inconsistent with some showing reasonable suspension and some showing almost none at all (Figure 3-16).

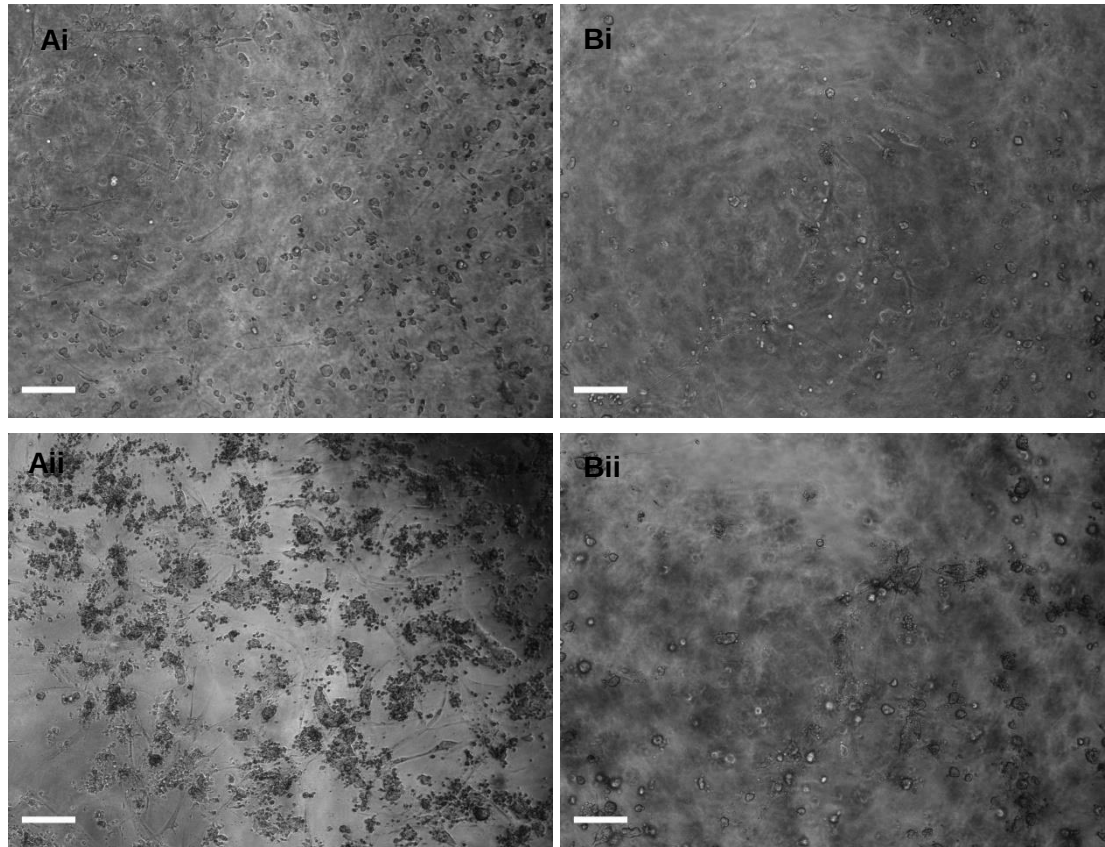


Figure 3-14: Fewer MCF7 cells sink to the bottom of the well in 6mg/ml than 3mg/ml BME
 Representative images from 4 replicates taken at 4x using Nikon Eclipse widefield microscope. MCF7 cells in co-culture with MSCs on the bottom of the well in 3mg/ml **(A)** and 6mg/ml **(B)** BME 3D-TE co-culture with MSCs on the bottom of the well, at 24h **(i)** and 144h **(ii)**. Scale bars 200 μ m

eGFP readings throughout the experiment did not indicate any net overall growth or death of the MSCs (Figure 3-13B), with MDA-MB-231s in 6mg/ml appearing to reduce in number at 96h before recovering at 144h while the other conditions did not appear to deviate beyond well-to-well variation over time.

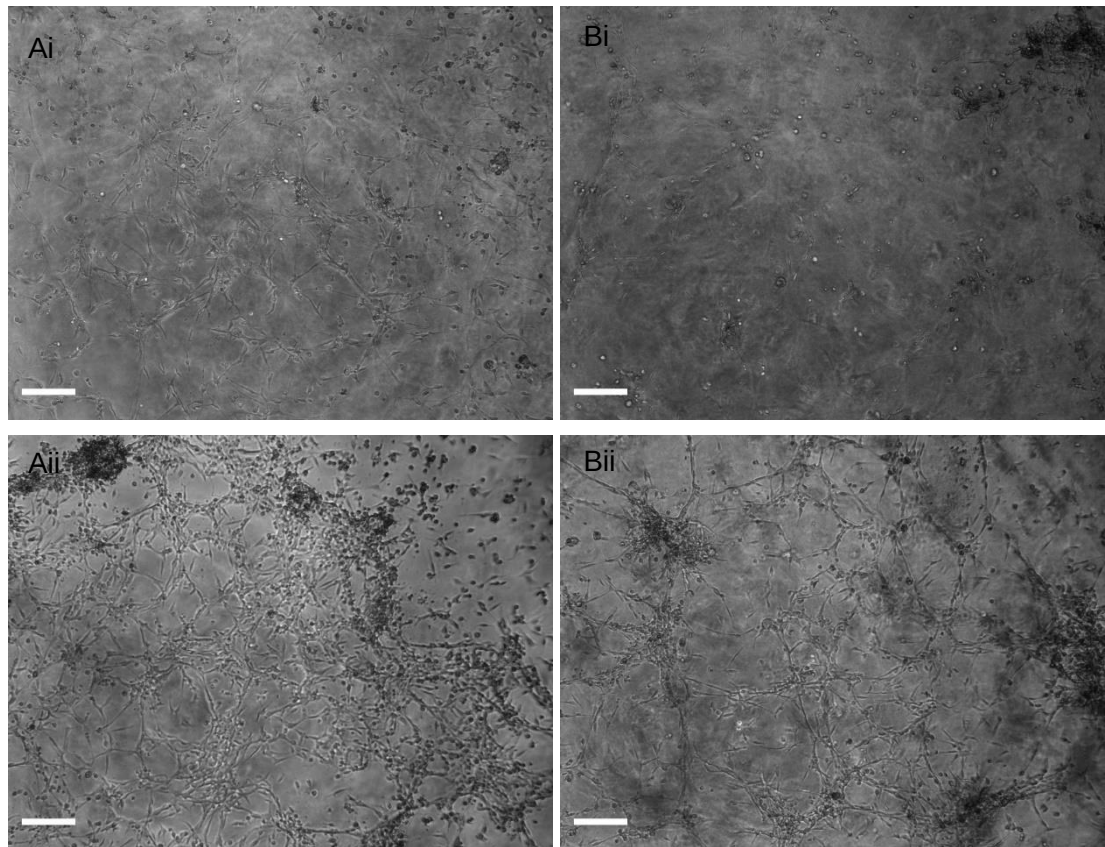


Figure 3-15: Fewer MDA-MB-231 cells sink to the bottom of the well in 6mg/ml than 3mg/ml BME
 Representative images from 4 replicates taken at 4x using Nikon Eclipse widefield microscope. MDA-MB-231 cells in 3mg/ml (**A**) and 6mg/ml (**B**) BME 3D-TE co-culture with MSCs on the bottom of the well, at 24h (**i**) and 144h (**ii**). Scale bars show 200µm

These results suggest that doubling BME concentration from 3mg/ml to 6mg/ml improves suspension of incorporated cells but does not affect proliferation effects. Both cancer cell lines are able to grow in co-culture with MSCs at both concentrations in the 3D-TE. By extension it can be inferred that under these conditions the sinking of some cells does not affect experimental outcomes.

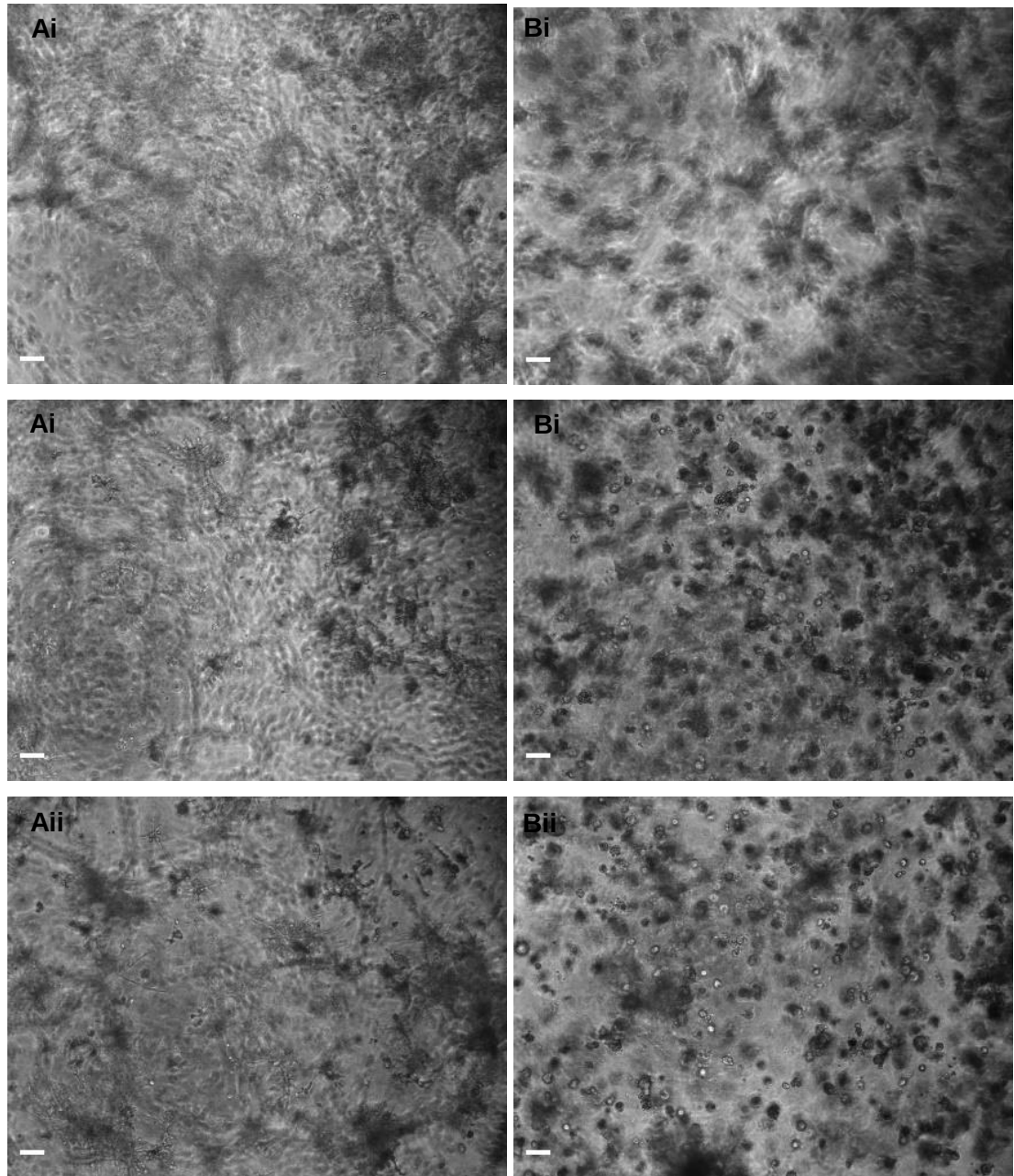


Figure 3-16: **When co-cultured with MSCs, MCF7s are better suspended in 3D than MDA-MB-231s. Increased BME concentration improves suspension of both cell lines.** Representative images from 4 replicates taken at 4x using Nikon Eclipse widefield microscope. MDA-MB-231 (A) and MCF7 (B) cells in co-culture with MSCs at 144 hours suspended in 3D-TE. (i) are typical of the two outcomes visible in 3mg/ml BME wells. (ii) are typical 6mg/ml wells. Scale bars 100 μ m.

3.1.7 Assessment of 3D-TE Suitability for PC-membrane Transwell Format

Experiments with the peptide hydrogel are normally performed in the inserts of a 24 well plate transwell arrangement, with medium bathing the gel in the insert from the outer well. In contrast the BME used for 3D-TEs in the early stages of this project continues to absorb liquid after it has set. Seeding of a typical 96 well plate 3D-TE experiment each well is as follows:

- Each well is seeded as 100µl of ice-cold liquid BME
- This sets when heated to 37°C
- After the BME has set an overlay of 50µl medium is added
- For AlamarBlue assays a further 100µl of liquid is eventually added

After the experiment is concluded it is clearly visible that the entire new volume of each well is a gel and that no state separation between the gel and the added liquids exists.

The 3D-TE is well established as an experimental model and so ideal for initial experiments to determine features that might be useful to include in an eventual peptide hydrogel model. However, if the BME of the 3D-TE continuously absorbs the liquid from the outer well of a transwell setup it cannot be used in such an arrangement. Had this been the case it would have been necessary to delay any testing of indirect co-culture effects until the peptide hydrogel model was sufficiently established within the purposes of the project to be used instead of the Cultrex™-based 3D-TE.

A small experiment was therefore performed to assess the feasibility of larger-scale experiments involving the 3D-TE in a transwell arrangement. Additionally, transwell inserts include a membrane as their bottom surface. This membrane may be polycarbonate or PET, the latter of which is described as more optically clear but the former of which was available in the laboratory. The experiment therefore served to test the optical clarity of polycarbonate membranes and determine whether PET membranes should be used instead.

24 well plate transwell inserts (Sigma Aldrich #CLS3412) were seeded according to 3D-TE protocol (at 6mg/ml on the basis of earlier optimisation experiments) and, at the same time as the addition of the overlay in the inner well, the outer wells were filled with 0.6 ml TGA medium (outer well volume suggested by manufacturer). Four wells were seeded in this manner with 25k MCF7 Fluc cells per well and another four with

no cells. An equivalent standard 96 well plate culture was set up at the same time for comparison.

Microscopy was planned to monitor the distribution, survival and growth of the cells in the transwell arrangement, but cells could only be identified when they were right at the bottom of the insert on the membrane (Figure 3-17), likely due to the depth of the transwell (though temporary transfer of the transwell to a 6 well plate where it sat right on the bottom did not help significantly) and the limited transparency of the polycarbonate membrane (the available 24 well transwell plates at the time of setup had polycarbonate membranes, but the supplier, Corning, recommends PET membrane for microscopy, and inserts with PET membranes will be used when the assay is performed in 96 well plate format). The experiment was monitored by visual inspection of the gels' viscosities and behaviours.

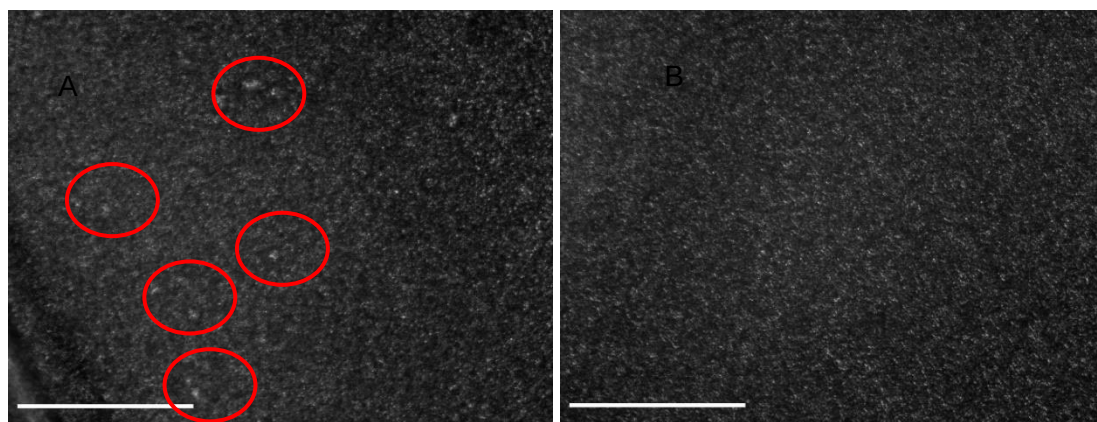


Figure 3-17: Polycarbonate transwell insert membrane severely limits visibility of cells within in microscope images. Representative images from 4 replicates taken at 20x using Nikon Eclipse widefield microscope. 3D-TE in transwell setup with (A) and without (B) MCF7 cells. Image taken from immediately above the transwell membrane. Scale bar 200 μ m, red circles highlight visible cells.

After 6 days of growth both the 96-well and transwell gels appeared gel-like and could be briefly inverted without flowing out of the well/insert. Gels in inserts appeared slightly more fluid on observation of tilting, but not enough to be certain that a difference existed or raise concerns that future experiments in this format were not possible.

While imaging was very challenging, and testing of alternative membrane material intended for imaging was clearly required, it seemed that the transwell setup is not inherently unsuited to the 3D-TE. A next step was to determine whether any assays of cell growth could be performed in this format.

3.1.8 Assessment of Co-culture Effect in 3D-TE in PET-Membrane Transwell

In the previously-described experiment (Section 3.1.7) it was determined that the Transwell plate format (used for peptide gel and necessary for any indirect co-culture experiments) was not incompatible with the 3D-TE format, but that transwell inserts with polycarbonate membranes significantly hindered ability to image the insert's contents by microscopy. An experiment was therefore performed with two goals:

- to assess compatibility of PET membranes (Corning, #3382) in transwell inserts, described by the manufacturer as more optically transparent than polycarbonate membranes, with microscopic imaging
- to determine whether the proliferation-promoting effect of co-culture is present in transwell format

MDA-MB-231 Fluc cells seeded at 25k cells per well with MSCs constitutively expressing eGFP at 0 or 12.5k cells per well in standard 96-well plate. In transwell inserts for 96-well plate, the same conditions were seeded except with volumes and cell counts halved so some general comparisons can be made between the two to check for any effects resulting from the transwell format. The different height and transparency of the plates means direct comparison of values between the two plate arrangements is not possible, but it is still worth comparing general trends and effects between the plates.

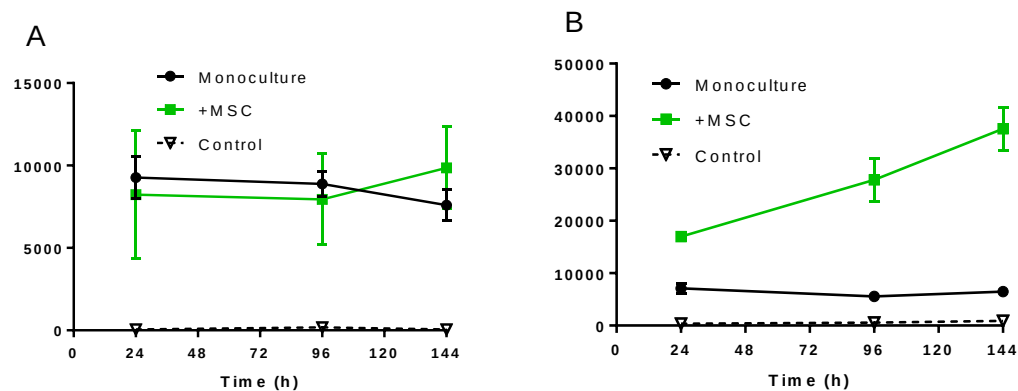


Figure 3-18: **Proliferation-enhancing effect of co-culture can be observed in transwell format, but is not always evident in standard plate format.** Growth of MDA-MB-231 cells in standard Brand 96-well plate (A) and 96-well transwell plate (B) in 3D-TE assay. Error bars show standard deviation based on 4 technical replicates.

MDA-MB-231 cells (Figure 3-18) appeared not to be affected by co-culture in the standard plate, in contrast to the previous experiment which was set up with identical conditions (Figure 3-11). In the transwell plate however the proliferation-enhancing

effect of co-culture seen in earlier experiments was restored. As previously, eGFP readings suggest MSCs survive but do not grow (Figure 3-19).

Compared to the polycarbonate membranes of the 24-well transwell plates tested earlier (Figure 3-17), the PET membranes of the 96-well transwell plates considerably improved microscope imaging capacity. For MDA-MB-231s the transwell setup showed no sign of impeding proper suspension of the cells in the BME, and may have improved it (Figure 3-20). Unexpectedly it seemed that inclusion of MSCs with MDA-MB-231s in either format reduced the number of properly suspended cells, which is not possible to explain by the order in which the cells were seeded (and subsequently the time they had available to sink before the BME gelled). In monoculture in standard wells the majority of cells which remained suspended were in large clumps, while in the transwell inserts individual cells were better suspended.

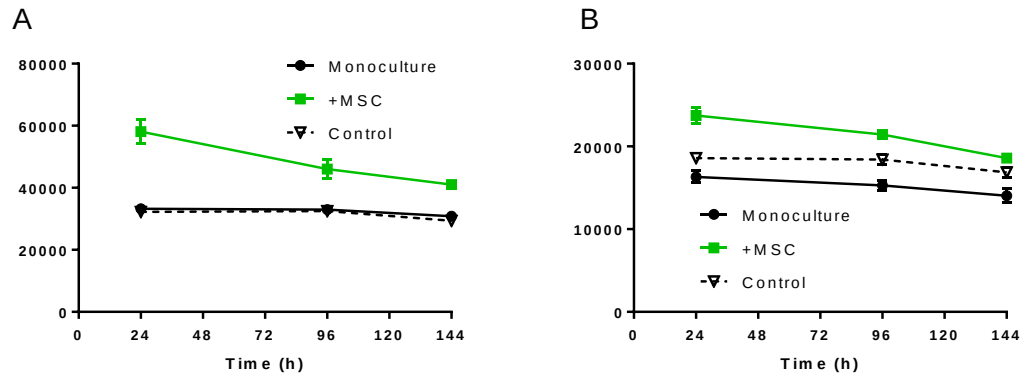


Figure 3-19: MSCs in co-culture with cancer cells survive without growing in both transwell and standard plate formats. eGFP readings from 3D-TE of MDA-MB-231 cells with or without eGFP-tagged MSCs in transwell (A) or standard (B) plates. Control refers to wells without cells of any type. Cells grown in 3D-TE assay. Error bars show standard deviation based on 4 technical replicates.

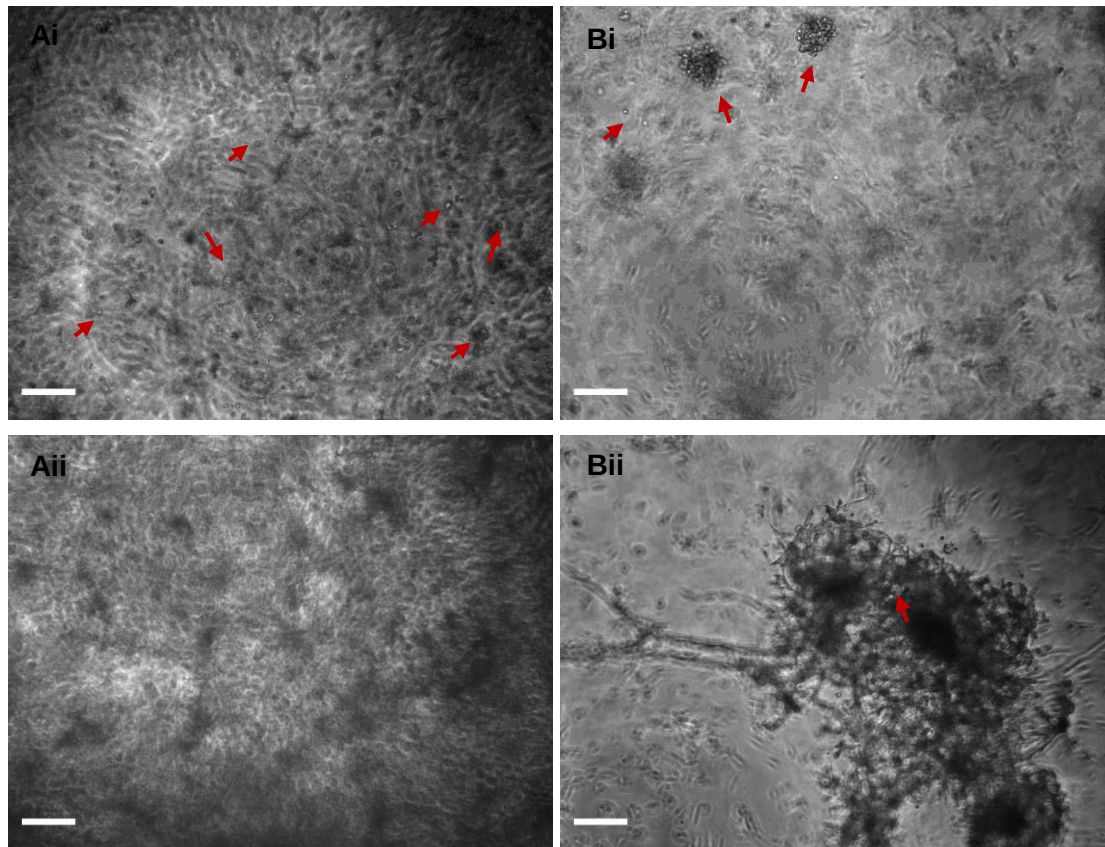


Figure 3-20: MSC co-culture but not transwell plate format causes sinking of cancer cells in 3D-cTGA assay. Representative images from 4 replicates taken at 4x using Nikon Eclipse widefield microscope. MDA-MB-231 cells grow in 3D-TE in transwell insert (**A**) or standard 96-well-plate well (**B**) in monoculture (**i**) or co-culture (**ii**) after 144h. Scale bars show 200µm. Images taken within the gel such that cells which are in focus have not sunk; arrows indicate expamples of in-focus cells and clusters.

These results highlight occasional unexpected inconsistencies between similar experiments but, combined with previous experiments, do demonstrate that the transwell format can replicate general effects seen in other assay formats as well as suggesting that the 96-well transwell format is generally applicable for the 3D-TE, which allows for indirect co-culture experiments.

3.1.9 Use of Immortalised MSCs as an Alternative to Primary MSCs

Use of non-immortalised MSCs is associated with a considerable number of practical problems which limit their usability in optimisation and would continue to be problematic if they were to become a recurring element of the model in its intended usage. Even when healthy and growing normally, primary MSCs will take a week or more to reach an ideal confluence for passage/use when passaged at a ratio of 1:3, meaning that particularly considering the requirement to preserve early-passage stocks a very limited number can be produced from a given patient sample. These cells are also vulnerable to anoikis when passaged at higher ratios and may resume growth very slowly if not optimally confluent before a 1:3 passage, in addition to beginning to slow in their growth as passage numbers beyond 10 or 11 approach. In comparison with immortalised MSCs which can be passaged at 1:5 without showing signs of anoikis or alterations in growth speed, and can also be passaged far beyond 10 passages without alterations in behaviour, it would be preferable to avoid using primary MSCs if possible.

An experiment was therefore performed to determine whether an immortalised MSC cell line had the same effect in co-culture (in the 3D-TE assay) as non-immortalised, eGFP-labelled MSCs on growth of MCF7 and MDA-MB-231 tumour cell lines.

A 3D-TE assay was performed in 6mg/ml Cultrex according to protocol. Cells used were MCF7-tdTomato, MDA-MB-231-tdTomato, TE67 (immortalised MSCs, unlabelled) and MSC-eGFP (non-immortalised MSCs, as previously used). Cancer cell lines were seeded at the previously optimised 25,000 cells per well; MSCs were seeded at half of this concentration as previously established. Seven conditions were seeded: a cell-free Cultrex control, MCF7 in monoculture, with immortalised MSCs or with eGFP MSCs; or MDA-MB-231 in monoculture, with immortalised MSCs or eGFP MSCs. Cancer cell line growth was measured using tdTomato fluorescence, and non-immortalised MSC growth was measured to confirm consistency with prior experiments using eGFP fluorescence. Images of the bottom of each well and a point further up into the gel (the same distance from the membrane in each well, aiming for a depth which will capture cells which are neither very close to the bottom nor very close to the top of the gel) were taken using a brightfield microscope. Fluorescence imaging was not available until day 7 – on this day an image from each condition was taken with brightfield, tdTomato and eGFP channels.

Consistent with previous results, eGFP readings did not change significantly throughout the experiment, but were higher in wells containing MSC-eGFP than any other (not shown).

TdTomato readings showed that while monoculture readings were higher than empty well readings and increased somewhat over time, inclusion of either MSC type increased proliferation with no significant difference between immortalised and non-immortalised MSCs (Figure 3-21).

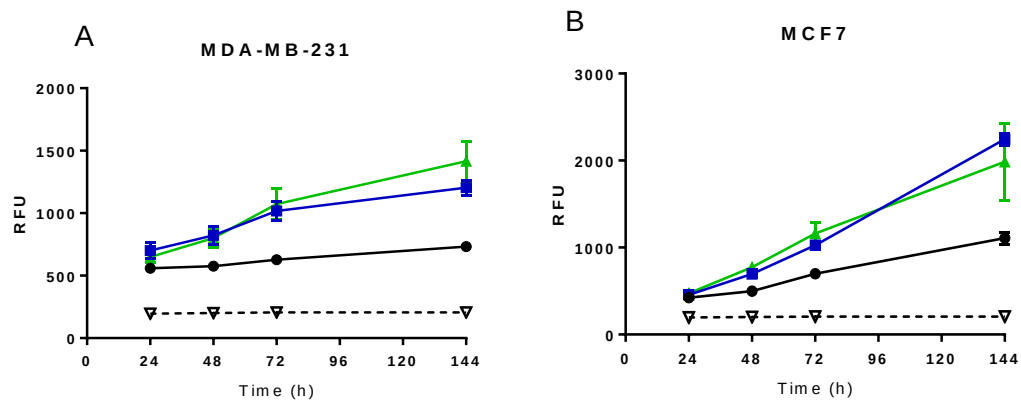


Figure 3-21: Immortalised and non-immortalised MSCs promote MCF7 and MDA-MB-231 growth to a similar extent Growth of cancer cell lines (MDA-MB-231 (A) and MCF7 (B)) in 6mg/ml 3D-TE, in co-culture with immortalised (blue line) or non-immortalised (green line) or in monoculture (solid black line), GFP expressing MSCs, measured via constitutive tdTomato fluorescence. Dashed black line shows shared cell-free control. Error bars show standard deviation based on 5 technical replicates.

When images were taken, the focus was first aligned to the bottom of all wells, and then offset by the same amount for all wells, so if sinking was equal between conditions the 'up' images would show similar cell populations.

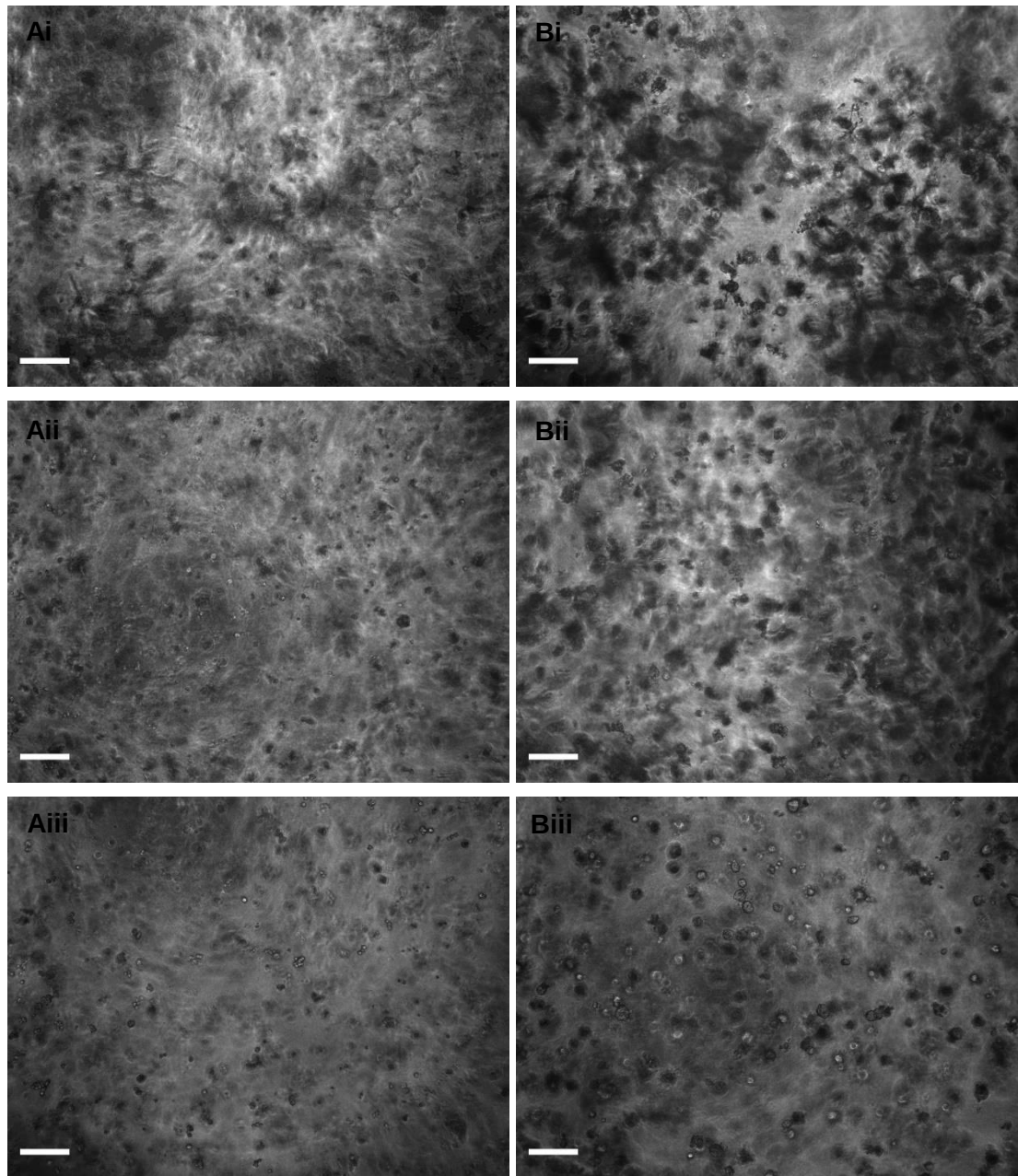


Figure 3-22: **Visual differences in cell distribution can be observed between different co-culture conditions** MDA-MB-231 (A) and MCF7 (B) cells grown in the hydrogel for 6 days with non-immortalised (i), immortalised (ii) or no MSCs (iii) in 3D-TE. Representative images from 5 replicates taken using Nikon Eclipse widefield microscope from within the gel, not at the bottom of the well. Scale bars show 200 μ m

In terms of proliferation effects the results of this experiment show great similarity between the two MSC cell types in co-culture with both cancer cell lines, which are also consistent with earlier results. However, there is apparently some difference between the two MSC types as the non-immortalised MSCs cause the cells to sink

over time, whereas the immortalised ones do not, and a different clustering pattern is visible for both cancer cell lines between the two MSC types (Figure 3-22).

Since TdTomato, can also be detected in dying cells, the previous experiment was repeated using luciferase to ensure that the growth observed was real.

25,000 MCF7-Fluc cells with or without 12,500 immortalised or non-immortalised (eGFP labelled) MSCs per well were seeded in a 3D-TE in a 96 well plate alongside no-cell control wells (five wells per condition per timepoint). Luciferin luminescence readings were taken at 24, 72 and 120 hours (wells were not reused). Images were always taken from the wells intended for 120h readings of luciferase activity for consistency.

Luciferase assay results showed that MCF7 cell numbers were increased compared to monoculture for both types of MSC co-culture, and that the immortalised and primary (eGFP) MSCs do not differ in their effect on the MCF7s' growth (Figure 3-23).

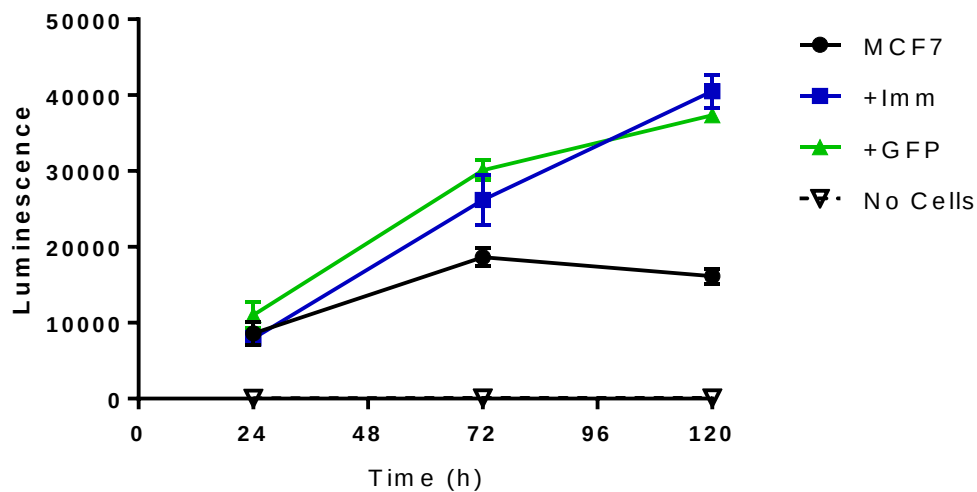


Figure 3-23: Luciferase readings confirm similar growth-promoting effect from the two MSC types
Luciferase readings over time when MCF7 cells are grown in monoculture or in co-culture with primary (eGFP labelled) or immortalised MSCs in 3D-TE assay. Error bars show standard deviation based on 5 technical replicates.

Images taken at 120h are consistent with luciferase assay results, showing many living cells (Figure 3-24). The cells appeared evenly distributed throughout the well in all conditions both vertically and in the XY plane.

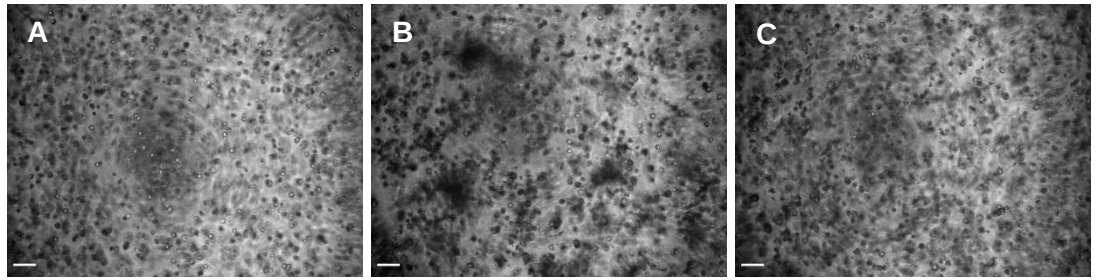


Figure 3-24: **Cells remain fairly evenly distributed on repeat of comparison of co-culture conditions.** Representative 120h images from 5 replicates: MCF7 in monoculture (A), primary MSC co-culture (B) and Immortalised MSC co-culture (C) Scale bars show 200µm

These results suggest that in terms of growth-promoting effects, immortalised MSCs are not significantly different to primary MSCs. Further experiments were needed to test how similar the two MSC types are in terms of gene expression.

3.2 Establishing Usability of the Peptide Hydrogel in a New Laboratory

For any model produced using the peptide gel to be useful it must be consistent between laboratories and over time. With the peptide powder manufacture process having been transferred to a new company and still being optimised for product purity, comparison of the physical and cell-supporting properties of the peptide gel between batches was necessary. Any pH differences between the powder batches particularly had potential to alter the amount of NaOH needed to achieve gelation; the amount of NaOH added was therefore compared as one measure of batch-to-batch similarity. Degradation products or otherwise incomplete octapeptides might alter the ability of the powder to form a gel, and so differently stored batches were also compared. Finally, distribution of cell growth within seeded gels was considered in addition to quantity of cell growth, as it was evident from the results in very early optimisation (not shown) that inconsistently viscous gels produced differential cell growth between areas of the same well.

6 gels were produced: 2 each from the aliquot of batch 32597 previously used ('old A'), the main aliquot of batch 32597 stored in a separate freezer for several months ('old B') and the newer batch 38296 B2 ('new'). While all had been stored at -80°C in sealed plastic bags with air expelled and silica drying sachets, the exact freezers and frequencies of access differed. Gels were made up at 10 mg/ml according to protocol and comparisons were made between the batches, particularly concerning stages such as NaOH addition where there is sometimes variation even within batches.

MCF7 Fluc cells were then seeded in one gel (into 2 wells) from each batch at 1.25×10^5 cells per well, and grown for 7 days with images taken every two to three days, the same days as the medium was replaced.

While the batches had fairly dissimilar powder properties (Table 3-11) when carefully observed during weighing, once dissolved they were indistinguishable and none were unusually difficult to dissolve using the protocol developed by the Merry group for the 'Old A' batch. At seeding, the new batch was noted to be slightly less viscous than the others were, but otherwise the gels remained very similar and required exactly the same quantity of NaOH.

Table 3-1: **Observations of Peptide Batch Properties**

Batch	Powder Observations	NaOH Required
Old A	Somewhat dense and grainy	75 μ l
Old B	Fluffier, more static	75 μ l
New	Flaky and powdery	75 μ l

Initially after seeding (on day 1 when the gels had had a chance to gel completely) there were some slight difference in visibility when observing cells through the gel during microscopy with the same exposure time, and visual difference in gel texture (including small bubbles appearing only in old A). However, with the exception of the bubbles, there was as much variability between wells as much as between gels (Figure 3-25). Growth was at an approximately normal rate for MCF7s in the peptide gel, with the cells becoming very numerous by day 7 (Figure 3-26). Visually there was little difference in cell cluster shape or size between images, and the number of clusters visible in each image was not noticeably more different from batch-to-batch than from well-to-well of the same batch.

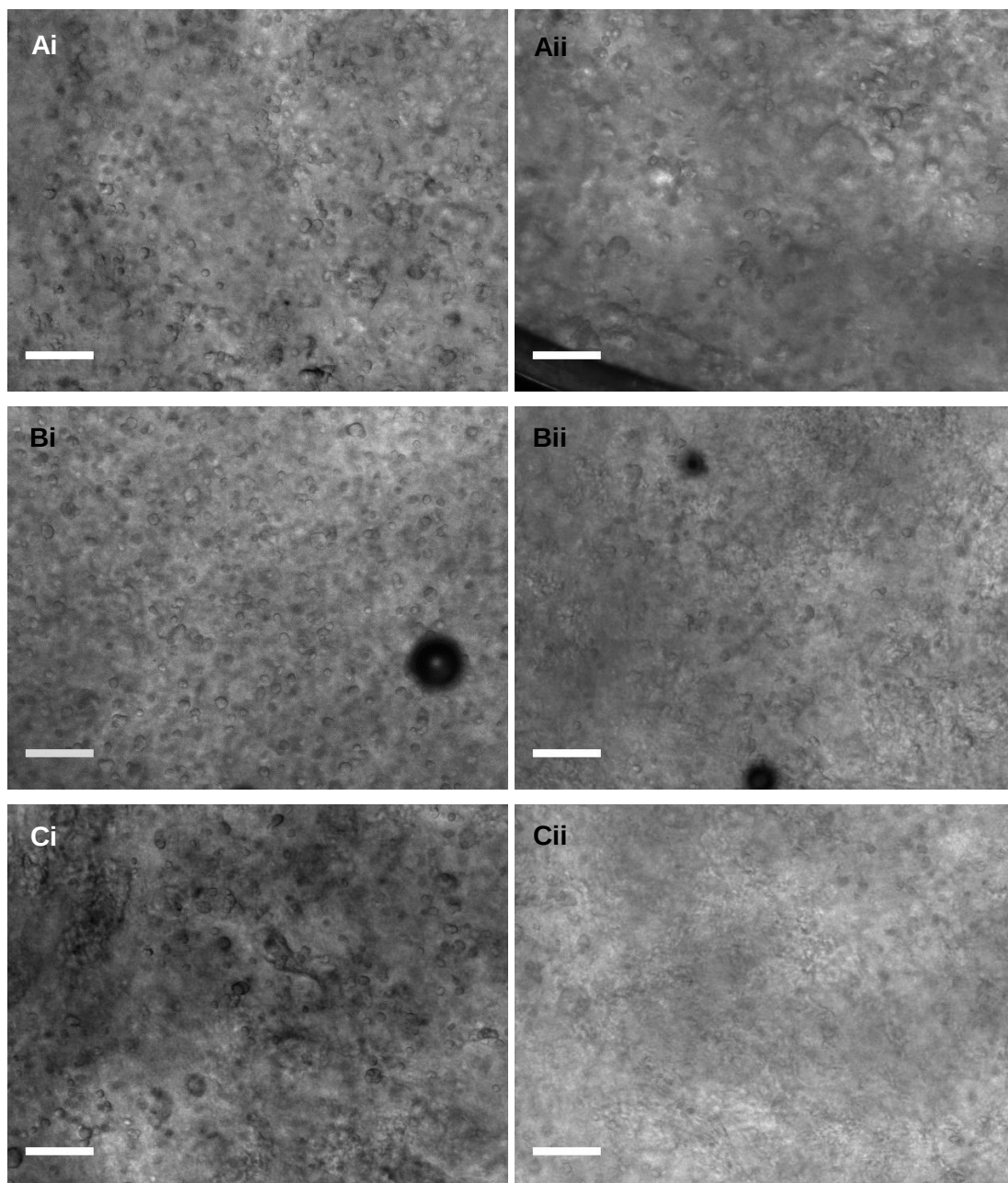


Figure 3-25: Initial MCF7 growth in different peptide gel batches varies to a similar extent between replicates and between batches Images taken at 4x using Nikon Eclipse widefield microscope. Representative images of duplicate wells of MCF7s grown in New **(A)**, Old A **(B)** and Old B **(C)** batches of peptide gels, taken after 1 day of growth in 3D in phenol red free RPMI medium. Scale bar 200 μ m.

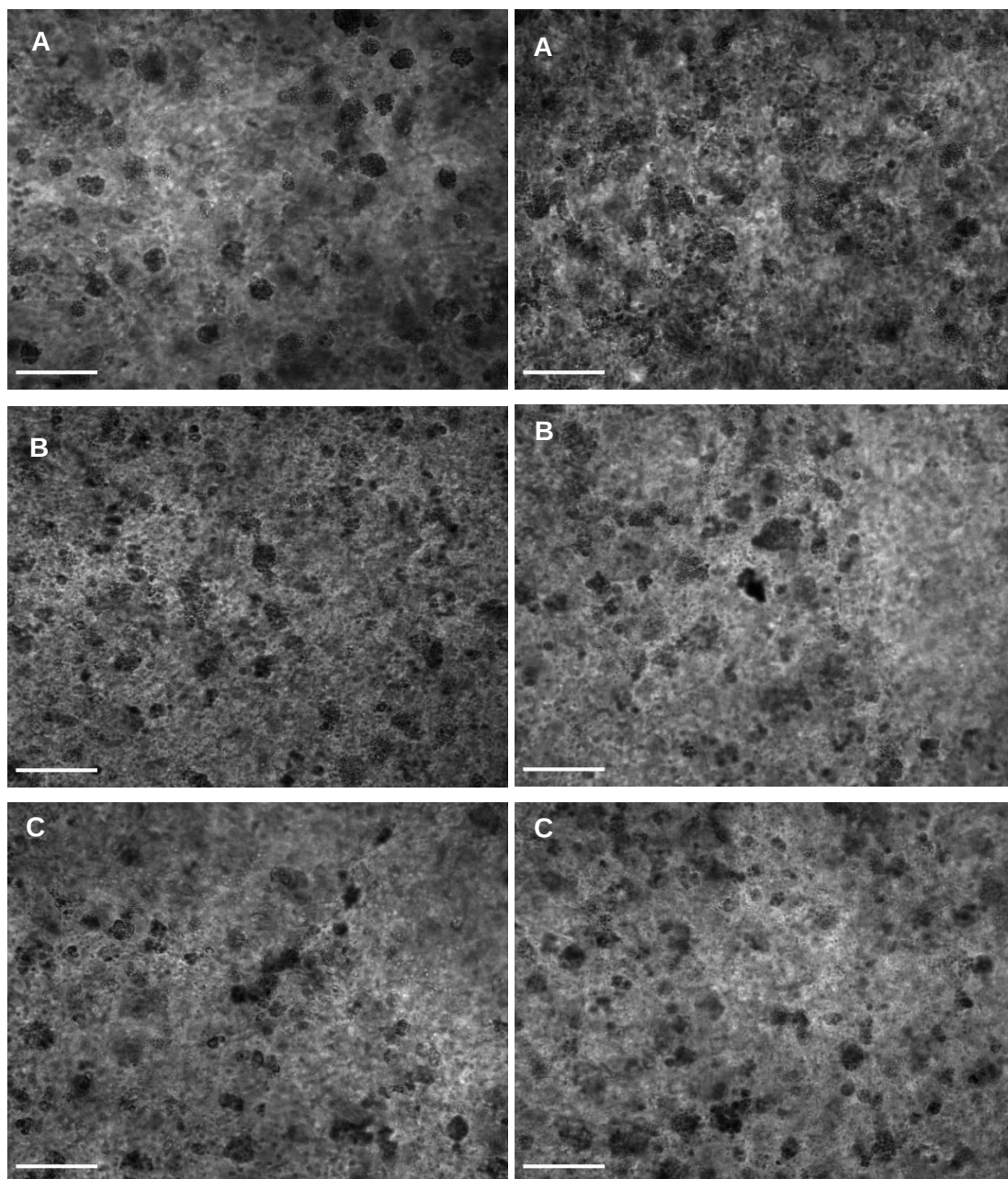


Figure 3-26: **Long-term MCF7 growth in different peptide gel batches varies to a similar extent between replicates and between batches** Images taken at 10x using Nikon Eclipse widefield microscope. Representative images from duplicate wells of MCF7s grown in New (**A**), Old A (**B**) and Old b (**C**) batches of peptide gels (phenol red-free RPMI medium), taken after 7 days of growth in 3D. Scale bar is 200 μ m.

In terms of requirement for gel-to-gel consistency these results are encouraging. There are no clear differences in behaviour after dissolving the peptide, amount of NaOH required to gel, ability to support MCF7s and visual appearance of the gel itself and so no further optimisation is likely to be necessary to make new or differently-stored batches compatible with the existing protocol. However, there may be problems with

mixing or pipetting which results in well-to-well variation from a single gel as seen particularly in the case of the new gel in which one well has noticeably more clusters of cells than the other. Another possibility is that images need to be taken from more areas of each well as in this format there are often areas of a well that grow more cells than others. Specifically, there may be differences between areas of the gel close to the centre versus areas nearer to the sides of the insert.

3.3 Growth of PDX-derived Cells in 3D *in vitro* Models

PDX-derived cells, primary cells taken from patients and grown in mice, have previously primarily been grown *in vivo* or in 3 mg/ml BME-based format. The peptide gel, meanwhile, had previously only used with established cell lines. Therefore a pilot experiment was performed to investigate growth of PDX-derived cells in the highly supportive and well-established Cultrex-based 3D tumour growth assay. Given that higher concentrations of BME than are ordinarily used were required to retain cancer cell lines in suspension in the 3D-TE (section 3.1.4, page 50), this experiment compared typical (3mg/ml) and increased (6mg/ml) BME concentrations for growing BR8 PDX-derived cells in 3D-TE, with the PDX disaggregated according to its own protocol and then seeded in six replicates each of the two 3D-TE concentrations in accordance with 3D-TE protocol. BR8 is derived from a triple-negative breast tumour and might therefore be expected to be equivalent to the MDA-MB-231 cell line.

Since proliferation was not visually obvious by microscopy during 6 day experiments and luciferase assay to test for proliferation could not be used in the unlabelled PDX-derived cells, the culture period was extended (without any additions to the medium) to 12 days, after which the cells could be seen clearly to have grown from single cells into small clusters which appear to be of similar sizes between the two BME concentrations (Figure 3-27). When images are taken above the bottom of the well, cells suspended at that height will appear in focus – the absence of in-focus cells therefore reflects poor suspension of the cells in the BME. It can be seen that the 6mg/ml BME keeps the cells in 3D better than 3mg/ml BME; dark shadows indicating cells in out-of-focus planes are present in images from both concentrations, but in-focus cells can only be seen at 6mg/ml. This confirmed that the xenograft was able to grow rather than merely survive in the 3D-TE, and highlighted a possible need to extend the timescale for future experiments with PDXs.

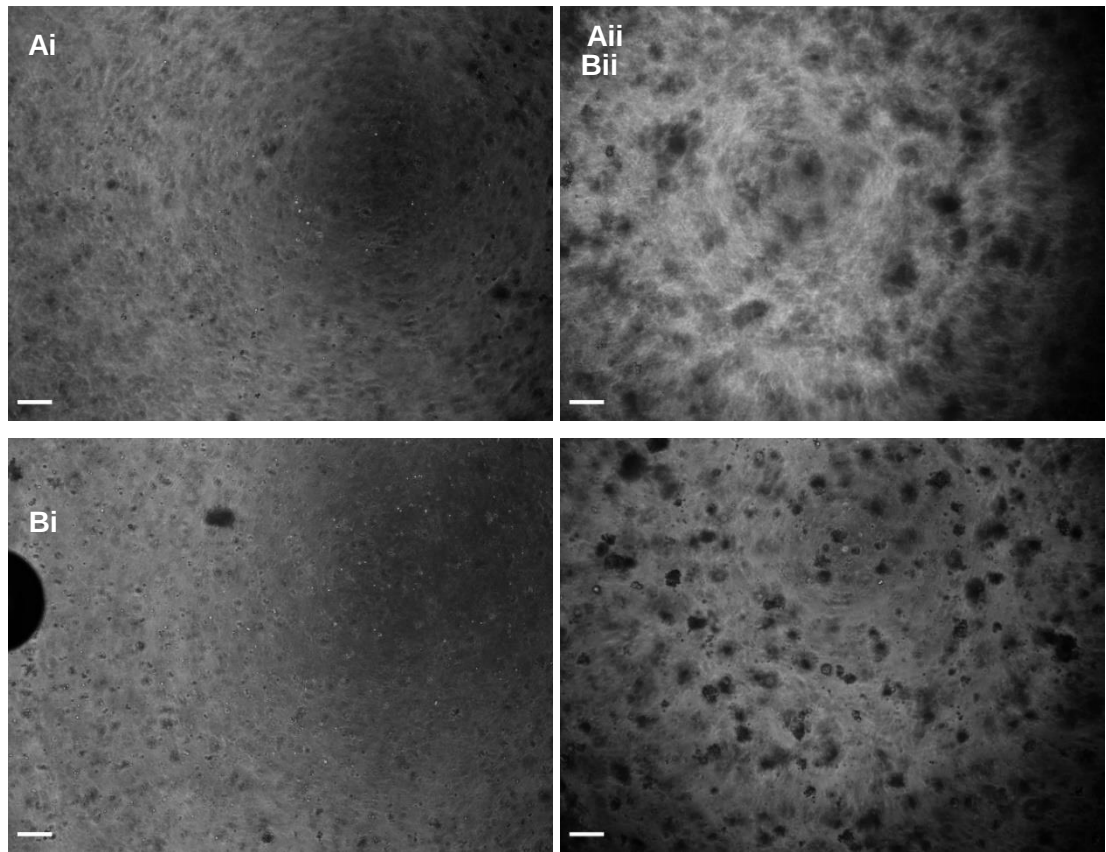


Figure 3-27: BR8 PDX-derived cells are better retained in 3D in 6mg/ml than 3mg/ml 3D-cTGA. Representative images from 6 replicates taken at 4x using Nikon Eclipse widefield microscope at a mid-depth in the gel. Dispersion in the matrix appears much better in 6mg/ml BME (**B**) compared to 3mg/ml BME (**A**). (**i**) shows day 1 growth while (**ii**) shows day 12 growth; the difference in dispersion between BME concentration is more pronounced following 12 days of growth but nonetheless clearly evident at day 1. Scale bars are 200 μ M

3.4 Conclusion

The above-described experiments have determined that:

- **Co-culture with stromal cells does have a supportive effect for the cancer cells when grown in 3D.** Breast cancer cell lines co-cultured with MSCs proliferated faster than those in equivalent monoculture in 3D in BME, consistent with 2D experiments and the work of other groups. It remains to be seen whether this applies in the peptide gel which is a quite different 3D environment to the signalling molecule- and binding site- rich BME. These experiments also highlighted a necessity to ensure that cells within the 3D model are distributed throughout, rather than sinking to a 2D arrangement at the bottom.
- **A 3D *in vitro* environment can support growth of BR8 PDX-derived cells.** BR8 PDX-derived cells were able to grow in 3D in BME; while this does not guarantee successful growth in the theoretically much less supportive peptide gel, it suggests that if the PDX-derived cells fail to grow in the peptide gel, addition of ECM molecules may be sufficient to restore growth as the 3D environment is not inherently a barrier to growth.
- **The peptide gel can be produced and imaged to an adequate standard with the available equipment.** Following some optimisation it was possible to entirely generate and use the peptide gel within the new lab, without observable differences from those produced using equipment for which the protocol was written. Microscope imaging is feasible for peptide gel in transwell inserts as long as PET membranes are used.
- **The peptide gel can support the growth of cancer cell lines for over a week.** MCF7 cells grew rapidly in the peptide gel to form many clusters of cells, indicating that the peptide gels, when generated in the EVPC laboratory setting, are able to support growth of reasonably durable cells.

These are all promising initial steps towards the assembly of a useful model to replace animals for passage of PDX-derived cells. The following steps were to combine these three elements (PDX-derived cells, peptide gel and stromal cell co-culture) and characterise the resulting model. The potential of the gel to support passage of the

PDX-derived cells is particularly important and a key concern investigated in the later chapters of this thesis, particularly as it will be required for expansion and maintenance of PDX-derived cells for other experiments within the project.

4 Methods for Characterisation of Cells Grown in Peptide Hydrogel Model

The ability to monitor and characterise cells grown in the peptide gel is important not only for optimisation processes such as in this project, but also for the gel's long term practical usability. Key starting points were metabolic assays and live/dead staining to monitor the growth and condition of the cells. The use of qPCR to test the species of cells within the gel additionally provided preliminary evidence for broad compatibility with other nucleic acid-centric assays. Compatibility with flow cytometry and *in situ* immunostaining was then tested to confirm that the gel could be used alongside common assays providing information about protein expression.

4.1 Identifying A Suitable Metabolite-based Growth Assay for Use in Peptide Gels

4.1.1 Assessing Compatibility of Transwell Format with Luminescence-based Assay

Fluorescence and luminescence assays such as Luciferase and AlamarBlue are commonly used for both cell lines and PDX-derived cells, though use of Luciferase assay requires genetic modification of cells. In Chapter 3, these assays were successfully used to allow high-throughput readouts of cell number and survival in a 96-well format, using black-sided plates to prevent strong signals from one well adding to that observed in neighbouring wells. This cross-well signal detection was particularly of concern for Luciferase assays which will produce signal at all times during the reading process, and not only when illuminated by an excitation frequency. In order to apply these assays in the transwell assay format normally used for studies involving the peptide hydrogel, it was important to assess whether black-sided, transparent-bottomed transwell plates were required. These would be difficult to obtain and costly and thus, in order to ensure that the procedures we were developing were as widely applicable as possible, we investigated whether high signals in individual wells interfered with signals in neighbouring wells. Luciferase was tested initially, as high luciferase signals would be most likely to affect neighbouring wells, and while AlamarBlue is more practical for PDXs (as genetic modification is not required for AlamarBlue assay) luciferase assay is very useful for control or optimisation experiments using cell lines.

A black, clear-bottomed 96 well plate and an otherwise identical entirely clear plate were seeded with MDA-MB-231 fluc cells – wells within each column were seeded identically as technical replicates and the columns were seeded in such a way as to give wells expected to give intense readings in varying proximities to wells expected to give less intense readings. Cells were seeded at 5k cells/well with luciferin substrate added at day 4 (low reading) or at 2k per well with luciferin substrate added at day 6 (high reading) and all readings were taken on day 6 when every seeded well had had luciferin added.

While there was a slight increase in detected fluorescence for some wells of the clear plate compared to the black plate, it was much less than anticipated and insignificant compared to the scale of difference which would need to be seen to indicate a real biological difference (Figure 4-1). The readings measured were in the range that would be expected in both 2D and 3D experiments with the cell lines to be used in future cell-line-based experiments, with the most intense ones for this experiment among the highest encountered in this project. Increases of 50% to 100% were observed from monoculture controls to co-culture conditions in previous experiment, and so this increase of around 10% to 20% in the lowest values and much less in the highest is subsequently reassuring that higher readings which might produce a ‘false positive’ effect are unlikely but that care should be taken when interpreting small differences in luciferase assay results.

This relative lack of interference observed is likely a result of the Fluorstar Omega plate reader being less susceptible to neighbouring well interference than anticipated, though not entirely unaffected. This demonstrated the necessity for any future experiments involving significantly altered experimental setup (such as a different plate reader or much brighter or more wide-ranging readings) to retest to ensure interference remained low.

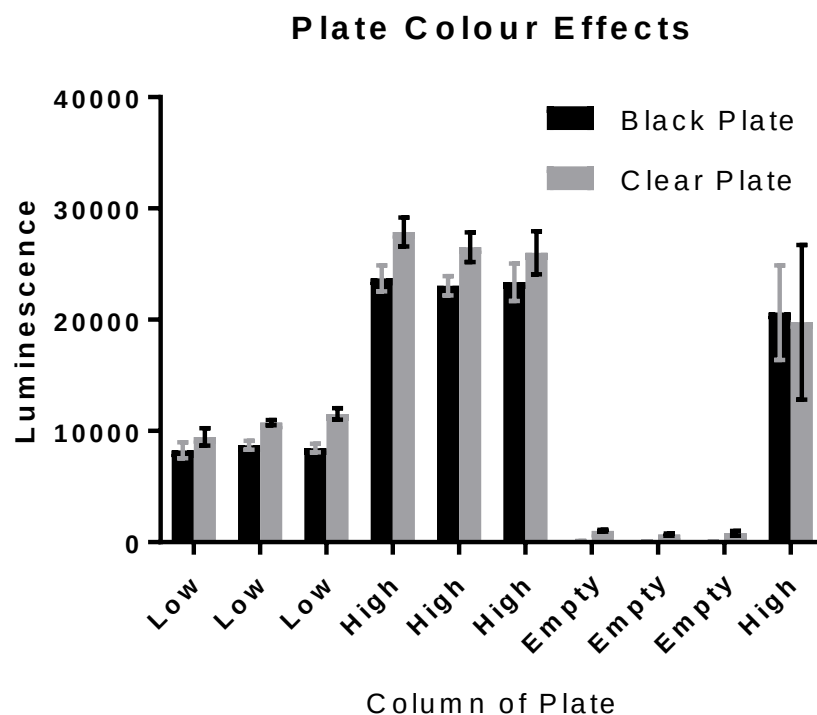


Figure 4-1: Use of a transparent-walled plate allows minimal signal detection from neighbouring high signal wells in low signal wells Comparison of neighbouring well effects between black-sided and clear-sided 96 well plates. MDA-MB-231 cells grown in phenol red-free RPMI medium for 4 days. Error bars are standard deviation calculated from 6 technical replicates. Neighbouring bars for the same plate colour represent neighbouring columns of readings from the plate.

Having established that the transparency of the plate would not limit usability of Luciferase assays within previously used ranges, the next step was to compare Luciferase assay with other growth assays to identify the most appropriate method for assessment of cell growth in 3D.

4.1.2 Comparison of Usefulness of Metabolite-Based Assays in Peptide Gel

Assays such as AlamarBlue and luciferase had previously not been used in the peptide gel model, or in the transwell insert format used for the peptide gel. This particular arrangement of gel and transwell insert was likely to affect the ability of assays such as AlamarBlue and luciferase to detect cell growth/survival; reagent must penetrate the gel to measure metabolic activity of the cells within and subsequently cannot be removed with the ease of a 2D assay, and the transwell setup alters the height of the volume whose fluorescence or luminescence is to be measured. An experiment was designed for the following purposes:

- to test basic function of these assays in the gels in transwells

- to identify whether re-using a single well for multiple timepoints is feasible (as this would increase the number of timepoints and technical replicates that can be used in each experiment)
- to assess the effect of MSC indirect co-culture on MCF7s growing in peptide hydrogel in a transwell insert.

On day -3 (three days before seeding of MCF7s in peptide gels), wildtype MSCs were seeded at approximately 15000 cells per well (equivalent to a 1:3 passage which would normally take around 1 week to become confluent) in 8 wells of a 24 well plate. These MSCs were cultured in MSC medium until day 0.

On day 0 MCF7 Fluc cells were seeded in phenol red-free RPMI in four 10mg/ml hydrogels, each seeded into four wells such that multiple different gels formed the technical repeats for each compared condition. The seeding density was 5×10^5 cells per gel, and was initially performed with the inserts in an empty 24 well plate. As part of the final media change of day 0, the inserts were moved to the plate in which the MSCs were seeded on day -3. An RPMI wash ensured any remaining MSCM (which included phenol red) in the wells containing MSCs could not affect the MCF7s.

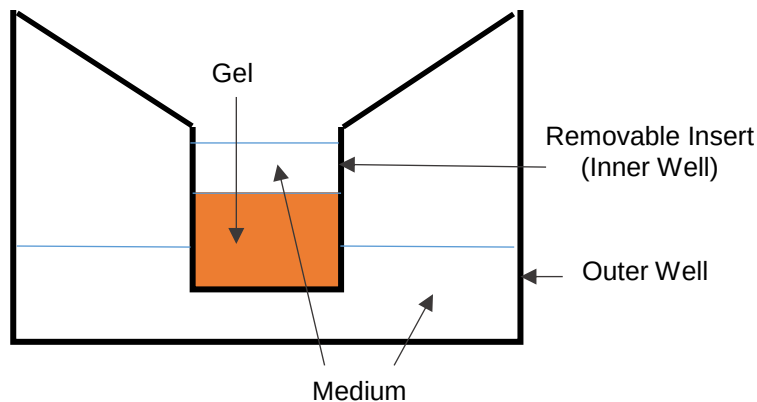
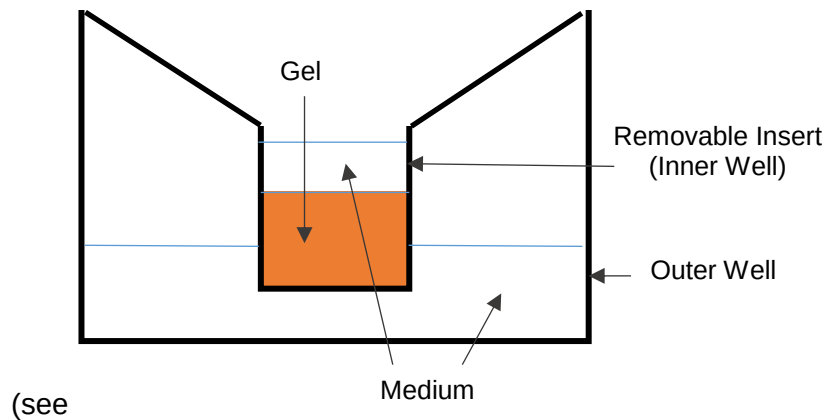


Figure 4-2: *Diagram of transwell arrangement used to grow cells in peptide gel*

This produced 16 inserts containing MCF7s in peptide gel; 8 for which the outer wells



(see Figure 4-2) contained MSCs growing in phenol-red free RPMI and 8 for which the outer wells contained phenol-red free RPMI alone, for four total conditions:

- + MSC in outer well, readings days 1 and 4
- + MSC in outer well, readings day 4 only
- Monoculture, readings days 1 and 4
- Monoculture, readings day 4 only

Each condition was represented by four inserts –two were used for AlamarBlue assays while the remaining two were used for luciferase assays.

On day 1 microscope images were taken for all wells within the inserts (plus additional images to check that the MSCs were still present and assess whether they showed any signs of growth, given they hadn't grown in previous experiments such as shown in Figure 3-11B and Figure 3-13B) and growth assays were performed on 4 +MSC and 4 –MSC wells. These inserts were transferred to an empty 24-well plate so that AlamarBlue would measure only MCF7 growth, with medium in the outer well to avoid drying the gels while performing the assays. The procedure for readings on days 1 and 4 is outlined in Figure 4-3; as much medium as possible was pipetted out of the inserts and 2 wells per condition were incubated with luciferin, while 2 wells per condition were incubated with AlamarBlue. Reagents concentrations were as described in Methods (2.2.2). 4 additional inserts (with gel and cells but no reagents) were transferred to the plate as a control to ensure the gel itself did not affect readings. All wells were read at wavelengths corresponding to both luciferase activity and

AlamarBlue. Readings were taken at 15, 30, 45, 60 and 90 minutes, incubating the plate at 37°C 5% CO₂ between readings as for normal growth.

After the 90 minute readings, the gels with added reagent were washed 3x with HBSS, then all inserts were replaced in the incubation plate containing the MSCs. A change of medium in all wells was also performed.

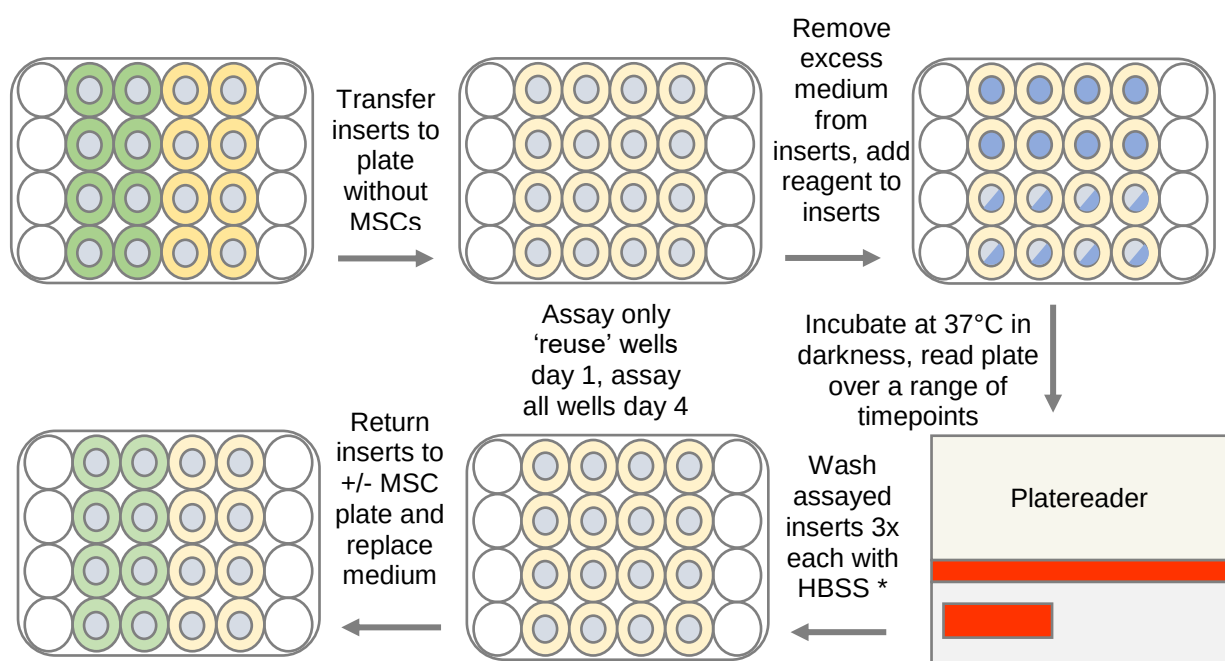


Figure 4-3: Flow diagram outlining data collection stage for comparison of Alamar Blue and Luciferase assays for peptide gels

The plates were then incubated again until day 4. Microscope images were taken as for day 1. All inserts were transferred to the temporary plate so that AlamarBlue readings would reflect only the MCF7 cells. Both AlamarBlue (ex 544 em 590) and luciferase reads were taken for the whole plate to determine how much detectable reagent remained in wells used on day 1. AlamarBlue and luciferase assays were then performed as on day 1, in both the wells used on day 1 and the wells reserved for day 4, at the same time intervals.

Day 1 AlamarBlue and luciferase assay reads taken at different timepoints following reagent addition showed that ideal incubation times (in which an increase in measured parameter could be detected without a disproportionately long increase in incubation time) were at 60 minutes for AlamarBlue (Figure 4-4A) and less obviously at 45 or 60

minutes for luciferase (Figure 4-4B). Luciferase assay reads also suggest an effect from co-culture at this early timepoint.

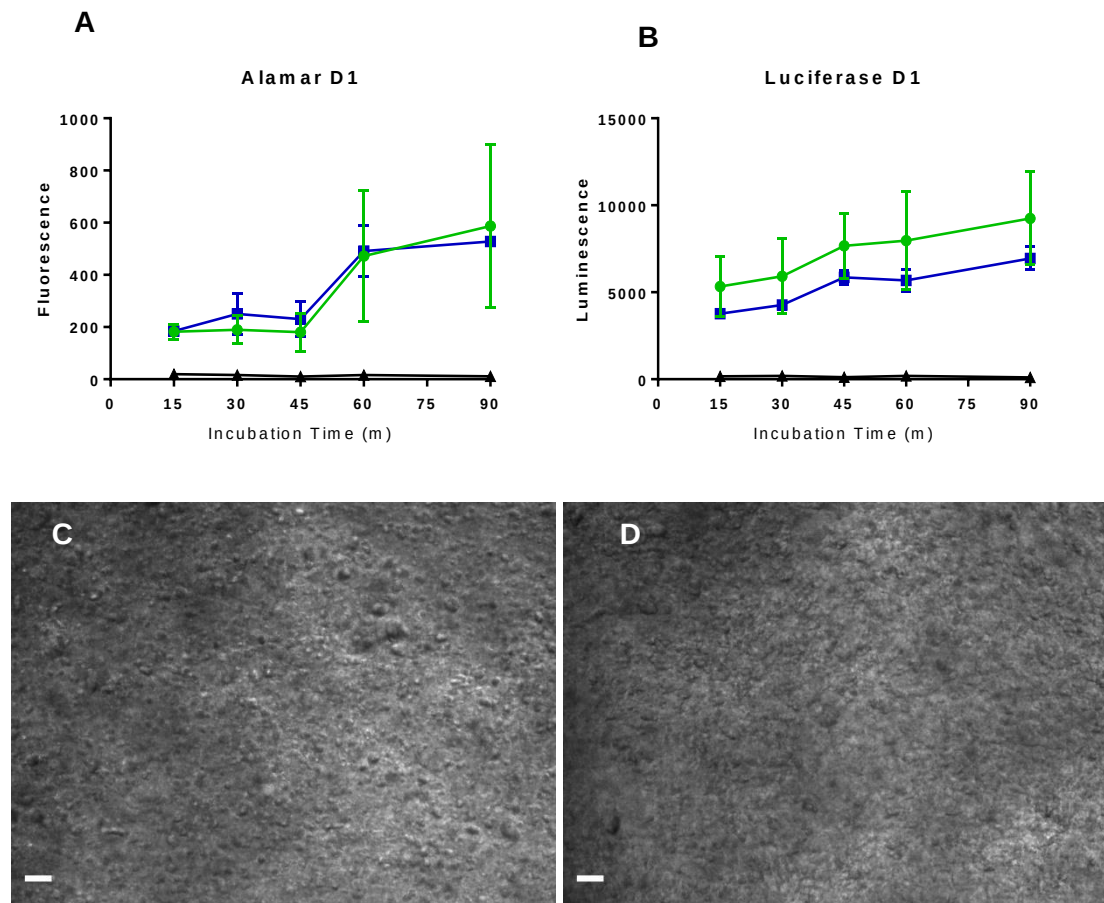


Figure 4-4: Ideal incubation times for MCF7 cells in peptide gel are 60m for AlamarBlue assay and 45m for Luciferase assay Alamar blue (A) and Luciferase (B) reads of MCF7 metabolic activity following increasing incubation times after 24 hours of growth in 10mg/ml peptide hydrogel with (green) or without (blue) MSC co-culture. Error bars show standard deviation calculated from 2 technical replicates. (C) Microscope image of MCF7 in indirect co-culture with MSCs in 10mg/ml peptide gel on day 1 (D) Microscope image of MCF7 in monoculture in 10mg/ml peptide gel on day 1. Representative images from 2 replicates taken using Nikon Eclipse widefield microscope at 4x magnification, scale bars 100µm

Microscope images showed no clear difference between conditions on day 1 (Figure 4-4). By day 4 some differences were visible (Figure 4-5), particularly with re-used monoculture wells appearing to have less growth than the other columns. Both co-culture conditions were more consistently well-populated than their respective monoculture conditions.

Assay results on day 4 were broadly consistent with these microscope images. The luciferase assay appeared to give much less variation within experimental conditions and therefore was able to completely distinguish the four conditions at day 4 (reading

at 60 minutes), with the reused wells showing the least growth and co-culture wells showing the most (Figure 4-6). The AlamarBlue assay was less conclusive (specifically because one point had a very large error) but also showed that the reused monoculture wells showed the least growth (Figure 4-6).

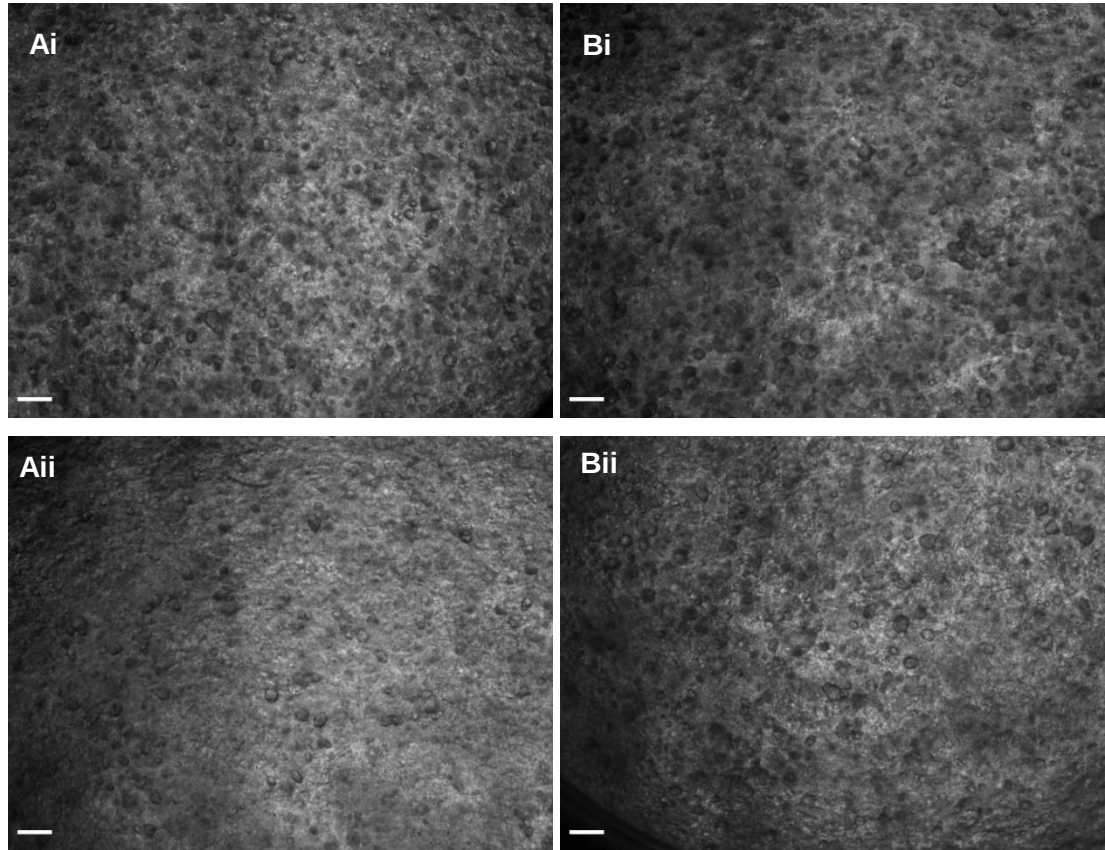


Figure 4-5: MCF7 cells survive and can be imaged in wells containing residual growth assay reagents from previous assays Representative images from 2 replicates on day 4 of MCF7 cells with (i) and without (ii) MSCs in indirect co-culture in 10mg/ml peptide hydrogel in phenol red-free RPMI in wells having been previously used for growth assays (luciferase and AlamarBlue have similar effects) on day 1 (A) or not (B). Scale bars show 200 μ m, images taken using Nikon Eclipse widefield microscope.

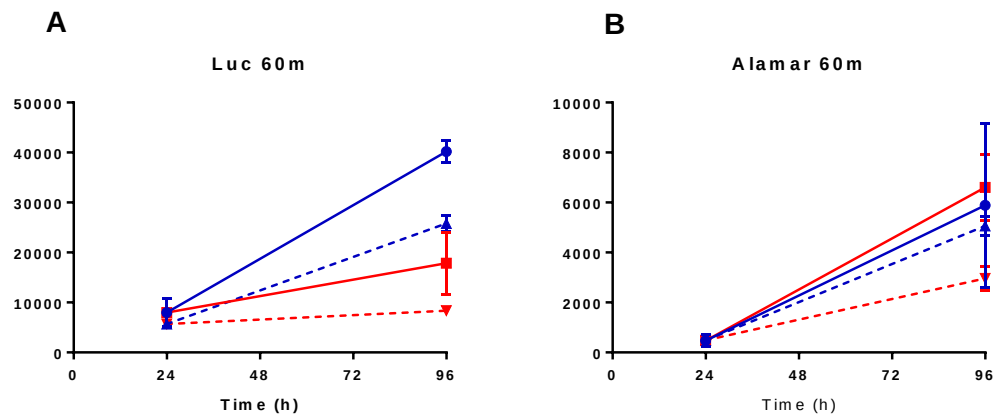


Figure 4-6: Luciferase assay reveals enhanced growth of cancer cells in co-culture not detectable by Alamar Blue assay Day 4 luciferase assay reads detecting MCF7 metabolic activity following increasing incubation times after 24 hours of growth in peptide hydrogel with (solid lines) or without (dashed lines) MSC co-culture. Blue lines show wells used for the first time at this timepoint, while red lines show wells previously used for measurements at the day 1 timepoint. Error bars show standard deviation calculated from 2 technical replicates.

A similar experiment was then performed to compare AlamarBlue with Prestobblue. The error bars on the AlamarBlue assay results were very large, which is not ideal for a PDX-based model in which starting material limits the number of technical replicates. PrestoBlue is advertised as having both shorter incubation times and greater sensitivity than AlamarBlue, though the mechanism by which this is achieved is not publicly known [172]. It was hypothesised that this altered composition might perform differently and possibly more consistently than AlamarBlue.

While the luciferase assay appears more consistent than AlamarBlue, having smaller standard deviation, it would not be possible for unmodified PDXs and was therefore omitted from this comparison. This experiment also provided an opportunity to assess using a greater number of technical replicates whether variation between replicates is consistently large, or if the variation observed for some wells in the previous experiment was an unusual event. Experimental setup was as before, with the following changes:

- PrestoBlue (20µl of solution per well provided at 10x) was used in the place of Luciferin.
- Based on the previous results a slightly shorter range of incubation periods was tested.
- All wells were assayed on both day 1 and day 4
- Three technical replicates were used per condition

Presto Blue signal was detectable more rapidly than AlamarBlue but after 1 hour incubation the reads were within similar ranges (Figure 4-7). Presto Blue also appears to show much greater consistency between technical replicates.

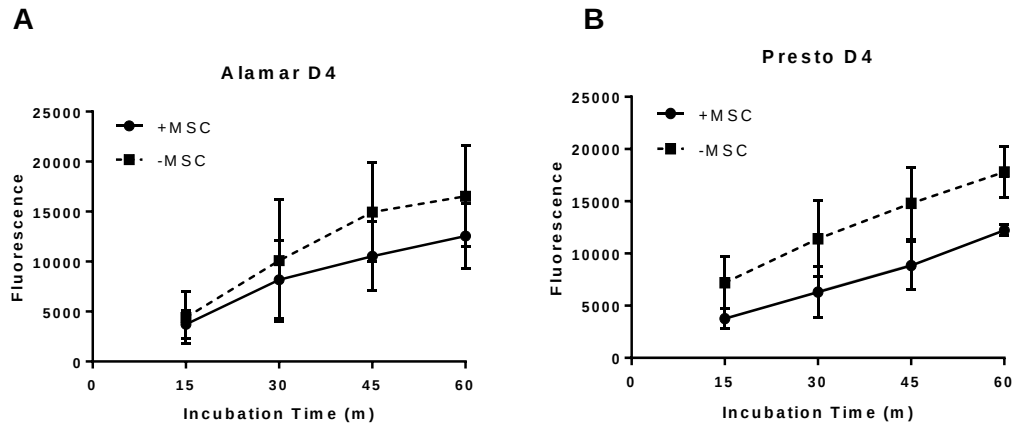


Figure 4-7: PrestoBlue gives reduced standard deviation in technical replicates compared to AlamarBlue Response of AlamarBlue (A) and Presto Blue (B) fluorescence to incubation times up to 1 hour when measuring growth of MCF7 cells after four days in peptide gel with or without indirect MSC co-culture. Error bars show standard deviation calculated from 3 technical replicates.

In this case co-culture with MSCs appears to slightly reduce proliferation, as measured by both assays (Figure 4-8).

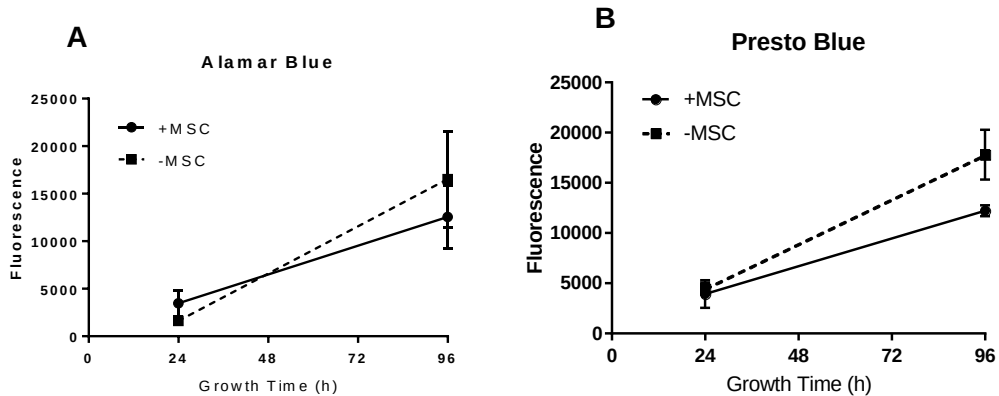


Figure 4-8: PrestoBlue assay continues to show reduced standard deviation compared to AlamarBlue following 4 days of growth Growth of MCF7 cells with or without MSCs in 10mg/ml peptide gel as measured by AlamarBlue (A) and Presto Blue (B) after 60 minutes reagent incubation. MCF7s grown in 10mg/ml peptide gel in phenol red-free RPMI with or without MSCs in indirect coculture. Error bars show standard deviation calculated from 3 technical replicates.

It was unexpected that the AlamarBlue readings for re-used wells, which represent identical experiments, showed different outcomes in the first and second set of experiments described here. In the experiment which compared luciferase and AlamarBlue, co-culture appears to increase growth (Figure 4-6), while comparison of

PrestoBlue and AlamarBlue, in which the AlamarBlue side of the experiment is set up exactly as it was for comparison with luciferase, shows the opposite result; reduced growth in co-culture compared to monoculture (Figure 4-8), suggesting that overall neither result is likely to be significant.

Four key conclusions are evident from this experiment:

- Any effect on MCF7 growth from indirect co-culture with MSCs cannot be determined with this limited number of replicates
- Even with washing, both luciferin and AlamarBlue have a toxic or otherwise growth-inhibitory effect on the MCF7s on a timescale of days
- AlamarBlue, PrestoBlue and luciferin nonetheless appear to be capable of measuring growth in the peptide gel (with 60 minutes as an ideal incubation time for AlamarBlue and PrestoBlue, and 45 minutes for luciferase)
- Two technical replicates per condition gives an excessively large standard deviation, particularly for AlamarBlue. Three appears to be an improvement but is still not ideal.

This suggests that ideal future assays would need to be performed using at least four technical replicates of each condition and fresh wells for each, though re-using wells does not appear to reduce ability to distinguish between different test conditions. Past examples of experiments comparing cancer cell growth with or without MSC co-culture in 3D have used BME as a support to achieve 3D distribution, raising the question of whether this effect would still persist in 3D were this growth factor- and binding-site-rich environment not present, in a 'blank slate' 3D environment more comparable to 2D culture [171]. Given inconsistent results and the possibility that indirect and direct co-culture inherently differ, these results did not confirm whether or not support of cancer cells by MSCs in 3D is dependent on the basement membrane extract typically used for such assays. However, unlike the inherently complex BME, the peptide gel format would allow for probing this question where called for.

Overall these results suggested that Presto Blue was the best option for reducing variation between technical replicates without requiring the cells whose proliferation is to be measured to be labelled, with the additional benefit of potentially being suited to a shorter incubation time and therefore less time spent with the cells exposed to the full assay concentration of reagent before the reagent is washed off.

4.2 Staining for Live and Dead PDX-Derived Cells in Peptide Gel

Another assay to be included in assessment of passage effectiveness within the gel was Live/Dead staining. Live/Dead staining gives information not about the metabolic activity of a well as a whole, as the assays described in section 4.1.2 investigated, but about the specific locations of live and dead cells in their 3D arrangement.

Before performing prolonged passage using optimised passage techniques, it was necessary to determine whether the Live/Dead staining kit previously used within the Merry Group in the gels was compatible with gels containing PDX-derived cells. This would make it possible to determine the condition of the PDX-derived cells growing in peptide gels, and identify whether time spent growing in peptide gel affected survival. Particularly of interest was a comparison of BR8 cells, which grew very rapidly into a large number of large cell clusters, and BB6RC160 cells, which in initial tests grew sparsely and slowly into small clusters at most.

The following samples were stained with live/dead stain:

- BB6RC160 cells grown in 10mg/ml and 8 mg/ml gels at passage 2 for 12 days
- BB6RC160 cells grown in 10mg/ml gels, unpassaged, for 15 days (derived from a different in-mouse passage)
- BR8 cells grown in 10 mg/ml gels, unpassaged, for 8 days

Growth times were selected based on expected and observed growth.

In all cases, the gel collapsed when extracted from the insert but staining and imaging of cell clusters did not appear to be negatively impacted by this collapse. In BB6RC160-seeded gels, some staining of both live and dead cells was visible, with the cells being distinguishable from the gel but not always clearly defined within clusters (Figure 4-9). Live/dead stain was consistent with what was visible in brightfield. In one BB6RC160 passage (P+2), brightfield showed that cells appeared unhealthy and fluorescence was difficult to identify as cellular shapes (Figure 4-9A and B). In the other (P+0), neither live nor dead stains were particularly evident, consistent with the fact that brightfield appeared to show mostly debris (Figure 4-9C).

In BR8 cells however, staining was bright and clear in both fluorescent channels, even with short exposure times around 1 second, and showed distinct live cells and

occasional dead cells in agreement with brightfield images, with imaging possible at 4x, 10x and 20x (Figure 4-10).

BB6RC160 survival appears to be limited in the gels. Low dead cell counts may simply reflect low overall cell counts from which dead cells can originate, relative to BR8 which grows very rapidly, producing a very large number of live cells and a small proportion of dead cells and very rapidly overcoming the structural integrity of the gel. It appears therefore that PDXs themselves do not present any barriers to clear and informative Live/Dead staining, but that a great deal of variation exists between PDXs when it comes to cell growth and survival in the gels. As BR8 is a triple negative breast cancer PDX and BB6RC160 is luminal, oestrogen supplementation at physiological levels may be required to improve BB6RC160 growth and survival, although it was not required for BR15 which is also ER positive.

In conclusion, Live/dead staining appears to be a highly informative technique which will not require further optimisation for work in the peptide gels with the PDXs. It appears highly consistent with estimated cell conditions judged from brightfield imaging and explains that the difficulty in passaging cells derived from certain PDXs is likely to be due to very low numbers of intact, live cells.

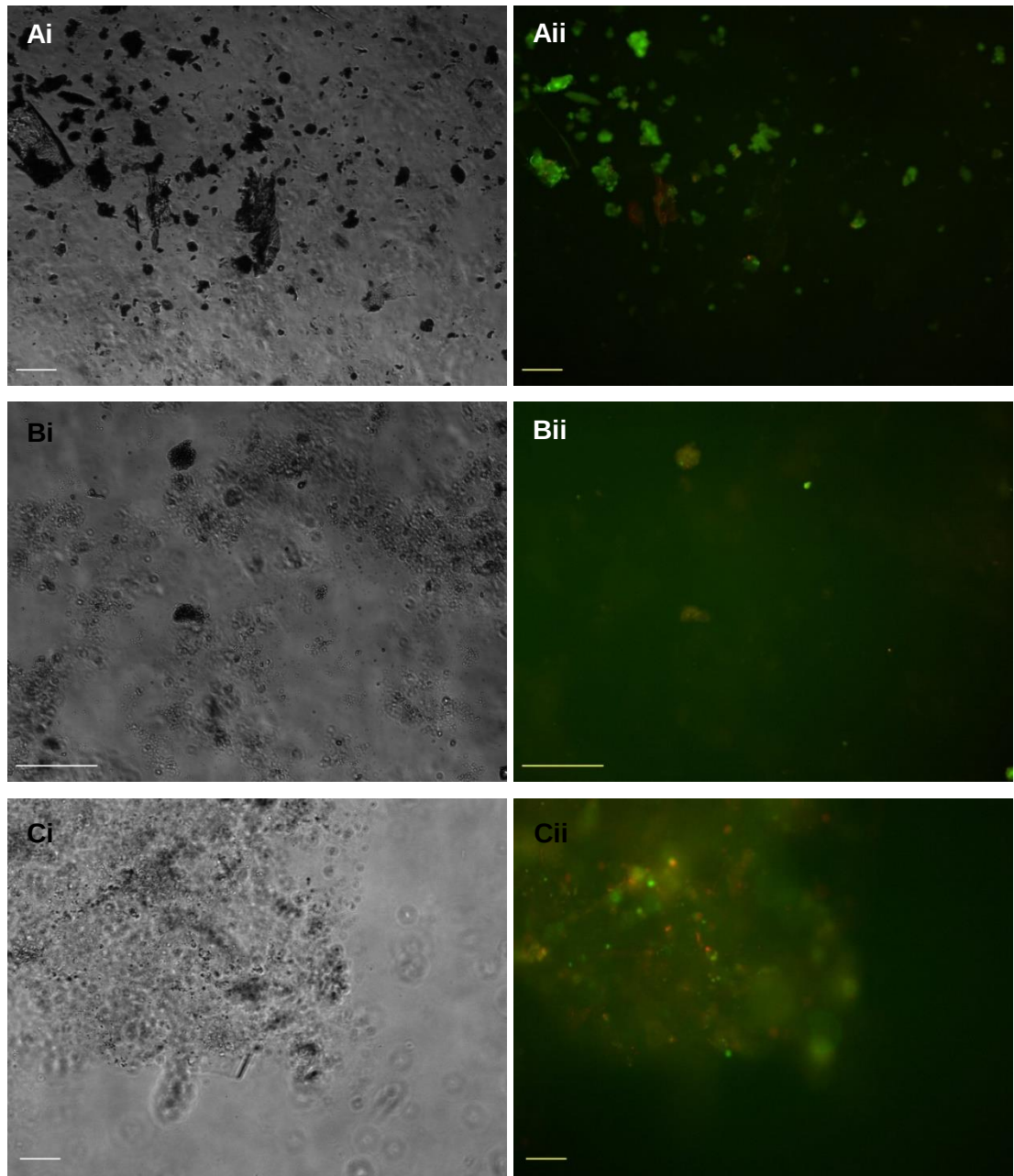


Figure 4-9: **Live/Dead staining of BB6RC160 cells grown in peptide gel shows sparse, scattered live and dead cells** Representative images from 2 replicates of BB6RC160 cells at P+2 grown for 12 days (**A and B**) and at P+0 for 15 days (**C**). Brightfield channel (**i**) and Live/Dead (green/red) channels (**ii**) are shown side-by-side. Scale bars are 100 μ m.

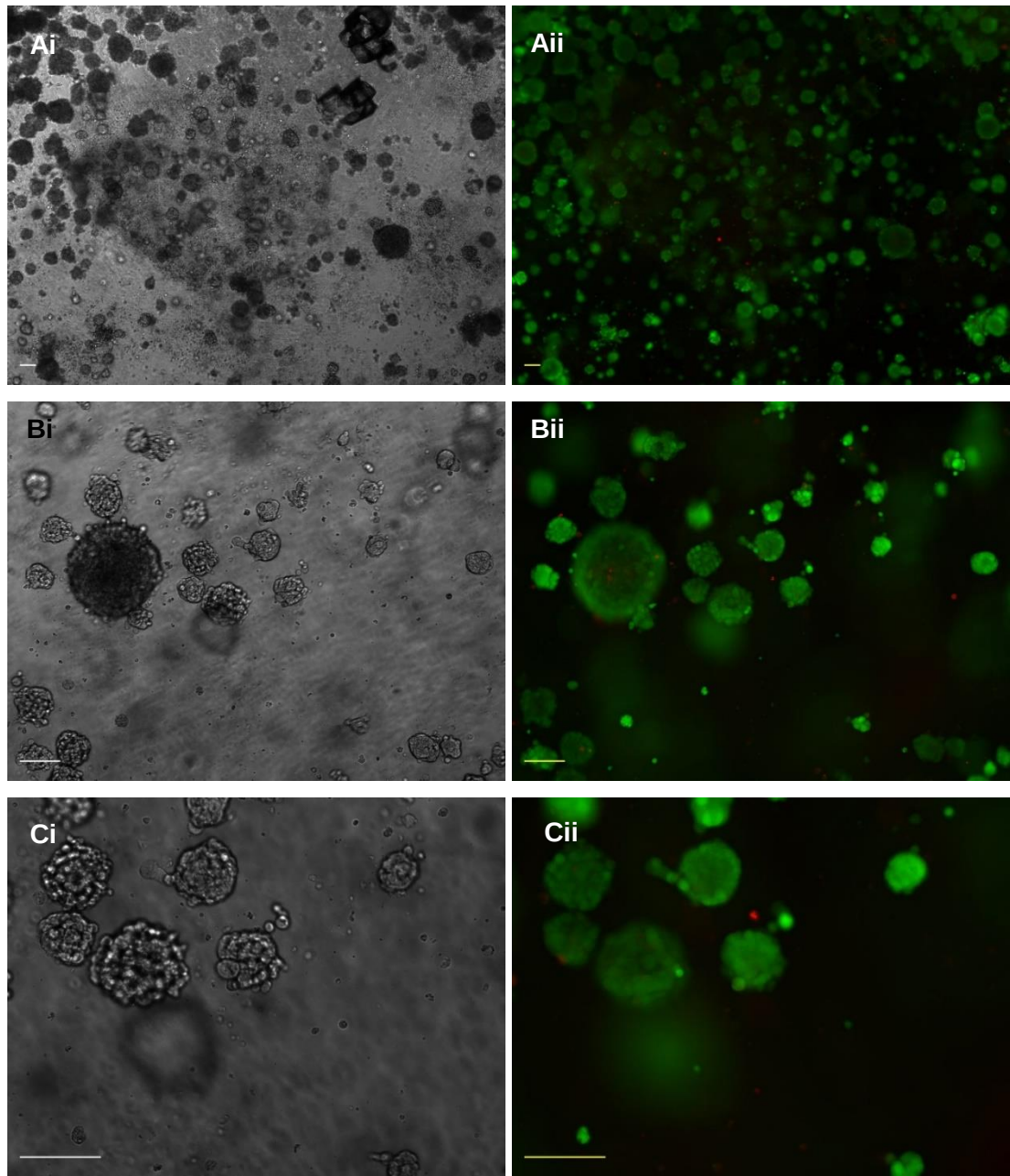


Figure 4-10: Live/Dead staining of BR8 in peptide gel is very clear and shows a large majority cells to be live and growing in near-spherical clusters Representative images from 2 replicates of BR8 PDX cells grown in gel at P+0 for 8 days, imaged at 4x (A), 10x (B) and 20x (C) magnification. Brightfield channel (i) and Live/Dead (green/red) channels (ii) are shown side-by-side. Scale bars are 100 μ m.

4.3 Ensuring that Human, not Mouse, Cells Survive Passage in Hydrogel Given Potential for Murine Contamination

The key goal of this project is to generate a model in which PDX-derived cells can be passaged over relatively long periods of time in the peptide gel. One potential problem may arise from the fact that the original tumour taken from the mouse to seed the gel will be contaminated with a significant percentage of murine cells. These are stromal cells which infiltrate the tumour and replace the human stroma rapidly following PDX establishment [173]. It is necessary to ensure that any cells which originate from a PDX and are being passaged over long time periods include at least a proportion of human cells following passage. Both the human (cancer) and murine (stromal) components may grow, and if the murine cells grow faster this would be a considerable obstacle to use of the model.

An experiment to establish that the species of surviving cells can be tested in material taken from the peptide gel was performed with Catherine Probert. Because of the species difference between the cancer cells and the contaminating cells, this could be done by amplifying a species-specific gene sequence with mouse- or human- specific primers in q-PCR. If compatible with samples taken from gels, this technique would be a quick, accessible method which could be used by any group wanting to test or optimise the model for their own PDXs. The primers used were based on those used by Ealba and Schneider [166] for the human ribosomal protein L19 gene. Due to differing bases at key points (bolded) they will only amplify DNA of the specified species.

Human Forward: CTGATCATCCGCAAGCCTGTGAC**G**

Human Reverse: G**CC**GGAAGGGCAGGCAC**AT**

Mouse Forward: CTGATCATCCGCAAGCCTGTGACT

Mouse Reverse: G**AC**GGAAGGGCAGGCAT**AT**

These primers had, prior to their use in this project, been validated within EVPC using controls of known species in a range of mixed ratios including generation of a control curve which continued to detect contamination as low as 10%. This experiment served to test the usability of DNA samples originating from cells grown in peptide gel for

qPCR assays such as this, and as an initial test of what species were present at differing passages.

The generation of the cell samples used in this project are described in section 5.1 and particularly 5.1.1 (beginning page 115). In addition to the no-template control (NTC), murine and human controls, these samples were:

- **BR8 P0**; direct from mouse having never been in gel, to judge the relative amount of cells of each species going into the gels in the first instance, and as an example of a real, mixed sample without any potential for changes due to the gel
- **BR8 P5**; a late-passage comparison to the above, to identify any ratio change over passage
- **BR8 P5***; as above, but extracted using a different DNA extraction kit, to confirm that this had no effect
- **BR15 P1**; to confirm that results are not exclusive to BR8-derived cells
- **BR15****; derived from the outer well of a BR15-containing insert

DNA was extracted using QIAmp DNA mini kit (with one duplicate BR8 P5 sample additionally extracted using GenElute™ genomic DNA miniprep kit for comparison) from cell pellets of excess PDX cells at passage between gels and immediately after disaggregation from the PDX itself. Extracted DNA concentration was determined using nanodrop, and 50ng DNA per well was amplified using the above primers.

Three technical replicates of each DNA sample/primer combination were performed.

Amplification was performed as described in Methods (section 2.4.1, page 33) followed by a melt curve from 60°C through to 90°C. Average Ct values were as shown in Table 4-1 below, with Figure 4-11 showing these values as fractions of human and murine cells (A) as well as melt curves demonstrating that no primer-dimer or off-target products are present (B and C). Consistency between technical replicates and clarity of negative versus positive results was good (Figure 4-12).

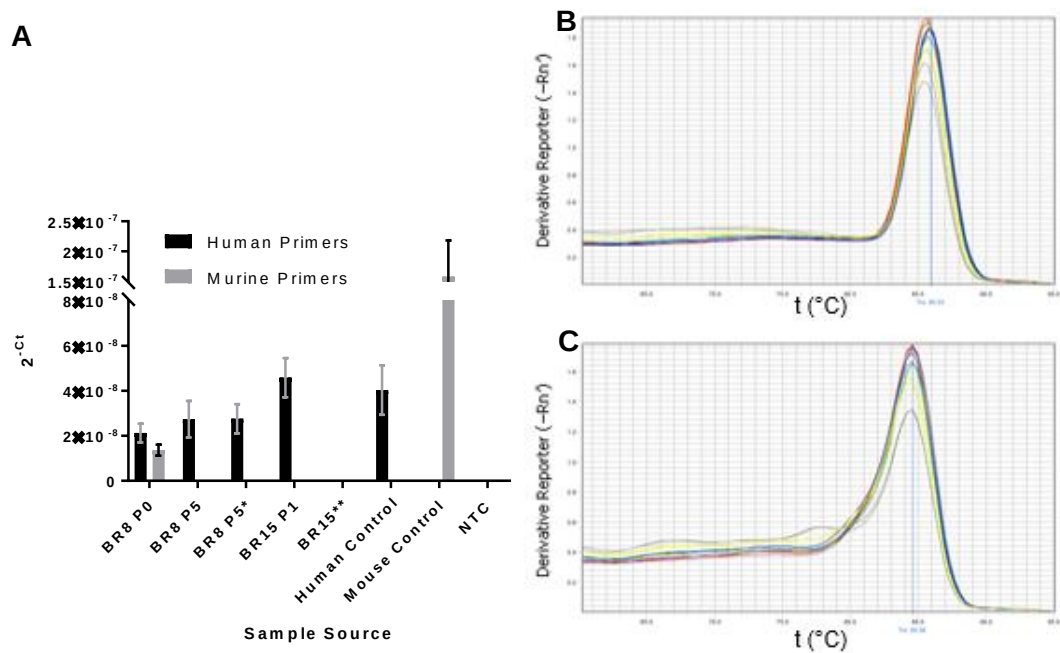


Figure 4-11: **qPCR can be used on samples taken from cells grown in peptide gels, and suggests contaminating mouse cells may be lost over passage** (A) 2^{-Ct} values for qPCR of DNA samples from mouse- and human-specific primers, qPCR performed in technical triplicate. * Indicates sample extracted using Sigma DNA extraction kit, ** indicates unknown sample taken from BR15 with suspected infection (section 5.1.1, Approach 6, page 128). Top quarter of Y axis adjusted to accommodate Mouse Control values while leaving other samples clear. (B) Melt curves for amplification of all samples using human primers (C) Melt curves for all samples using mouse primers.

Table 4-1: Ct of amplification of samples and controls using mouse- or human-specific primers 'X'
denotes primers with which the specified sample could not be amplified to the threshold

Sample	Human		Mouse		Species Present
	Ct	(2 ^{-Ct})	Ct	(2 ^{-Ct})	
BR8 P0	25.5	2.1E-08	26.1	1.4E-08	Significant human and mouse
BR8 P5	25.2	2.6E-08	X	X	Entirely human
BR8 P5*	25.1	2.8E-08	35.7	1.8E-11	Human, possible some mouse
BR15 P1	24.4	4.5E-08	37.8	4.2E-12	Human, possible some mouse
BR15 **	X	X	37.8	4.2E-12	Minimal human, minimal mouse (unknown)
Human Control	24.6	3.9E-08	38.1	3.4E-12	
Mouse Control	X	X	22.7	1.5E-07	
No-Template	X	X	38.6	2.4E-12	

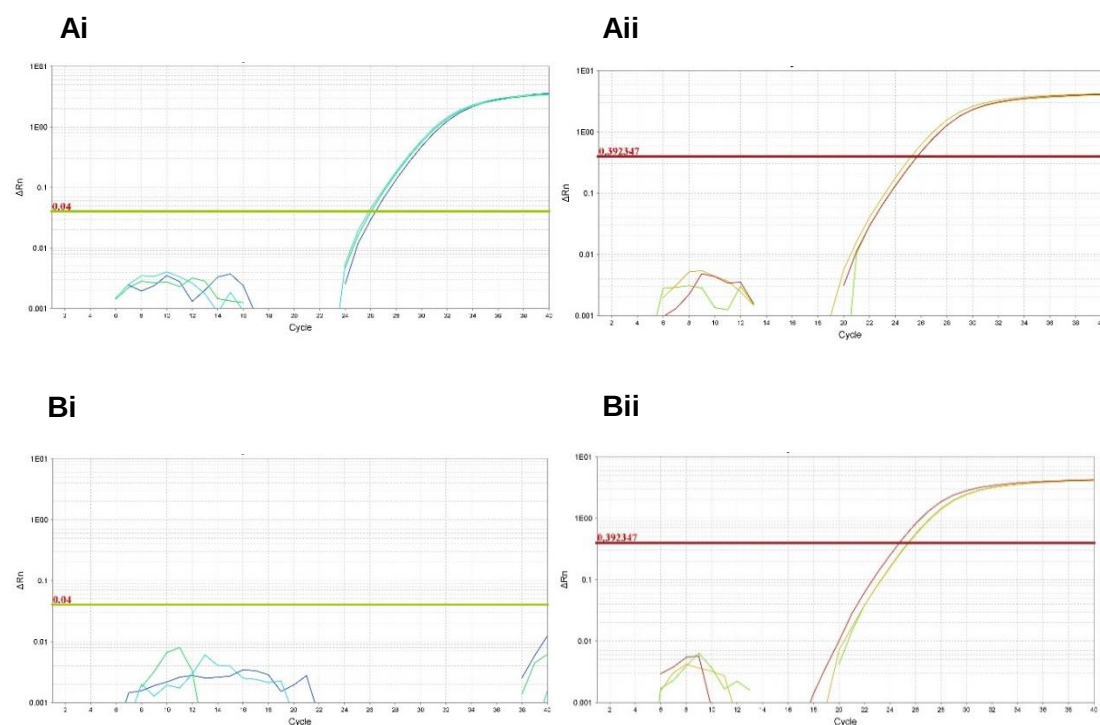


Figure 4-12: Example amplification curves show clear species testing results from DNA taken from PDXs or from PDX-derived cells passaged in gels. (A) shows qPCR results from DNA from un-passaged cells, while (B) shows cells passaged five times in the peptide gel – (i) shows murine primer amplification while (ii) shows human primer amplification.

These results suggest that immediately after removal from the mouse, the disaggregated PDX contains significant contamination by mouse cells (around half of cells are murine), but this contamination decreases to very low levels (less than 10% at most) throughout passage in the gel (Figure 4-11). There is a large range of Ct values between the positive and negative controls where no sample Ct values fall, relative to the spread of values between replicates, allowing fairly confident conclusion of which samples contain cells of which species. The two BR8 P5 samples handled using differing DNA extraction kits were in full agreement, suggesting that the peptide gel model is broadly compatible with commercial DNA extraction kits and use of these differing kits should have no effect.

That the assay was able to identify BR15**₂; derived from the outer well of a BR15-containing insert which PDX-derived cells ought not to be able to access from the insert and therefore strongly suspected to be an infection, as being neither human nor murine, was a good indicator of the assay's flexibility in accurately reflecting species composition.

The outcome of this testing, particularly the implication that only human cells will persist, was very informative and promising for future work. While the level of contamination in the straight-from-mouse BR8 P0 sample was high, it was all but undetectable in the P5 samples. It is not unexpected that cancer cells, already adapted for survival at all costs, would out-compete stromal cells which are bound by a multicellular organism's controls on speed and timing of replication. This suggests it will not be difficult to improve the humanness of the model in comparison to passage in mice, and reduces concerns associated with obtaining a pure human cell population at the end of passage. It is possible that this is simply consistent with behaviour of MSC-cancer co-cultures in BME, in which MSCs are observed not to grow – they need not necessarily die to be rapidly lost in passage. Alternatively a mixture of activated and inactivated stromal cells may be required to maintain the population [174]. On the other hand, if the reason that the contaminating murine cells have died is because they are not transformed, cancerous cells this may be indicative of problems to come if it proves necessary to directly co-culture human stromal cells alongside the cancer cells. Importantly, this experiment additionally demonstrated the compatibility of the peptide gel model with qPCR and specifically with testing of sample species by qPCR.

Both the human and NTC controls gave a Ct value for mouse primers. However, no Ct values fell between 27 (which all positive controls were below) and 35 (which all negative controls were above), giving a very clear distinction between positive and negative results and indicating that any Ct value above 35 is definitely negative. Further repeats to determine how consistent these values are between PDXs and between biological replicates were later performed in Section 5.2, page 152.

4.4 Exploration of Methods to be Used to Test Heterogeneity

4.4.1 Investigation of High Resolution Melting for Heterogeneity Testing

One concern when passaging PDX- or patient-derived cells for long periods is loss of heterogeneity, in which the variation between cell types in the patient tumour is lost due to selective pressures of the new setting. As this heterogeneity may contribute to treatment resistance, it is important to retain as much as possible to ensure that the behaviour of the generated cells (for example a drug response) is reflective of how the patient's tumour would respond as a whole, rather than just the response of a subset of tumour cells. High resolution melting was explored in this experiment as a method to detect heterogeneity at the gene level. It was used to identify whether heterogeneity existed in pre-passage samples, and if so whether it persisted into later passages.

High resolution melting (HRM) takes advantage of the differing energy requirement to melt different base pair combinations to identify (if not distinguish) point mutations in short PCR products. Dyes binding only double-stranded DNA will become progressively less bound in a sample of PCR-produced ds-DNA as it is melted into single strands, producing a melting curve reflective of the bases in the amplified region. These vary slightly between point mutants and can be compared with known mixtures of positive and negative controls to establish the prevalence of an allele within the sample; depending on the genes involved, HRM has been found to detect mutated DNA at 1-12.5% levels [175-177].

It would be expected that a homogenous sample in which all cells were wild-type, homozygous mutant or heterozygous mutant would match samples of 0, 100% and 50% positive and negative controls respectively. Heterogeneous samples on the other hand might have melting profiles intermediate between these shapes.

HRM was therefore performed, in collaboration with James Hassall, in order to establish whether it would be a feasible technique to identify, measure and compare

heterogeneity in PDX-derived tissue before and after passage in peptide hydrogel. This would require, on a broader scale, cells retrieved from passage in peptide gel to be compatible with the reagents and techniques and to produce an interpretable curve. In terms of this project it would additionally require at least one of BR8 and BR15 to show a mutant allele frequency other than 0%, 50% or 100%. This would indicate heterogeneity in the sample which could then be tracked for maintenance or loss over passage in the peptide gel. A mutant allele percentage indicative of homogeneity in non-passaged samples isn't necessarily a cause for concern, as the mutation frequencies in the breast cancer population are not exceptionally high, but it would necessitate identification of a panel of possible mutations which is not achievable on the timescale available.

Two regions of PIK3CA were identified as frequently mutated in breast cancer based on information from COSMIC; one in exon 10 (where exon 1, not included in the protein, is counted; otherwise exon 9) and one in exon 21 (again counting exon 1; otherwise exon 20). These very small regions have mutation rates of approximately 10% and 15% respectively in patients.

HRM Primers for these two regions were available (Section 2.4.2 page 33), already optimised and accompanied with positive and negative controls, so it was possible to perform a pilot experiment in order to establish whether it would be a feasible technique to identify, measure and compare heterogeneity in PDX-derived tissue before and after passage in peptide hydrogel.

DNA from the five non-control samples described in Table 4-1 were initially amplified by PCR in a reaction including EvaGreen dye as described in Methods (Section 2.4.2 page 33).

Each sample was run with primers for each mutation hotspot (exon 10 and exon 21) in duplicate plus two NTCs, three positive controls and three negative controls (HEK as negative, MCF7 as exon 10 positive, HCT 116 as exon 21 positive) per primer set.

BR15** was not human and could therefore not be amplified at all. BR8 P0, BR8 P5, BR8 P5* and BR15 P1, all shown in 4.3 to contain human DNA, amplified successfully and were identical to the negative control HEK in exon 10. Exon 21 could not be amplified in any sample.

Exon 10 is therefore not a useful target site for these PDXs (though it may yet be for others), and E must originate from some form of contamination. Exon 21 was later repeated with a different master mix.

A separate species testing experiment confirmed E was definitely not human and therefore it was omitted. As C only differed from D in DNA extraction kit used, and previous experiments showed it to be comparable to D, it was also omitted. Exon 21 primers were therefore only re-tested on samples A, B and D, using a different reaction mixture as described in Methods (Section 2.4.2 page 33).

On this occasion, positive controls were included as 50% and 12.5% mixes with the negative control, representative of intermediate cases between homogenous homozygous wildtype cases and homogenous homozygous mutant cases.

It was found that the melt curves of the exon 21 mutation hotspot amplified from A, B and D all resembled the 50% mutant/wildtype mixture very closely. While this may represent consistent, maintained heterogeneity, it is also indistinguishable from homogenous heterozygosity, a far more likely outcome and not useful at all for monitoring presence or maintenance of heterogeneity.

This experiment demonstrated that PDX-derived cells retrieved from passage in peptide hydrogel are suitable for DNA extraction followed by PCR and HRM, suggesting broad compatibility with DNA-centric molecular biology techniques and the potential for heterogeneity testing using HRM in general. Both primer sets detected homogeneity in all samples; wildtype in exon 10 and with a heterozygous mutation in exon 21. These two particular mutation hotspots are therefore not useful for monitoring heterogeneity in samples derived from these specific PDXs. It was concluded that while HRM is very likely to be sufficiently sensitive to detect heterogeneity and its maintenance, a large panel of optimised, cancer-relevant amplification sites would be required to have a good chance of obtaining meaningful results. This would not be an implausible goal, and might be particularly useful for cancers associated very strongly with mutations at specific sites of specific genes, but would require a body of work far beyond the scope of this project, particularly given the relatively extremely low density of breast cancer mutations in regions other than those probed here. While many such mutations exist they are both rare enough and sufficiently well spread out in the genome that a PCR product suitable for HRM might capture a mutation in around 3%

of breast tumours at best; even a fairly large panel of primers might identify mutations in only a relatively small fraction of tumours [178].

4.4.2 Investigation of Immuno-Staining and Imaging for Heterogeneity Testing

Immunostaining and imaging of gels was required to allow assessment of cell growth and surface marker expression during optimisation, and therefore was important not only during this project's initial development of a model using breast cancer cells, but also for any later work required to adapt the model to other tissue types or purposes.

An experiment was performed to test immuno-staining procedures for peptide hydrogels and to observe cell types and numbers present after 7 days of growth when MCF7 cells are co-cultured with immortalised MSCs.

2 wells each were seeded according to peptide hydrogel protocol with no cells, 5×10^5 MCF7 cells or 5×10^5 MCF7 cells and 2.5×10^5 immortalised MSCs. They were grown according to protocol for 7 days. Images were taken on days 1, 4 and 6 to confirm the presence of cells.

These gels were stained on day 7 with CK18, actin (phalloidin) and nuclei (DAPI) as described in Methods, Section 2.2.5 page 31. Immediately after staining, three 10x images of each experimental condition and one of the control were taken using Nikon widefield microscope using channels for Cytokeratin 18 (eGFP channel), Phalloidin (tdTomato channel), DAPI and brightfield. A z-stack image and some manual images of the same XY position at different Z heights were included among these images.

20x magnification images were taken the next day of mono- and co-culture wells, though the small gel fragments in the co-culture wells drifted during exposure times and made the images blurry. 40x images of monoculture were also taken.

8 days later, the gels having been stored in PBS at 4°C, a confocal microscope was used to take images of the gels at 10x magnification; exposure times of 0.2 to 2 seconds were used.

The gels were very fragile, which somewhat limited imaging quality in the following ways:

- The gels could not be moved from the 24-well plate
- The gels remained in PBS and therefore drifted slightly during long exposures

- Fragmentation prevented imaging of the intact gel

However, imaging was achievable and fragmentation may in fact have improved visibility by reducing gel depth – many fragments were still large enough to represent a large number of cell clusters and their arrangement relative to one another clearly. There was a clear difference between cell cluster shapes when comparing monoculture gels with co-culture gels (Figure 4-13, both images) suggesting that co-culture may have an effect on 3D arrangement of cells.

The gel itself was visible in brightfield and may be showing some staining in the DAPI channel but generally images were quite clean and evenly stained, with the main barrier to visibility being the inherent 3D nature of the system forcing some aspects out of focus (Figure 4-14).

Observing images taken using a confocal microscope separately for the monoculture shows imperfect staining of the centre of some cell clusters with antibody, whereas Phalloidin, a much smaller molecule, penetrates completely (Figure 4-15).

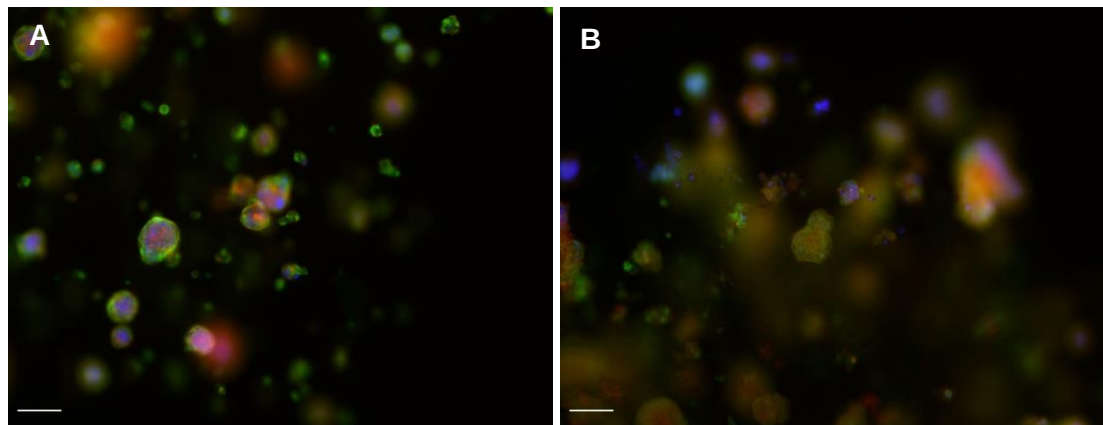


*Figure 4-13: **Cell cluster shape appears to differ between MCF7 monoculture and co-culture with MSCs** Representative images from 2 technical replicates of MCF7 grown for 7 days in peptide gel in RPMI with (B) and without (A) immortalised MSCs. Stained for DAPI (blue), cytokeratin 18 (green) and Phalloidin (red). Images take using Nikon Eclipse widefield microscope, scale bar 100µm.*

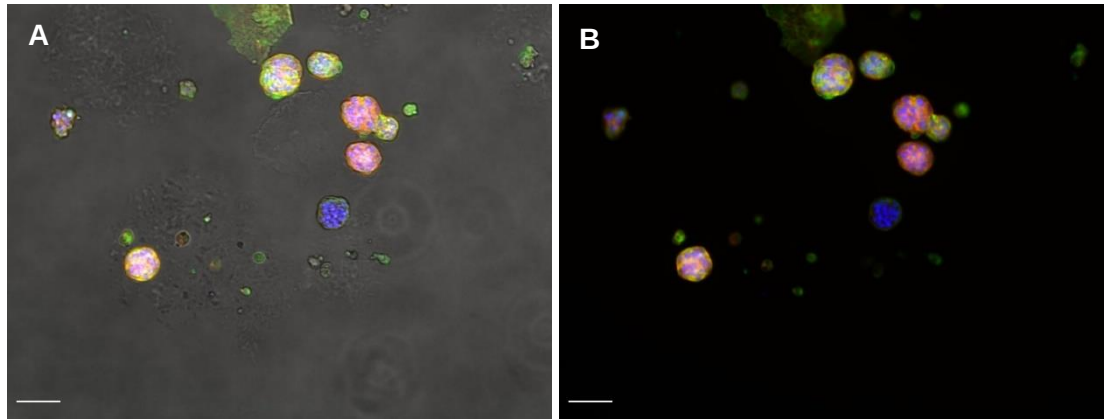


Figure 4-14: **Stain strength and cluster penetration varies between cell clusters but clearly picks out cells from gel/background** Representative image from 2 technical replicates of MCF7 cells grown for 7 days in monoculture in peptide gel in RPMI showing fluorescent channels plus brightfield (**A**) and only fluorescent channels (**B**). Stained for DAPI (blue), cytokeratin 18 (green) and Phalloidin (red). Images take using Nikon Eclipse widefield microscope, scale bar 100 μ m.

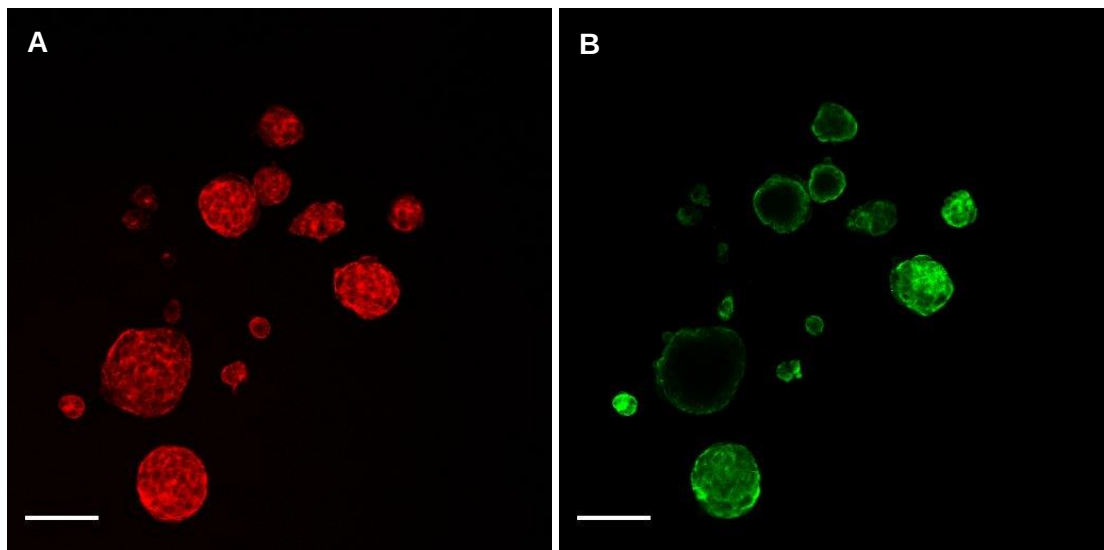


Figure 4-15: **Phalloidin stain penetrates cell clusters completely while anti-CK18 antibody penetration is incomplete** Phalloidin (left) and CK18 (right) channels of the same image. Cells are MCF7 monoculture grown in 10 mg/ml peptide gel in RPMI for 7 days using confocal microscope at 10x magnification, scale bar shows 100 μ m

These three stains and the protocol used here appeared to work effectively, were not excessively challenging and with optimisation to improve gel integrity should be possible to adapt to other stains for a range of features such as HER2 and other

treatment-relevant hormone receptors. A higher percentage triton in the blocking/antibody dilution buffer may be necessary to improve antibody staining. Additionally, the microscope used has not previously been used for this purpose and appeared to perform well without any additional techniques or training compared to techniques and functions used for imaging other experiments.

Both cytokeratin-negative cells (MSCs) and cytokeratin-positive cells (MCF7s) survived in the co-culture, which had not previously been confirmed for this combination of cell types in co-culture in the hydrogel. Cytokeratin-negative cells were more challenging to confidently identify as eGFP-channel fluorescence is not always evident (particularly in the centres of cell clusters) even in the monoculture where all cells should be CK18 positive. There are some clear single-cells in the co-culture wells which would certainly have stained with CK18 antibody if they contained CK18 and yet appear not to show any eGFP-channel fluorescence, indicating that these cells are definitely MSCs. The changes in growth with MSC inclusion also seem to support previous data suggesting that stromal cell inclusion produces significant changes which may affect experimental and patient outcomes.

4.4.3 Investigation of ImageStream X Compatibility for Heterogeneity Testing

Imaging flow cytometry has potential to be a powerful tool for single-cell interrogation of cells samples which could, with optimisation, give an extremely detailed comparison of direct-from-PDX cell samples with those which have been passaged in the peptide gel. This would allow a multi-faceted investigation into maintenance of heterogeneity at different stages of expansion.

A pilot experiment was performed in mutual discussion with David Onion to assess whether the disaggregation process used to prepare PDX for seeding in the gel create an adequately disaggregated suspension that can flow through the Imagestream X imaging flow cytometer and be usefully assessed by the device.

A BR8 PDX was disaggregated according to the standard protocol for doing so and the resulting cell suspension halved into two Eppendorfs. One was stained live with DAPI and anti-human HLA antibody, and the other fixed and later stained with propidium iodide for cell cycle analysis.

Flow cytometry data from the live sample showed that while disaggregation was sufficient to produce a good number of live, single-cell events (in addition to large

numbers of multi-cell clusters and small fragments), staining (both DAPI and human HLA) was only identifiable at low levels and likely reflected a need for further optimisation (Figure 4-16A and B).

The fixed sample showed better staining, and while only relatively few (around 1000) intact, in-focus single cells were detected it was possible to establish cell cycle information from these events, demonstrating a high cycling population reflective of a fast-growing tumour (Figure 4-16C and D).

While it was not possible to complete this experiment with a post-gel-passage sample for comparison, the outcome was nonetheless informative in that it demonstrated that the disaggregation technique used was adequate to generate interpretable events using flow cytometry equipment and that the BR8 PDX was compatible with the methods used, though staining of live cells might require some additional optimisation in the event it were to be taken further.

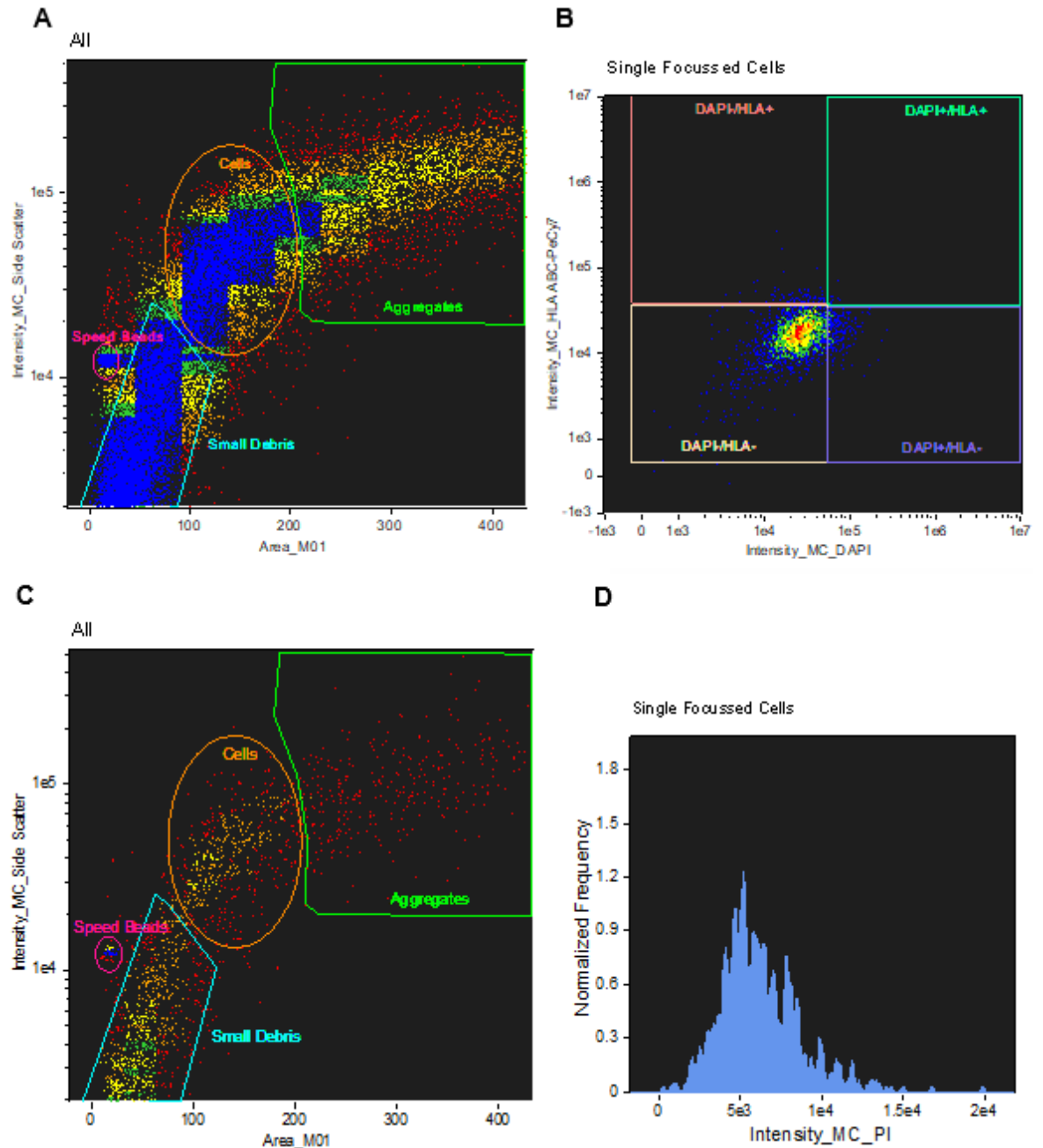


Figure 4-16: *Sufficient single-cell events can be detected by flow cytometry to perform further analysis on cells disaggregated from PDXs. (A) and (C) show distributions of single-cells, debris and aggregates in live and fixed samples respectively, while (B) shows weak DAPI and human HLA staining in live cells and (D) shows PI staining in fixed cells.*

4.5 RNA Sequencing assessment of Fibroblast Types to Improve Understanding of Co-culture Effects

One key question in design of *in vitro* cancer models including a co-culture component intended to mimic the effect of cancer-associated fibroblasts is which cell type to include, and whether that cell type continues to behave in the same way following co-culture with cancer cells. The literature is somewhat divided on the origin of the stromal cells found in patient tumours, with some of this debate attributable to differences in different tumour types [179], which therefore leads to ambiguity in terms of which origin cell type should be included if tumour-type-specific patient-derived CAFs cannot be sourced and/or practically combined with the model. As bone marrow-derived MSCs were available within the group, and have been shown to be capable of becoming CAFs when cultured with cancer cells [180], an experiment was performed to further probe the new identity and behaviour of these cells to better understand effects co-culture might have in new contexts like the peptide gel or in indirect co-culture. This experiment provided context to guide the use of alternative cell types if primary MSCs proved not to be either practical to use or supportive to cancer cells when removed from the highly supportive 3D-TE format.

A pilot RNA Sequencing experiment was performed with several purposes:

- Assess similarity of immortalised and non-immortalised TE67s
- Anticipate potential differences in co-cultures that may arise from differing cancer subtypes
- Further probe effects of co-culture on stromal cell types.

Non-immortalised MSCs and TE67 immortalised MSCs were conditioned for 3 days (after 24 hours to attach to the flask in their standard medium) with conditioned medium. Conditioned medium was generated by filtering MSC medium incubated with MCF7 cancer cell line, MDA-MB-231 cancer cell line, or the same MSC type for 3 days. MSC-conditioned media served as a control to allow for nutrient depletion without the effect of another cell type. RNA was extracted following conditioning of cells, using a Trizol extraction, and sent to Novogene for sequencing where 5 samples proved to be of adequate quality for sequencing:

- MSCs conditioned with MCF7-conditioned medium
- MSCs conditioned with MDA-MB-231-conditioned medium

- TE67 conditioned with TE67-conditioned (control) medium
- TE67s conditioned with MCF7-conditioned medium
- TE67s conditioned with MDA-MB-231-conditioned medium

Novogene provided BAM files, from which Counts files were generated using Galaxy and combined in Excel (along with column headers to define variables) to be further analysed using NetworkAnalyst. Due to the absence of true technical replicates the only comparison for which significantly differing gene expression could be identified was between TE67s and non-immortalised MSCs – here the matched conditioned media acted as technical replicates. Either there was very little difference between MCF7 and MDA-MB-231 conditioning, or it was simply insignificant compared to the difference between MSC types.

Three statistical methods were available for analysis within NetworkAnalyst – Limma, DEseq2 and EdgeR. Initially, the top 50 differentially expressed (DE) genes as identified by these techniques were compared to identify genes which all three techniques were in agreement upon. 6 such genes were identified:

- CADM3
- CCND2
- CHST2
- KIF11
- TERT
- WDR76

Given that overexpression of TERT is the deliberate genetic modification that differentiates these cell types, it is very reassuring to find that all three methods identify it as having changed between the cell types. CCND2, KIF11 and WDR76 are likewise associated with cell cycle processes, which might be indirectly affected by immortalisation, while the remaining genes, CADM3 and CHST2, are associated with cell adhesion and keratin sulfate synthesis respectively.

EdgeR was then used to further investigate changes in pathways and genes between the conditions due to its high ranking of TERT, effectively a positive control gene, among altered gene expressions, as well as its assumption that many genes would not be differentially expressed, which was considered reasonable in the context of the experiment [181].

An Over-Representation Analysis (ORA) Enrichment Network for the dataset (Figure 4-17) highlights the pathways (as opposed to individual genes) which are most differentially expressed between the two MSC types. While expected pathways such as those involved in DNA Replication and Cell Cycle were indicated as significant, other pathway changes such as in those involved in cardiomyopathies and rheumatoid arthritis were also prominent.

One possible explanation for the upregulation of genes relating to several cardiac conditions is that it may be a side-effect of the selection process used in genetically modifying the cells to overexpress TERT. As not all cells will take up the modified DNA, an antibiotic resistance gene is included alongside the exogenous TERT, so that the corresponding antibiotic can be used to kill any cells which have not taken it up. High expression or function of endogenous cell membrane transporters that can also remove these toxic molecules are therefore selected for in addition to TERT expression, and like many proteins they may be associated with a variety of seemingly unrelated processes, in this case the cardiac conditions identified by the RNA sequencing.

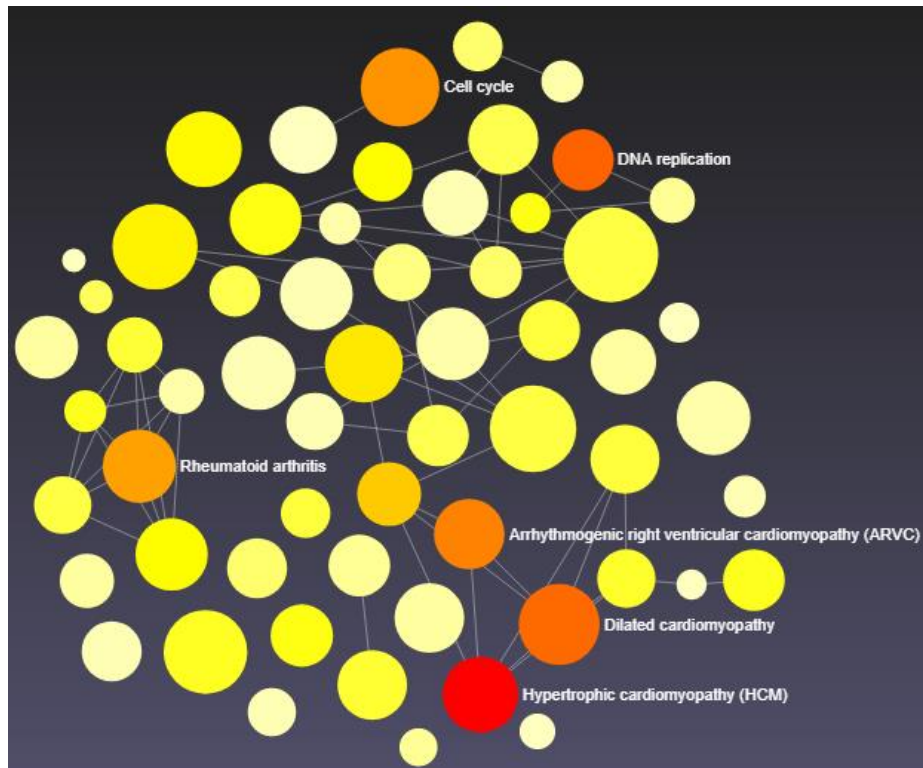


Figure 4-17: **ORA Enrichment Network** shows most differentially expressed pathways include **cell cycle**, **DNA replication** and **cardiomyopathies**. Red and darker orange circles show pathways with greatest differential expression between primary MSCs and TE67s, while white and lighter yellow circles show those with lesser differential expression.

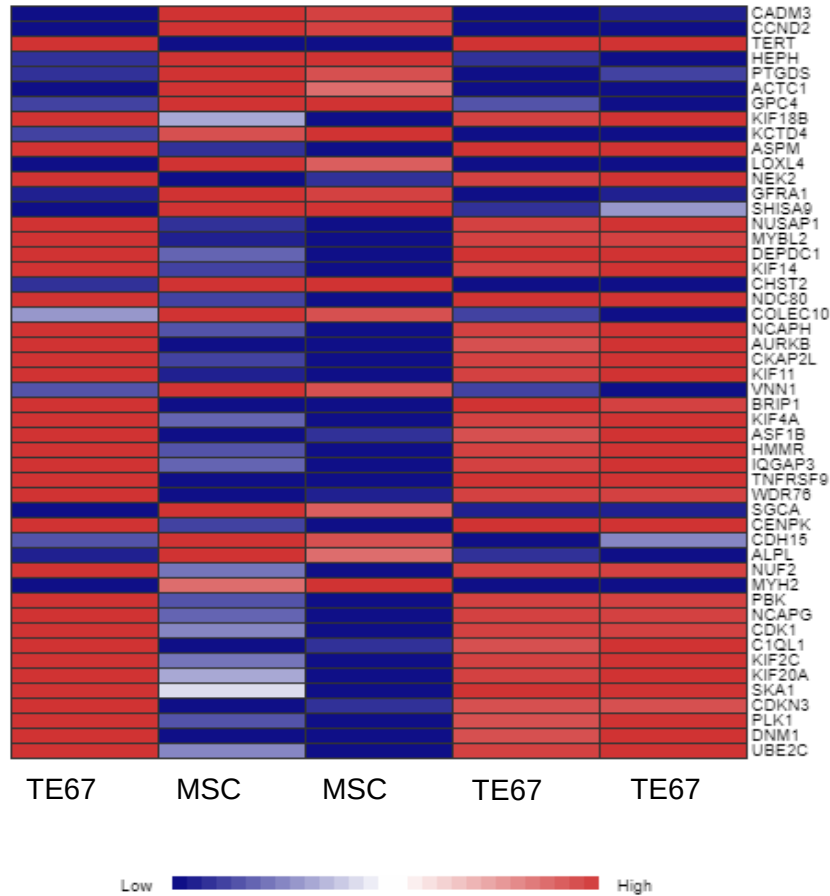


Figure 4-18: **GSEA Heatmap of most DE genes (comparing MSC and TE67) for all samples shows contrast between MSC types** Each sample labelled as the media-conditioning cell type in brackets.

Gene Set Enrichment Analysis (GSEA) Heatmap Clustering shows gene changes in specific, highly differentially-expressed genes for all five samples (Figure 4-18), and while the most visible pattern is the clear distinction between the MSC types as evidenced by earlier stages of analysis, it is also of interest that in some cases there are very clear differences between co-culture conditions for one MSC type but not the other, for example between differently-conditioned TE67s for SHISA9 and between differently conditioned primary MSCs for SKA1. This suggests that there may in fact be occasional effects from conditioning that vary between MSC types and therefore are hidden by the averaging of the two MSC types.

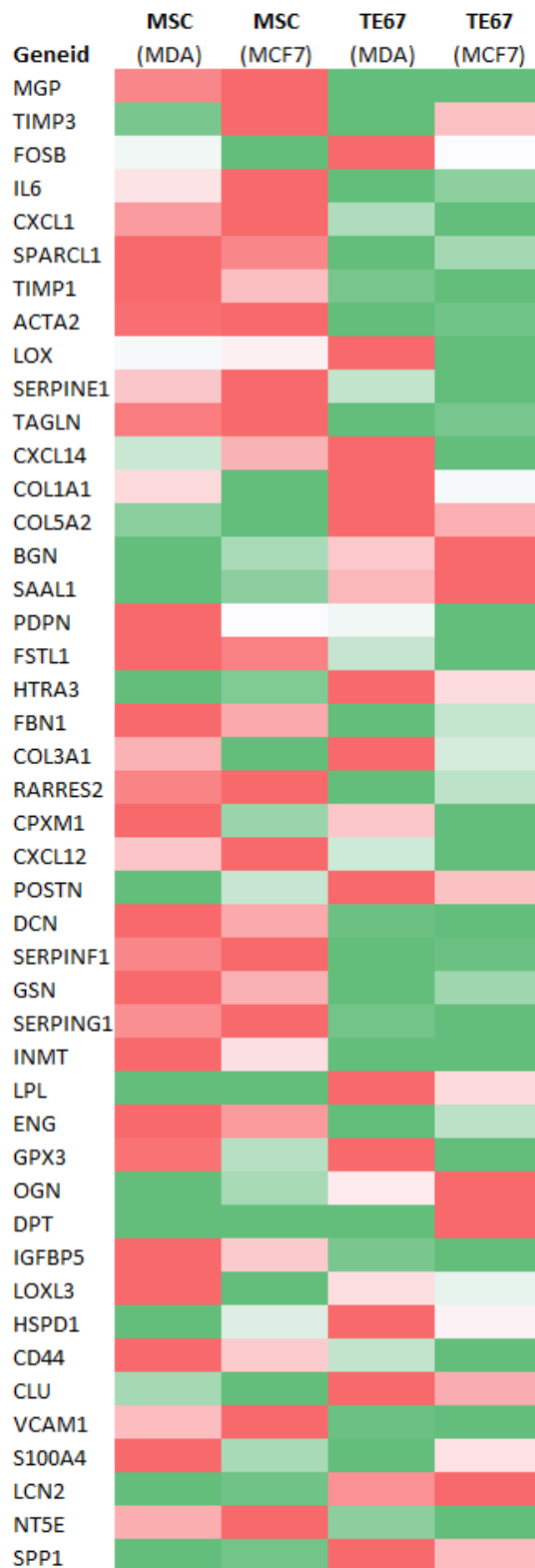


Figure 4-19: **Heatmap showing changes in expression of CAF-related genes highlights contrast between MSC types** Red shows higher counts and green shows lower counts. Topmost headers show the MSC type sequenced, while (MDA) in the column header indicates cells conditioned with MDA-MB-231 conditioned medium, while (MCF7) indicates equivalent conditioning by MCF7 cells.

It was of interest to take a closer look at a series of genes identified by Friedman *et al* as being particularly associated with CAFs (Figure 4-19)[182]. Four genes identified by Friedman *et al* were omitted from this analysis as they were not identified within these samples – this may reflect gene naming inconsistencies, extremely low copy number or lower depth of sequencing within this project compared to Friedman *et al*'s work. While many of these genes followed the general pattern of the dataset by differing primarily based on MSC type, it was interesting to note that S100A4 and PDPN, which Friedman *et al* identified as being linked to clinical outcome, did not clearly follow this pattern. Something that would be important to pursue further, given an opportunity to repeat this experiment in a less preliminary format with genuine technical replicates, is that many of these CAF-related genes are *more* highly expressed (red) in primary MSCs than in TE67s, indicating TE67s are less activatable.

A similar comparison was made using the same gene set to investigate whether any consistent effect from the tumour conditioning was observable. The percentage change from MCF7-conditioned to MDA-MB-231-conditioned counts were compared within MSC types, and then the two percentage changes for each gene were delineated as having different (one increased while the other decreased), similar (both increase or both decreased) or uncertain (counts were below 30 making percentage change very sensitive to precise count numbers) responses to conditioning. Of the 45 genes, 23 responded similarly to the different conditioning media in both MSC types, 17 responded differently and 5 were uncertain. A repeat with true technical replicates and non-conditioned controls for both MSC types would be required to make any firm conclusions, but the initial implication of this data is that the two conditioning types did not have a consistent effect between the MSC types.

In conclusion, while several potential avenues for further investigation can be identified, the only meaningful comparison that can be made from this experiment is between the two MSC types - relative to the permanent genetic alteration that distinguishes the MSC types, the effect of tumour conditioning is negligible. The extent to which any effect from different conditioning conditions can be observed depends on which analytical method (Limma, EdgeR, DEseq2) is used and the way in which the resulting data is presented. Additionally, the specific genes that are differentially expressed differ from analytical method to analytical method. TERT appears as a high-

ranked significant change regardless of method or presentation, which is consistent with expectations considering that the only expected difference between the two mesenchymal cell types was that one had been immortalised by introduction of exogenous TERT expression [183] while the other is not immortalised and only modified by addition of eGFP expression which would not be identified when seeking human RNA transcripts.

While the immortalised MSCs clearly differ from their non-immortalised equivalents, it is reassuring to know that the most prominent variations are among genes and processes that would be expected to be affected by the changes introduced to confer immortality. Changes to expression of growth factors and enzymes involved in generation of signalling molecules, which would be the most likely to affect how the MSCs affect cancer cells in co-culture, are not prominently evident regardless of the analytical method used.

4.6 Conclusions

In this chapter, the options available for characterising PDX-derived cells grown in the peptide hydrogel model were probed. The methods investigated were assessed for their compatibility with the peptide gel model and their ability to provide useful information in the context of the PDX-derived cells.

- For assessment of proliferation, PrestoBlue was identified as a useful reagent capable of producing reduced variation between technical replicates relative to AlamarBlue and not requiring potentially heterogeneity-reducing alteration of the PDX-derived cells as the luciferase assay does.
- For assessment of survival, Live/Dead staining proved both practical and informative. Staining was clear and easy to both perform and image, and showed excellent survival, as suggested by compatibility with prolonged passage, for BR-series PDXs. For PDXs with reduced survival and proliferation Live/Dead staining was in agreement with estimates inferred from brightfield imaging.
- For assessment of the species of surviving cells qPCR using species-specific primers gave unambiguous results and showed no sign of being affected by any gel contamination in the samples.

- For assessment of heterogeneity changes in passage several techniques were probed in a preliminary manner.
 - High Resolution Melting proved compatible cells grown in peptide gel, and in general theoretically feasible, but would require optimisation of a full panel of primers to be useful for the purpose of heterogeneity tracking. This would be a very interesting project with a wide range of additional valuable outcomes for cancer research particularly, but far exceeds the scope of this project
 - Immuno-staining and imaging again proved feasible but likely to require a larger panel of markers to be useful in the particular context of heterogeneity tracking.
 - Imaging Flow Cytometry using the Imagestream X was more informative than expected given the limited disaggregation of the PDX to small clusters rather than single cells as is typically required by flow cytometry methods. The combination of imaging and flow cytometry functions meant this technique was able to give a range of different types of information (including live/dead, cell-by-cell species, and cell cycle information) using pre-optimised stains, and so while more optimisation would be required to pursue this further, if full disaggregation can be achieved without unduly compromising viability this might be a very interesting route for future work to pursue.

Additionally, initial steps to test the suitability of different co-culture cell types and set-ups were taken, the outcomes of which suggested that secretion of signalling factors might be a better indicator of stromal cell relevance within the model than classical markers used to identify them, and that genuine co-culture (direct or indirect) would likely be far more relevant than tumour-conditioning.

5 Combining Core Elements of the Peptide Hydrogel Model

Having established typical parameters for growth of PDXs in 3D, effects of co-culture with MSCs on cancer cells and production of the peptide gel in the EVPC lab (Chapter 3), as well as development and adaptation of analytical methods (Chapter 4), enough context was available to combine these elements and interpret the results.

5.1 Growing PDX-derived Cells in Peptide Hydrogel

The core concept of the planned model is growth of PDX-derived cells in the peptide gel, and so this was the goal of the first combination of individually optimised elements. For this pilot experiment a triple-negative PDX, BR8, was selected, as these more aggressive tumours are less likely to need support from specific molecular additions such as oestrogen to survive.

An experiment was performed to test the hypothesis that the triple-negative breast cancer PDX BR8 can be grown in 10mg/ml peptide hydrogel without support from additional cell types or molecular additions beyond standard RPMI medium. 10mg/ml peptide gel concentration was chosen for this pilot experiment, for its resemblance in terms of stiffness to the matrix of invasive tumours, compared to lower concentrations used to mimic normal breast tissue.

BR8 tumour, freshly resected from the mouse host, was disaggregated as described in Methods. The cells were then counted and seeded at 5×10^5 cells/mL final gel in one hydrogel as per protocol for the peptide gel, while a second hydrogel was 'seeded' with medium only as a cell-free control. For each gel two transwell and two 96-well wells were seeded, the former with 200 μ l gel and the latter with 100 μ l gel (see Figure 5-A).

In addition to culture and passage with BR8, a triple-negative PDX, passage of BR15 PDX was also established. BR15 is hormone receptor positive and therefore likely to have more specific requirements, for example oestrogen, and was therefore used to indicate how broadly the approaches might apply when passaging a broader set of PDXs derived from a range of breast cancer subtypes. Separately, BR15 PDX was seeded under the same conditions as the BR8 PDX in this experiment, but exclusively

in the transwell plate to compare triple-negative BR8 growth with hormone receptor-positive BR15 growth.

By day five growth was very evident in the transwell and much sparser but nonetheless present in the 96-well plate. Some degree of reduced cell numbers would be expected due to the reduced seeding numbers, but by eye the number of clusters seemed reduced by more than this would account for. Day 8 showed a similar pattern with growth in both but far more limited in the 96-well plate, and sparser in BR15 than BR8 despite maximum cluster size being similar (Figure 5-1).

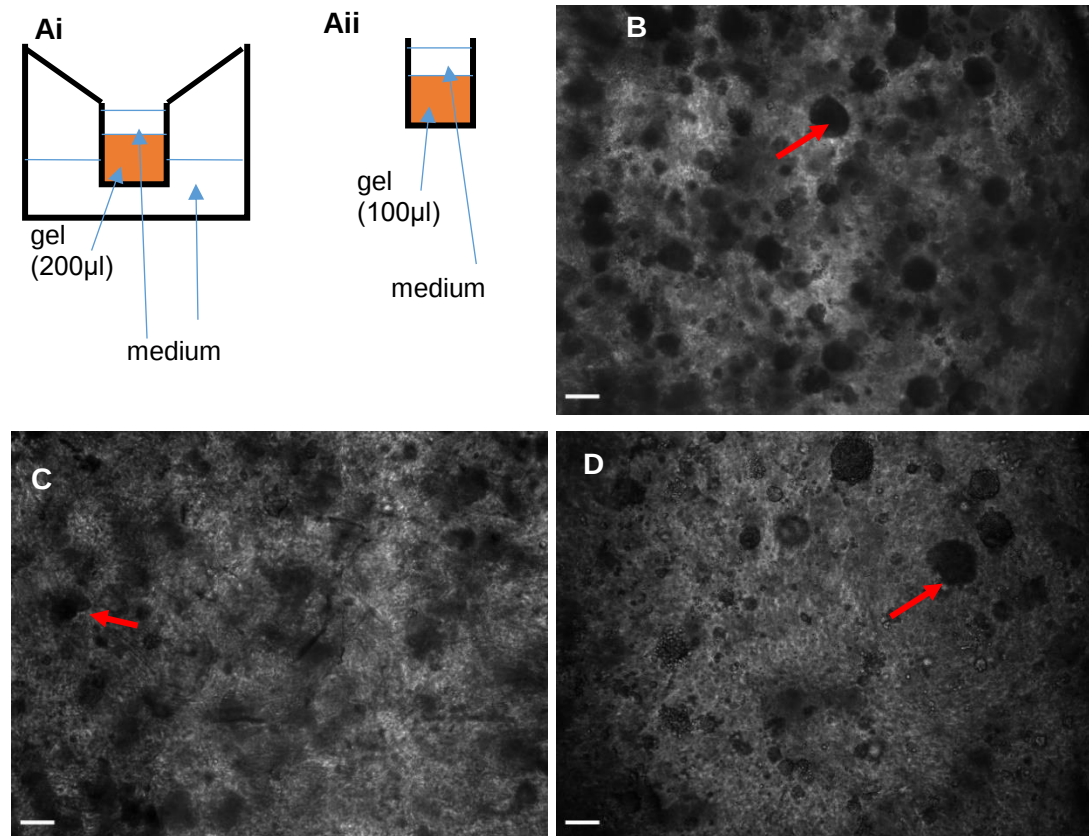


Figure 5-1: BR8 and BR15 PDXs grow in peptide gel without further additions. Diagrammatic representation of seeding of peptide gel in transwell (Ai) and 96-well plate (Aii) formats. Representative images from 2 replicate wells showing growth of BR8 PDX in transwell (B) and 96-well (C) setups and of BR15 PDX in transwell (D), all in 10mg/ml peptide gel after 8 days in RPMI, 4x magnification images taken using Nikon Eclipse widefield microscope, scale bar 200µm

After 11 days, many dark patches (some examples indicated with red arrows in Figure 5-1 and demonstrated to consist of mostly living cells in section 4.2, page 88) were

visible in the BR8 gels in transwell. These indicated significant growth; however, in many cases it was not possible to focus on the apparent cell clusters, possibly indicating that the optical properties or structural integrity of the gels has been altered by the cells over time (Figure 5-2). This was also the case with BR8 in 96-well plate and BR15 in transwell, though to a lesser extent.

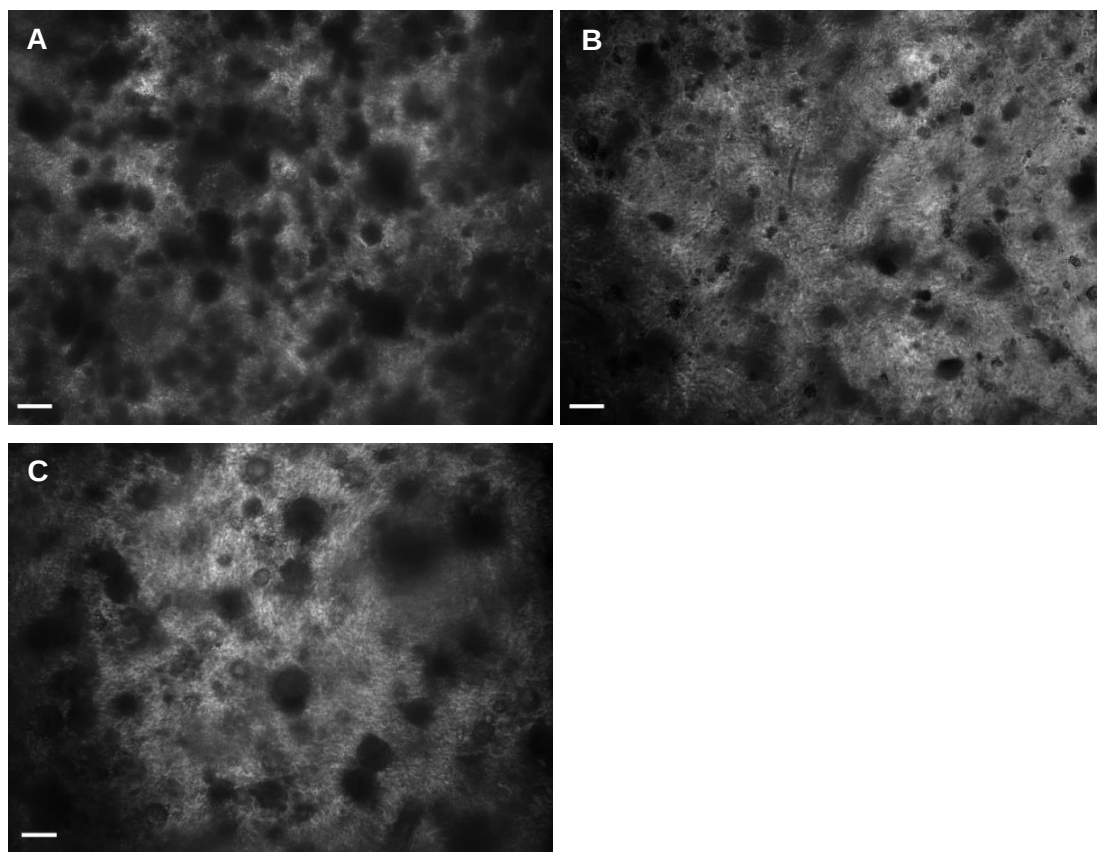


*Figure 5-2: **BR8 and BR15 PDX-derived cells continue to grow in peptide gel at 11 days, reducing optical clarity.** Growth of BR8 PDX in transwell (A) and 96-well (B) setups and BR15 PDX in transwell setup (C) in peptide gel after 11 days in RPMI, 4x magnification, representative images from 2 replicate wells taken using Nikon Eclipse widefield microscope, scale bars 200 μ m*

In conclusion, the seeding protocol for hydrogels is clearly highly compatible with survival and growth of the BR8 PDX but may require further adaptation to be usable in a 96-well plate. Alternative imaging approaches were required to obtain more information than a general assessment of occupancy beyond around day 8, and an assessment of how many of the cells are alive and proliferating is more informative where possible, whether by metabolic colorimetric/fluorescence assay or by live/dead stain.

The cells seeded in the transwell appeared to be, by day 11, occupying a large fraction of the gel volume and in large spheres that were visible to the naked eye, only possible if they had reached a considerable diameter, which might lead eventually to a necrotic core. The next step was therefore to develop a method that would allow them to be passaged. This would also demonstrate the viability of the cells within the clusters.

5.1.1 Optimisation of Passage for PDX-Derived Cells in the Peptide Gel

A practical and effective means of passaging PDXs from hydrogel to hydrogel is vital for the usability of the model and for testing in future use. Following initial growth of BR8 PDX in hydrogel, several approaches for passaging were tested. This was done through an iterative process, with variations to the protocol introduced at each stage and compared with the previous stage's most promising result. This ensured that the PDX remained available and provided a control.

Unlike passage of cells in 2D applications, obtaining a single-cell suspension was not specifically the goal of this process; when PDXs are transplanted from human-to-mouse or mouse-to-mouse, tissue pieces, smaller cell clusters and single-cell suspensions are all valid approaches depending on the specific application [184]. For example, use of tissue chunks retains a greater complexity of stroma, while use of single-cell suspension allows better replicability as more accurate cell counting, assessment of viability, and reseeded can theoretically be achieved.

The following aspects were kept constant throughout passage optimisation:

- All centrifugation steps at 300g for 5 minutes
- All incubation steps at 37°C
- Gels were re-fed every 2-3 days
- Medium applied was RPMI + 10% FBS + 1% L-glutamine
- Unless otherwise described, gels were passaged at day 14 or day 15
- All peptide gels were 10mg/ml gels from the same peptide powder batch (32597)

The key aspects modified in the approaches tested were:

- Enzymatic digestion incubation time
- Enzymes used for digestion
- Fraction of disaggregated cells passaged
- Volumes of enzyme solution (and therefore neutralising medium) used
- Number of centrifugation steps

The different approaches taken and how they incorporated these aspects are summarised in Figure 5-3 and described in more detail below.

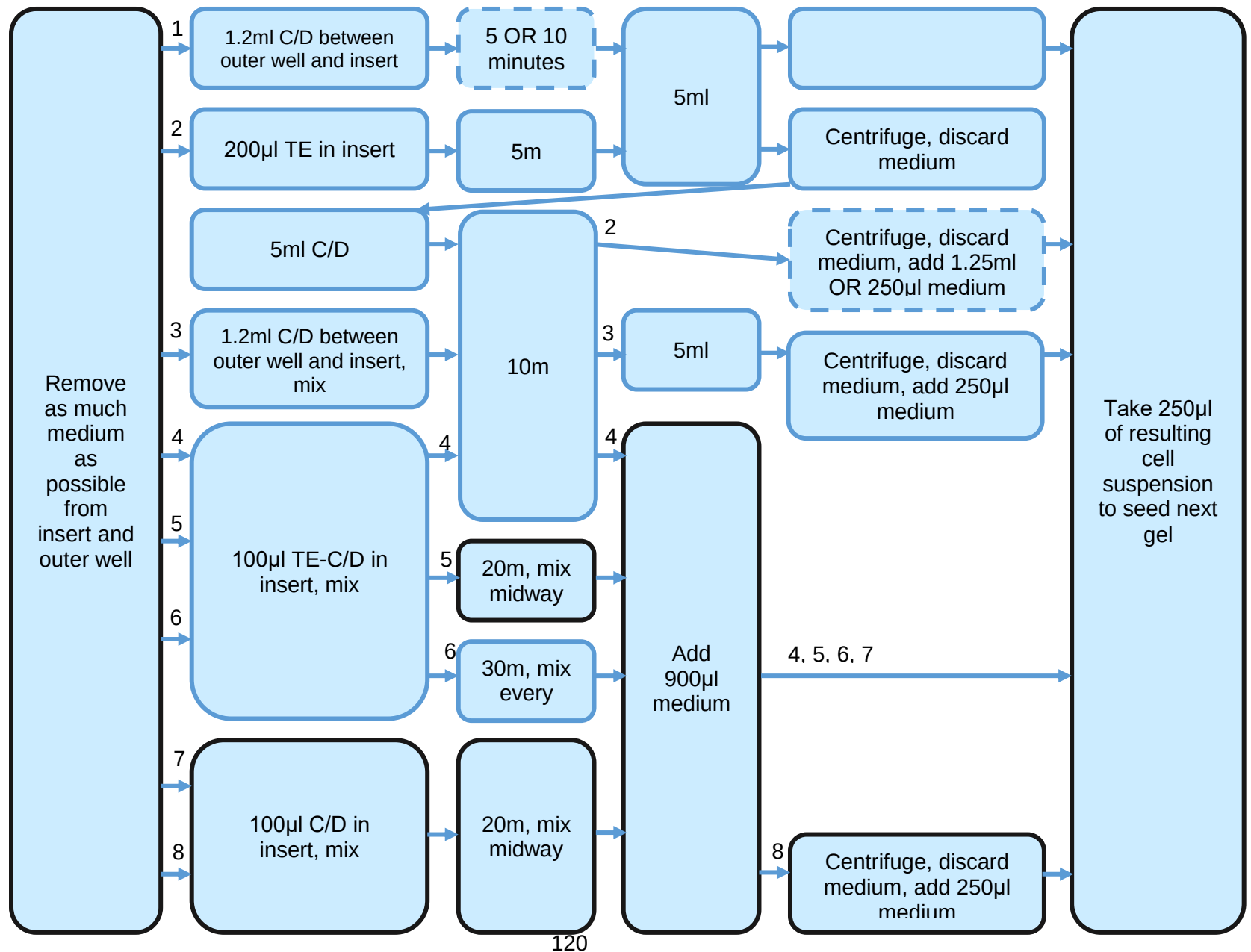


Figure 5-3: **Approaches used in optimisation of passage of PDX-derived cells in Peptide Gel**; C/D = collagenase/dispase, TE = TrypLE Express, TE-C/D = C/D made up with TE in the place of HBSS, Mix = pipette thoroughly to mix. Black outlines highlight optimised technique. The path for each approach is marked by its number.

Approach 1

At day 11 following initial seeding of the BR8 PDX described in 5.1, the two transwell inserts containing BR8 PDX in peptide gel were subjected to slightly different disaggregation attempts designed to be analogous to the protocol used for passaging cells in 2D. Approach 1 (see Figure 5-3) was used for both wells; for one, 5-minute incubation was used, and for the other 10-minutes.

Survival and disaggregation success were assessed, and the cells counted manually using trypan blue and a haemocytometer; cell counts were low but viability was good, and while many clumps were present, none were exceptionally large. Cells derived from the two versions of Approach 1 were seeded into a new hydrogel each and two transwell inserts were seeded for each condition. It was evident in both conditions that the new gels (Figure 5-4) contain fewer, larger clusters of cells than the un-passaged gels did (Figure 5-1B). This suggested that disaggregation of PDX-derived cells growing in gels, while both gentle and effective enough to allow the cell to be propagated into a new gel, was considerably less effective at separating the cell clusters than the disaggregation method originally used for the donor PDX.

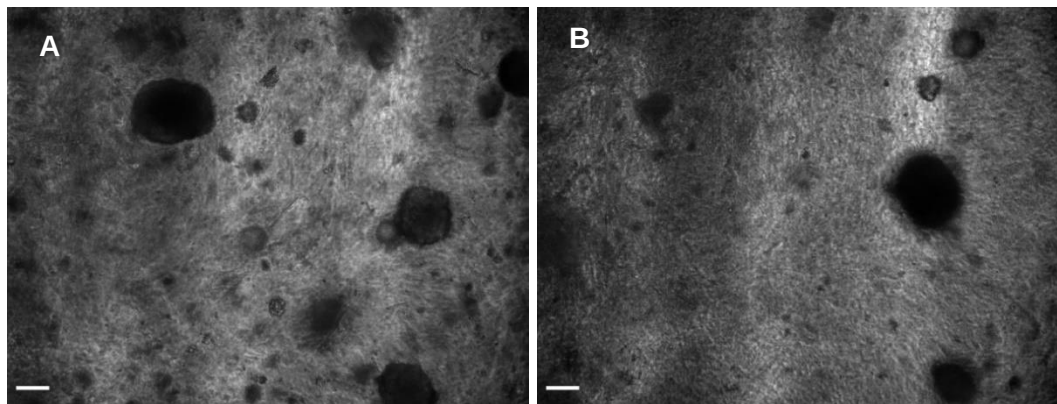


Figure 5-4: BR8 PDX cells can grow again in peptide gel following passage. 7 days of growth following first passage. Cells growing in RPMI after passage using incubation with disaggregation fluid for 5 minutes (A) or 10 minutes (B) 4x magnification, representative images from 2 replicate wells taken using Nikon Eclipse widefield microscope, scale bars 200µm

Approach 2

Using either variation of Approach 1 appeared to result in disaggregation that was incomplete but also gentle enough that sufficient cells could survive it to grow well in the next gel. A more thorough disaggregation step was therefore devised for a second passage (Approach 2, Figure 5-3).

As both the 5-minute and 10-minute incubation approaches from the first passage appeared adequately and similarly effective, wells from both were passaged to P2 five days apart, allowing for an opportunity to judge whether one or the other was earlier or later than ideal, and for a second attempt if the first killed the cells. The gel seeded from passage with 10-minute incubation was passaged at day 9 (into 'P2a') and the 5-minute incubation equivalent was passaged at day 14 (into 'P2b') (Figure 5-5).

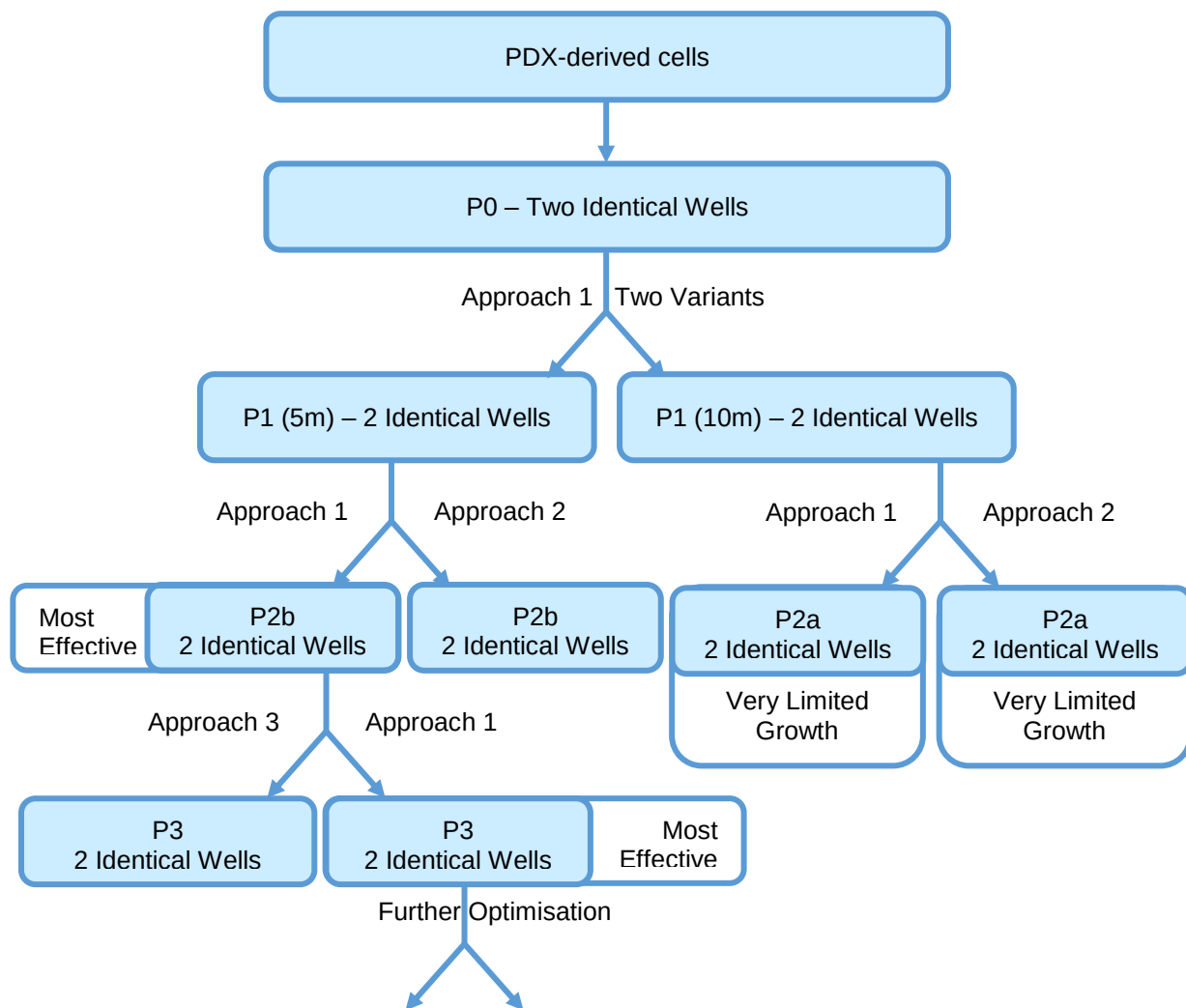


Figure 5-5: Flow diagram showing passages of each well during early stages of optimisation

Approach 1 was used to passage one well from each variation of Approach 1's initial test; the second wells were passaged using Approach 2, intended to break down the gel to allow better access of the disaggregation enzymes to the cell clusters. Survival and disaggregation success were assessed and the cells counted manually using trypan blue and a haemocytometer; counts were low but a high percentage were viable, and disaggregation was reasonable but not to single-cells.

Passage of the 10-minute Approach 1 variation wells (to P2a) produced only near-empty wells by both Approach 1 and Approach 2, possibly related to the fact that cell count had been fairly low, with no sign that either passage approach gave improved survival (Figure 5-6).

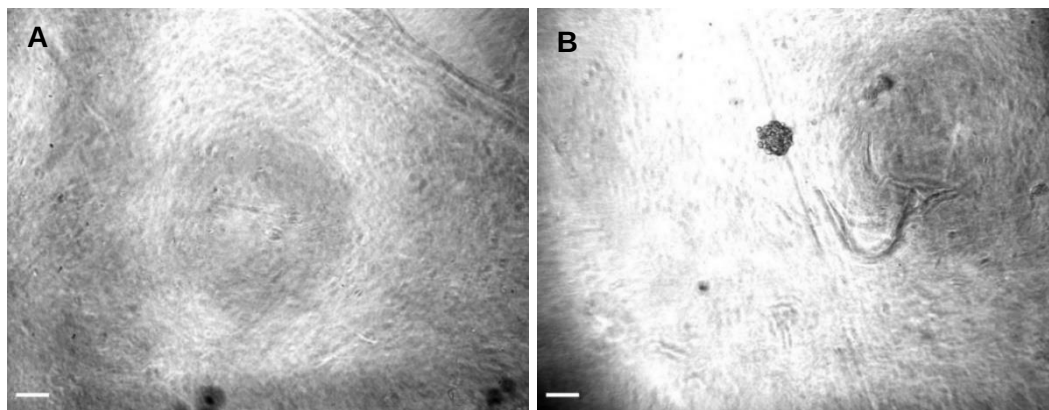


*Figure 5-6: **Growth following a second passage was extremely limited for both Approaches 1 and 2.** Growth of cells passaged twice, after 12 days of growth in new gels in RPMI medium. Passaged using Approach 2 (A) or Approach 1 (B). 4x magnification, representative images from 2 replicate wells taken using Nikon Eclipse widefield microscope, scale bars 200 μ m*

When passaging to P2b the passage protocols were as for P2a, except that cells were centrifuged again to reduce total volume to 0.5ml, giving a 1:8 instead of 1:20 passage. This aimed to improve growth at the next passage, as cell counts were as low as for P2a in which no growth was visible after five days. The increased seeding fraction, or possibly the additional time spent growing in P1, appeared to restore successful passage (Figure 5-7), with Approach 1 in P2b performing far better than Approach 2 in terms of the number of cells growing following passage.

Some texture or lighting differences were apparent in images taken of the wells, and were consistent between wells seeded with the same approach. It is uncertain whether this is due to effects of the cells on the gel (such as breaking it down or adding stromal molecules) or the cells themselves being more numerous higher in the gel and subsequently limiting the ability of light to pass through it.

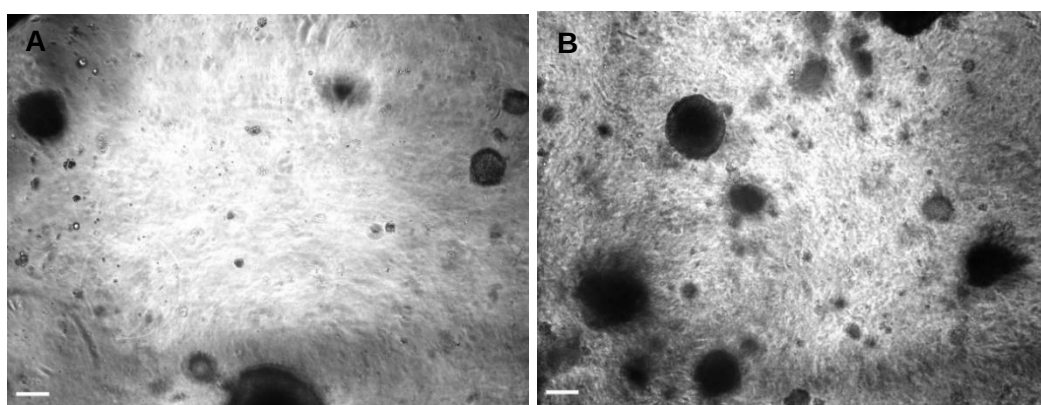


*Figure 5-7: **Given additional growth time BR8 can survive and grow after 2 passages, with Approach 1 performing better than Approach 2.** P2b in peptide gel in RPMI after 7 days of growth passaged using Approach 2 (A) or Approach 1 (B). 4x magnification, representative images from 2 replicate wells taken using Nikon Eclipse widefield microscope, scale bars 200 μ m*

Approach 3

The third passage of BR8 PDX used P2b's Approach 1 wells as they were very clearly growing better than those passaged using Approach 2. Approach 3 (see Figure 5-3) was devised based on Approach 1 – the 200µl of collagenase/dispase added to the insert was mixed thoroughly into the gel before incubating. After centrifuging, the supernatant was checked at two depths to ensure that cells were not being discarded in supernatant due to unexpected medium density changes, which might result from the gel's disintegration. Resuspended cell counts seemed approximately similar for both pellets and no living cells were observed in supernatant.

Following seeding into gels, large clusters of cells grew in both gels but very few small clusters were visible in Approach 3 gels relative to simultaneous Approach 1 gels (Figure 5-8); either disaggregation was much poorer or cells in smaller clusters did not survive. This method, however, did give far better survival than Approach 2, which involved many centrifugation steps, and so it was hypothesised that an approach without any centrifugation step might perform well.

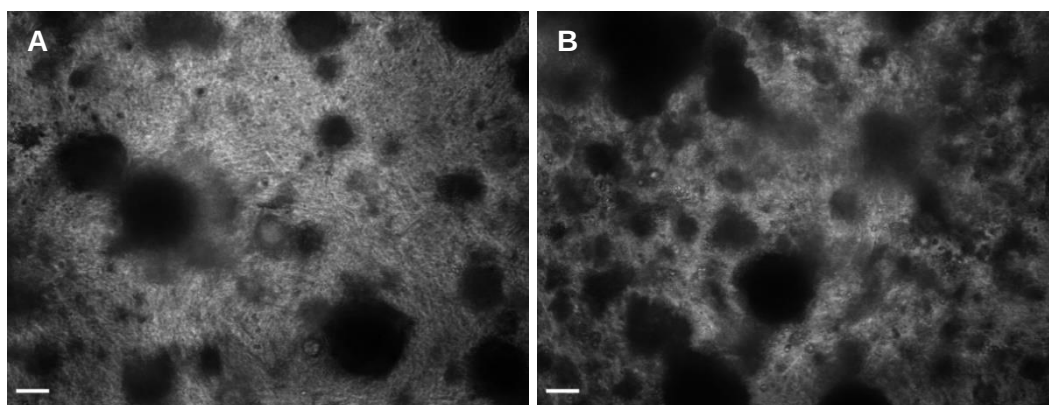


*Figure 5-8: **BR8 continues to grow in P3, with Approach 1 disaggregating more effectively than Approach 3.** Passage using Approach 3 (A) and Approach 1 (B) to passage BR8. Day 10 of growth in peptide gel with RPMI, 4x magnification representative images from 2 replicate wells taken using Nikon Eclipse widefield microscope, scale bars 200µm*

Approach 4

Given the very poor performance of passage involving an additional centrifugation step, a new protocol, Approach 4 (see Figure 5-3), was devised which eliminated the need for centrifugation. By this point, the BR15 PDX was ready to be passaged and so Approaches 1 and 4 were compared, to passage BR15 for the first time. Approaches 1 and 4 were also compared in the fourth passage of BR8 as BR8 and BR15 will not necessarily respond identically. Observation with a haemocytometer using trypan blue showed very few single cells using either approach, with possibly somewhat more consistently sized clumps for Approach 4.

Following 72h between refeeds on day 13, the medium (containing phenol red) was a very similar colour between the two passage approaches, whereas in previous passages Approach 1 had consistently been more acidic than the new Approach. This indicated that Approach 4 was on par with Approach 1 in terms of cell growth after passage, whereas Approaches 2 and 3 had not been. Microscope images likewise showed growth that was similar between the two approaches (Figure 5-9). In both approaches, some of the clumps were still very large, and so the next approach aimed to reduce this, which would improve consistency within and between gels. BR15 appears to have less growth in both conditions than BR8, possibly relating to the BR8 more aggressive triple-negative status of the BR8, or an early sign of contamination detected later in BR15.

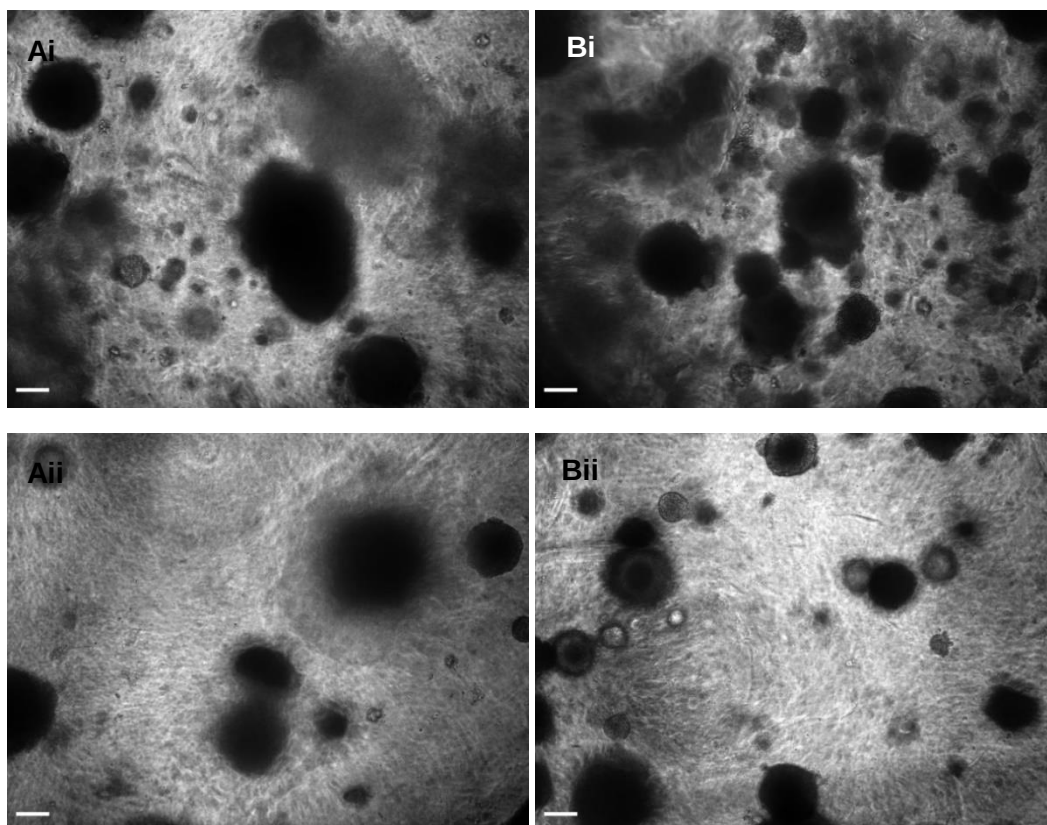


*Figure 5-9: **Approach 4** appears to be a similarly effective passage technique to **Approach 1**. Approach 4 (**A**) and Approach 1 (**B**) used to passage BR8 (**i**) and BR15 (**ii**) BR8 (P4) shown after 10 days of growth and BR15 (P1) after 11 days of growth in peptide gel with RPMI medium. 4x magnification representative images from 2 replicate wells taken using Nikon Eclipse widefield microscope, scale bars 200 μ m.*

Approach 5

In an attempt to produce smaller clumps and more single cells, at the next passage both PDXs (BR15 to P2 and BR8 to P5) compared Approach 1 with Approach 5 (see Figure 5-3), a modified version of Approach 4, in which the 10-minute incubation was replaced by a 20-minute incubation with mixing by pipetting halfway. Contamination prevented meaningful assessment of BR15 P2 but for BR8 it was clear even by day 13 that there was no observable difference between the medium colours, suggesting the extended incubation time did not harm cell viability significantly. The cell clusters growing in the gel became visible by-eye over time, so that the whole well can be checked for general cluster size and number trends. From 10 days onwards, Approach 5 appeared to have produced more consistent cluster sizes than Approach 1 (Figure 5-10), suggesting extended incubation improved disaggregation without harming cell survival.

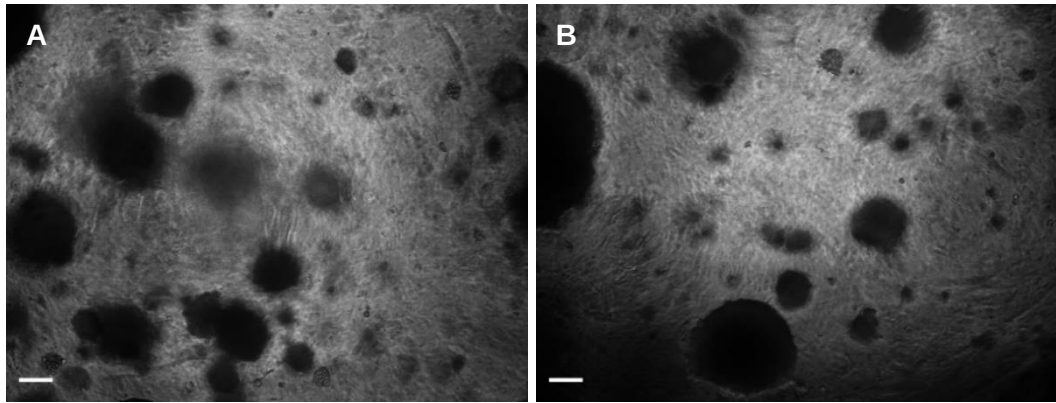


*Figure 5-10: **The extended incubation of Approach 5 produces more consistent cluster sizes than Approach 1.** BR8 passaged to P5 by Approach 5 (A) and Approach 1 (B). 10 days of growth in peptide gel in RPMI, 4x magnification representative images from 2 replicate wells taken using Nikon Eclipse widefield microscope, scale bars 200µm*

Approach 6

Following infection of first passaged BR15 a new BR15 P0 was seeded with additional empty gel controls. The wells including cells seemed unusually cloudy to the point of making imaging the wells at the same exposure time as the controls challenging, and this may have been due to cell debris or other non-cell materials from the PDX. Samples from this gel were retained for species testing by PCR and showed that it was neither human nor murine (section 4.3, page 92).

As increasing incubation time in TE/collagenase/dispase appeared to improve disaggregation, the effect of a further increase to 30 minutes total was assessed. BR8 was passaged to P6 with comparison of Approach 5 and Approach 6 (see Figure 5-3). The 30-minute incubation appeared not to improve disaggregation (Figure 5-11). The longer incubation may also have reduced survival, although this cannot be definitively concluded with just two wells per condition.

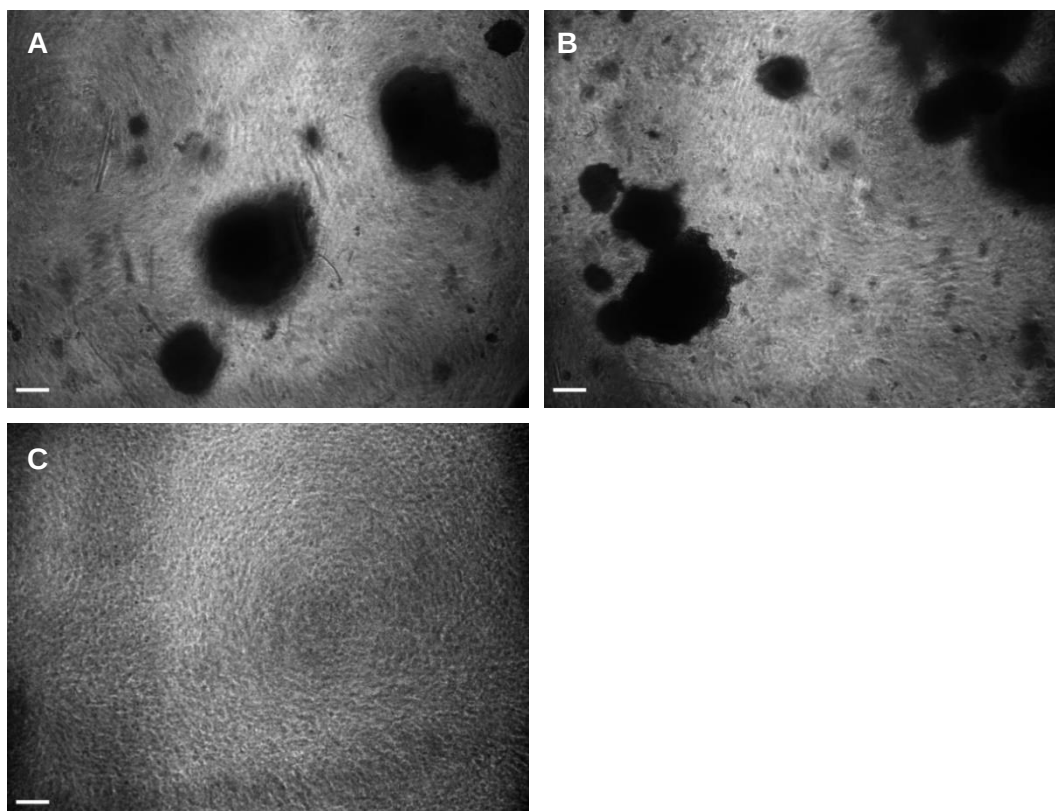


*Figure 5-11: **Further extending incubation beyond that of Approach 5 does not improve cluster size consistency.** BR8 passaged to P6 by Approach 6 (A) and Approach 5 (B) after 10 days of growth in peptide gel in RPMI. (C) A further passage by Approach 5 to P7. 4x magnification representative images from 2 replicate wells taken using Nikon Eclipse widefield microscope, scale bars 200µm*

The BR8 PDX was passaged to P7 using Approach 5 again (from the Approach 5 wells); however, no living cells were visible even after 8 days (Figure 5-11C). Possibly this was simply further than BR8 could be passaged. More likely, given the relatively successful previous passage, something introduced during passage killed the cells.

Approaches 7 and 8

As making up collagenase/dispase with trypLE express resulted in nearly 50ml of trypLE express being discarded every two weeks, further passages were performed using HBSS (Approach 7) to make up the collagenase/dispase solution and no changes were observed as a result of this.

Approach 7 and later Approach 8 (see Figure 5-3), which discards fewer cells and allows passage to continue for longer, were therefore used for future passage.

5.1.1.1 Summary of Conclusions from Experiments to Optimise Passaging Methods

Key findings from these optimisation steps were that:

- Centrifugation steps, as normally performed in a 2D culture passage, appear to significantly reduce the number of cells surviving into the next passage
- While there are theoretically target sites for trypsin in the gel, mechanical disruption alone is sufficient to separate cells from the gel if the cells are given sufficient time to grow before passage.
- Different approaches affected survival and disaggregation differently (Table 5-1)

It is possible that inclusion of trypsin as well as collagenase/dispase, at small enough volumes to not require re-addition of a centrifugation step, would improve gel clarity in later passages by reducing passage of the gel itself from previous gels into new ones.

Table 5-1: Effectiveness of Passage Approaches

Approach	Survival	Degree of Disaggregation Achieved
1	Good	To mid-sized clumps
2	Very poor	Mixed – some large clumps
3	Adequate	Less effective than Approach 1
4	Good	Looked good at cell count but grew very inconsistently
5	Good	More consistent than Approach 1
6	Good/Fair	Not purely single-cell, but good

5.1.2 Disaggregation for Single-Cell Suspension for Analytical Techniques

Disaggregation for passage will ideally reduce large cell clusters to small clusters rather than to a single-cell suspension, reducing selective pressure (as single cells may be vulnerable to anoikis) and therefore improving the likelihood that any heterogeneity present is maintained. Some analytical techniques such as single-cell RNAseq or flow cytometry specifically require a single-cell suspension however, and it was thought valuable to establish how achievable this would be using cells previously passaged in the peptide gel. Using the Approach 6 wells from P6, an attempt was made to refine disaggregation for single-cell purposes such as flow cytometry.

Two approaches were compared; **A** was gentler and resembled previous disaggregations, while **B** involved additional steps, including an additional dilution and centrifugation to remove gel and use of a strainer to remove large cell clusters.

Approach A

- Pipette well contents into 5ml collagenase/dispase
- Incubate at 37°C with mixing for 30 minutes
- Centrifuge at 300g for 5 minutes
- Discard supernatant and resuspend cell pellet in 1ml medium

Approach B

- Pipette well contents into 2ml medium
- Centrifuge at 300g for 5 minutes
- Discard supernatant and resuspend cell pellet in 5ml collagenase/dispase
- Incubate at 37°C with mixing for 30 minutes
- Rinse through 70µm nylon strainer with additional 1ml medium
- Centrifuge at 300g for 5 minutes
- Discard supernatant and resuspend cell pellet in 1ml medium

Both approaches resulted in a low cell count; however, while Approach A had a low cell count with fair viability and the majority of cells in 4-cell clusters or smaller, Approach B resulted in almost no cells visible at all. This indicated that future disaggregation attempts aiming at single-cell suspensions might need to start with a greater number of cells and, as for passage, require minimal centrifugation steps to avoid cell loss or death. While it was not possible to achieve a single-cell suspension here, for the core purposes of the project and many additional techniques, the existing disaggregation is adequate or even more ideal. Techniques such as single-cell sequencing would require further optimisation of disaggregation were they to be attempted, and this experiment has provided a starting point for that optimisation to begin if it becomes necessary.

The core process of expanding PDXs within the peptide gel was therefore established, allowing further refinement and interrogation of the model, both because of the increased availability of PDX-derived cells as a result and because it was now possible to investigate effects of longer-term growth of PDX-derived cells in the peptide gel.

5.1.3 Replacement of RPMI with Xeno-Free Medium

From the perspectives of both ethics and relevance, there is value in removing animal components from the model as far as possible. The peptide gel eschews both live mice and the mouse-derived BME of the most common alternatives for 3D passage, but for the majority of the project had been fed with phenol red-free RPMI supplemented with FBS, derived from cows and therefore containing bovine signalling molecules.

Replacing FBS-containing media is challenging for two reasons, firstly because even hardy cell lines may fail to grow well or at all in serum-free media, and secondly because the serum in the media is used to neutralise disaggregation enzymes during passage, ensuring no proteolytic activity is brought forward into the next passage. An alternative passage method might therefore be required for use with animal-product-free media formulations, either without use of disaggregation enzymes or with particularly generous dilution of disaggregated samples before centrifugation.

A xeno-free medium (XFM) supplied by PromoCell was identified as a possible alternative to the FBS-supplemented RPMI typically used with breast cancer cells in the peptide gel model. This XFM was specifically intended for growth of tumourspheres in 3D, which while not precisely matched to the goals of this project shared 3D architecture elements and would likely be supportive of growth of potentially delicate cells.

An experiment was therefore performed to determine whether this XFM was capable of supporting growth of the BR15 PDX in the peptide gel, essentially forming a fully animal-free model.

Two 10mg/ml peptide gels were seeded according to protocol with BR15 PDX-derived cells. Three inserts from each gel were seeded, and the second well of each swapped between conditions before medium was added to reduce any effects from variation between the gels. This resulted in three wells for XFM assessment and three control wells for a standard serum-supplemented RPMI control. The initial 250µl cell suspension volume was in standard supplemented RPMI to avoid the need for potentially damaging additional centrifugation steps to change medium, but all wash/refeed steps used XFM for XFM wells.

Following the D1 re-feed, gels were re-fed every 2-3 days, as is recommended in the protocol, and were imaged by brightfield microscopy to monitor growth. Images showed some variation between replicate wells but at day 11, when gels would

normally be considered for readiness for passage, there was a reasonable amount of growth in all wells and no clear difference between the XFM and the control (Figure 5-12).

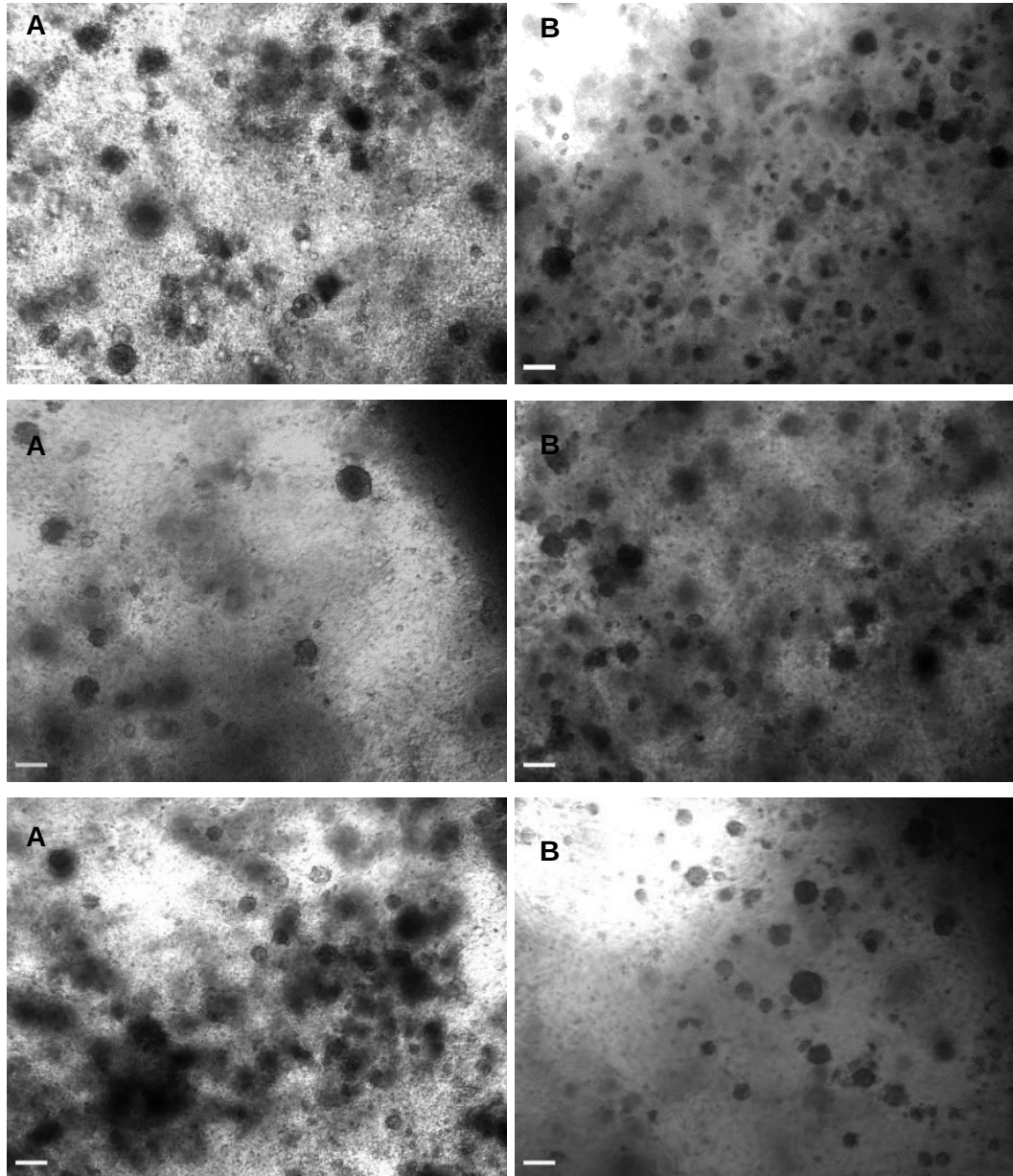


*Figure 5-12: **BR15 PDX-derived cells grow comparably in serum-supplemented RPMI and animal product-free XFM** One representative image from each of triplicate wells of cells grown in standard RPMI (A) or XFM (B) in 10mg/ml peptide gel for 11 days. Scale bar shows 200µm, images taken at 4x magnification on Nikon Eclipse microscope.*

On day 14 both of these conditions were passaged by a technique devised to eliminate the use of enzymes, which would require serum to neutralise following disaggregation; the contents of each insert were thoroughly mechanically disaggregated by pipetting and diluted in 1.5ml condition-appropriate medium.

These were centrifuged at 300g for 5 minutes to re-suspend in 250µl condition-appropriate medium, with the goal of reducing the amount of non-cellular material being passaged on and allowing the full well to be passaged into a new gel (equivalent to a 1:4 passage as one gel can, if seeded carefully, be seeded in 4 wells).

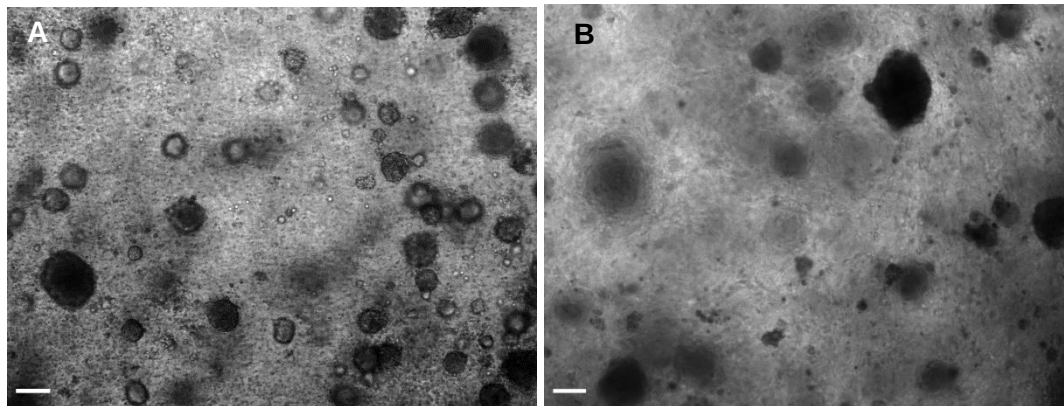


Figure 5-13: Following passage BR15 cells grown in XFM form sparser, less consistently sized clusters than in RPMI day 13, BR15 growing in 10mg/ml peptide gel in RPMI (A) and XFM (B) 13 days following passage. Scale bar shows 200µm, representative images from 3 replicate wells taken at 4x magnification on Nikon Eclipse microscope.

After 13 days of growth, it was evident that while the passage technique was capable of propagating in both media, the clusters growing in RPMI were both more numerous and more consistently sized than those grown in XFM were (Figure 5-13). That both media allow growth following this enzyme-free passage is promising, but the difference between the conditions suggests either that the XFM affects cluster size or that experimental consistency from well to well requires more work.

This experiment established that passage is feasible with only relatively minor adaptations to the optimised protocol when using serum-free medium, though optimisation would be ideal were this to be attempted for prolonged periods. This sets up the model for potentially entirely animal-free culture, an attractive prospect for many purposes, particularly where human-only or highly consistent signalling environments are necessary.

Having established that BR8 PDX grows well in the gel under a range of conditions, and that in spite of some problems with infections in later passages BR15 appears to behave similarly, it was of interest to broaden the range of PDXs trialled in the peptide gel.

5.1.4 Assessment of Growth Differences between PDXs

The peptide hydrogel model must be capable of supporting a wide range of tumour types, and from PDXs derived in a range of labs, and so a third breast cancer PDX was seeded into the gel to assess growth. This PDX was generated in Robert Clarke's group at Manchester Breast Centre, and therefore formed an interesting comparison to BR8 and BR15, which were generated within EVPC. Additionally, culture with phenol red containing versus phenol red-free medium was compared, to assess the effect of phenol red, which has been described as affecting breast cancer culture. It was hypothesised that the PDX, BB6RC160, would grow in gels with or without phenol red, but that growth might be faster when phenol red was included in medium due to phenol red's activity as a weak oestrogen [185].

The PDX was disaggregated according to protocol, but not using the Binder oven as had previously been used for disaggregation. An oven with a rocking mixer, in contrast to the Binder oven's rotating mixer, was used instead. Four wells were produced from one 10mg/ml gel. Phenol red-free medium was used for seeding, with phenol red then being used for washes (i.e. everything after the addition of the gel itself to the insert) and re-feeds in two of the wells.

The gels were re-fed and imaged every 2-3 days. The gels were passaged using Approach 7 after 23 days – one well of each medium type was passaged into two new inserts, while the second of each was retained. The resulting 'P+1' gels were imaged and re-fed regularly as before and were harvested by mechanical disruption to freeze after 21 days.

Growth appeared very limited compared to both cell lines and PDX-derived cells previously grown in the gel, particularly in the phenol red containing wells, which surprisingly showed near-zero growth. Growth was evident in the phenol red-free wells, but did not occur in as defined clusters as with previous PDX- and cell-line derived cells grown in the gels (Figure 5-14).

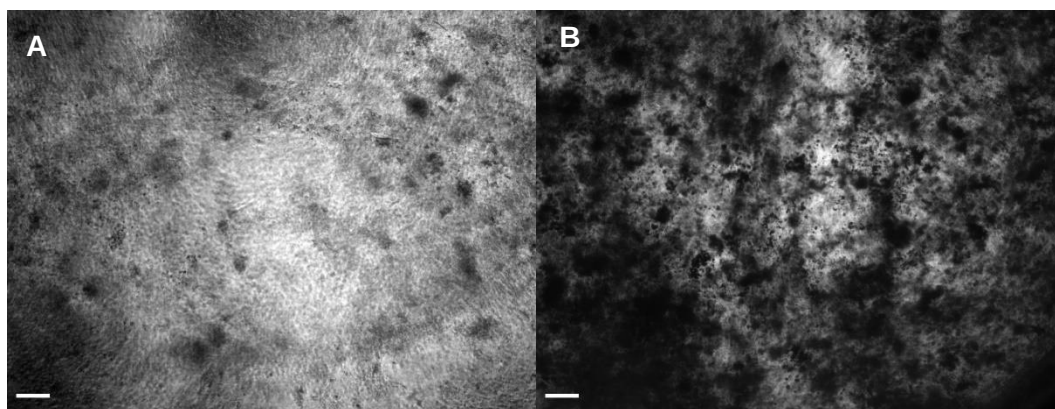


Figure 5-14: **BB6RC160 shows differential growth with versus without phenol red** BB6RC160 cell grown in peptide gel for 22 days with (A) and without (B) phenol red included in culture medium (passage 0). Scale bar shows 200 μ m, representative images from 2 replicate wells taken at 4x magnification on Nikon Eclipse microscope.

Before harvesting, it appeared that the gels in wells without phenol red had contracted significantly to form a smaller gel visible to the naked eye, the edge of which could be easily distinguished by microscopy (Figure 5-15).

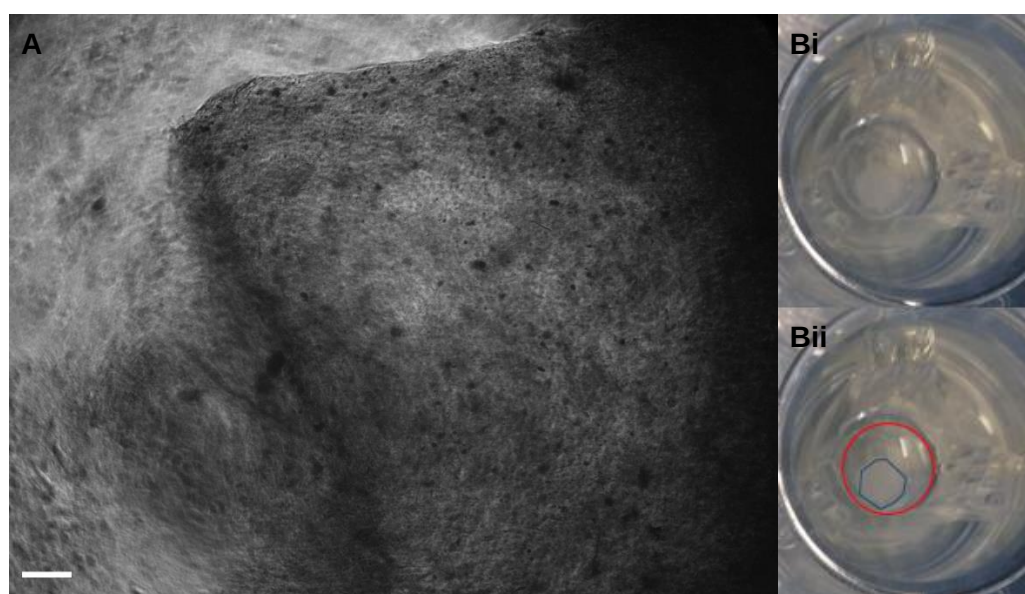


Figure 5-15: **Peptide gel appears to shrink when BB6RC160 is grown without phenol red.** (A) 4x Magnification image of shrunk gel edges taken on Nikon Eclipse microscope, scale bar 200 μ m (Bi) top-down image of gel in insert (Bii) overlaid outline of insert/starting gel size (red) and shrunk gel size (blue) on the same image

The extreme difficulty separating healthy cells from the minced tumour may have been due to poor temperature control and inadequate shaking in the oven used. Temperature controls on the oven used (with the rocking mixer) were inconsistently responsive and imprecise, and though a thermometer within the oven and manual checking ensured no extreme fluctuations were allowed to occur, the temperature was likely rarely at the ideal 37°C. Additionally the weaker mixing motion of the rocking compared to the normally-used rotating may have had an effect. Repeated

centrifugation was required to obtain the cells, possibly because of the reduced shaking, and may have damaged them, which would explain their very slow growth compared to BR8 and BR15 cells under otherwise similar conditions.

To establish whether improved disaggregation conditions could increase growth and/or survival of the PDX, or whether the PDX inherently grew less well in the peptide gel than BR8 and BR15, the Binder oven was used to disaggregate a second BB6RC160 PDX. Cell suspension was taken in fractions by centrifuging at 300g for 2 minutes, collecting the supernatant and replacing the collagenase/dispase. Fractions were taken at 1 and 1.5 hours, with the remaining tissue being strained through a 70µm nylon mesh, producing fraction 3 as is typical at 2 hours. Cell count and viability were assessed and while fractions 1 and 2 contained some cells, fraction 3's viability and counts were significantly better. Fraction 3 was therefore used to seed gels as before, continuing to compare phenol red-free and phenol red-containing medium.

Use of the Binder oven (with its rotating mixing motion and very precise, easily controlled temperature) appeared to improve the number of cells retrieved from the tumour but growth still appeared very limited compared to BR8 and BR15 (Figure 5-16). Additionally, it appears that the increase in growth without phenol red is still present to some extent in this repeat. From this point on phenol red-free medium was used, and where possible the Binder oven was used to disaggregate PDXs. Several further attempts at growing BB6RC160 (not shown; five total throughout the project) resulted in the same poor growth outcomes regardless of disaggregation method.

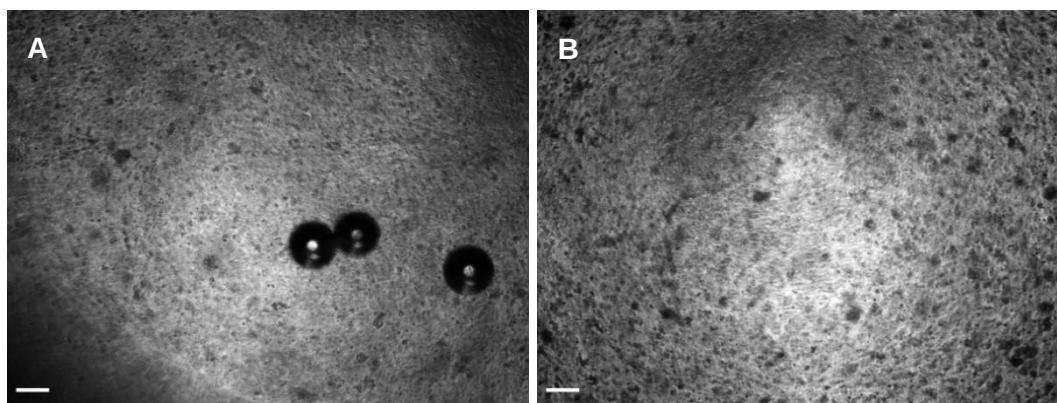


Figure 5-16: BB6RC160 growth in peptide gel remains limited when disaggregation is performed using Binder oven. Growth of BB6RC160 in 10mg/ml peptide gel after 13 days with (A) and without (B) phenol red. Scale bar shows 200µm, representative images from 2 replicate wells taken at 4x magnification on Nikon Eclipse microscope.

5.1.5 Co-culture of PDX with MSCs in Peptide Gel

With several key elements of the monoculture growth of PDXs in peptide gel established, the next key group of experiments was to expand the assessment of the effect of co-culture with MSCs from the 3D-TE model to the peptide gel model.

An initial exploration of the effect of MSCs on PDX growth in peptide gels was performed using cells derived from the BB6RC84 PDX, which had not previously been grown in the peptide gel and therefore would be interesting to observe in the monoculture control, and primary MSCs.

Two days prior to PDX seeding, MSCs were seeded into two wells of a 24-well plate at a ratio estimated to be likely to give slightly over a week of growth. BB6RC84 was disaggregated (as described in Section 2.1.2, page 27) and seeded into four wells of a single gel in transwell inserts and moved into the MSC-containing plate at the final day 0 media change, with two inserts being placed in co-culture and two in monoculture. Brightfield imaging was used to monitor growth throughout the experiment followed by AlamarBlue assay on the final day.

Growth of the PDX in both monoculture and co-culture appeared typical of BB6RC-series PDXs, with cells remaining dispersed in the gel and not forming large clusters (Figure 5-17). After 8 days of growth, some small clusters are visible.

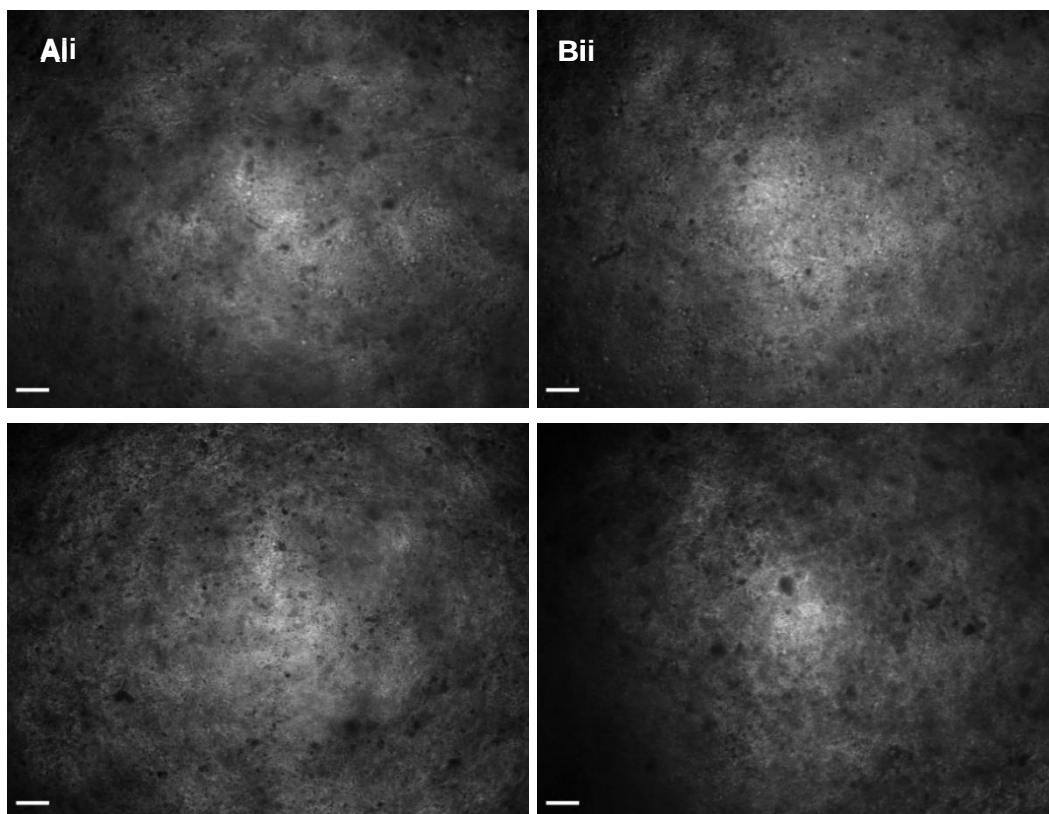


Figure 5-17: Visual difference in growth between BB6RC84 monoculture and co-culture with MSCs is negligible Representative images from 2 replicate wells of BB6RC84 PDX-derived cells grown in monoculture **(A)** and co-culture with MSCs **(B)** grown in 10mg/ml peptide gels in phenol-red free RPMI. **(i)** shows 24 hours of growth while **(ii)** shows 8 days of growth. Scale bar shows 200 μ m.

In spite of the limited number of cells and therefore wells available, it was possible to perform AlamarBlue assay at day 8 to give a quantitative estimate of how the two conditions compared. Fluorescence readings of 1590 and 1331 were observed for co-culture and 1093 and 880 for monoculture. This suggests that there may be a slight increase in growth in co-culture compared to monoculture, though further data would be needed to draw any firm conclusions regarding this.

Limitations in cell number and time available for this experiment prevented observation of growth effects beyond imaging and end-point metabolic assay – additional wells would have allowed live/dead staining and/or metabolic assays at multiple time points. Additionally, the PDX used for the experiment belongs to the BB6RC series, which appear not to be as compatible with the peptide gel as BR series PDXs. An experiment was therefore planned to compare growth of both BR8 and BR15 in peptide gel with and without indirect MSC co-culture, additionally incorporating a larger number of technical replicates to allow more quantitative assessment.

BR8 and BR15 PDXs were initially seeded in monoculture in peptide gels for expansion so that enough wells would be available for four replicates per condition. 24 hours before the first passage, TE67 immortalised MSCs were seeded in eight wells of a 24-well plate using a ratio equivalent to a 1:2 passage which would allow at least a week of MSC growth without inducing anoikis. DMEM was added to all wells to ensure any effects from phenol red affected all conditions equally.

When the PDXs were passaged, eight inserts were seeded from each PDX (four from each of two starting wells per PDX) and at the final media change transferred to the plate previously seeded with TE67 MSCs, with the media changed to Φ RF-RPMI. There was concern that earlier seeded wells from the same gel tended to contain more cells than later seeded wells, and that this would affect the experiment both on the basis of the P0 wells in which the cells were expanded and the P1 wells in which the experiment was begun. Wells were therefore swapped within PDXs but between co-culture conditions before the monoculture inserts were added to the MSC-containing plate, such that every condition contained a first, second, third and fourth well from P1, but those wells consisted of two inserts originating from well 1 at P0 and two from well 2.

PrestoBlue assay was used to monitor growth at days one, four and six (Figure 5-18); as PrestoBlue measures all metabolic activity, the inserts containing the PDX-derived cells in peptide gel were moved briefly to a read plate containing only Φ RF-RPMI to avoid the MSCs directly affecting assay results. The medium was removed from each insert, 20 μ l PrestoBlue was added to each and the plate was incubated in darkness at 37°C for 30 minutes. The Fluostar Omega plate reader was used to detect fluorescence. Following the day 1 and day 4 reads a 5-minute HBSS wash was used to remove the reagent from the gels. This HBSS was then replaced with media and the inserts returned to the TE67-containing plate for incubation until the next read. Additionally, images were taken on day 5 to give context to the PrestoBlue reads.

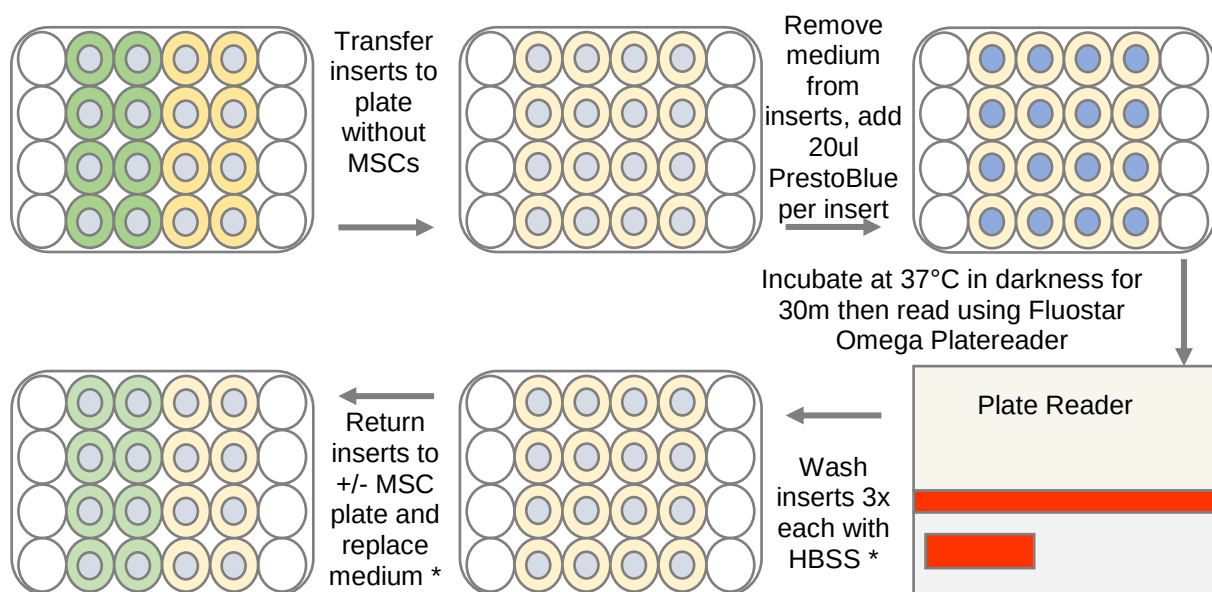


Figure 5-18: **Diagram summarising process of PrestoBlue plate read** Process followed on days 1, 4 and 6, with steps marked with * omitted on day 6

PrestoBlue results show no difference between growth in monoculture and in co-culture; all four conditions grow slowly in the first four days and then faster in the final two, as is typical of such assays (Figure 5-19). While exceptionally large variation between wells in BR15 conditions make it unclear whether the difference is real, it appears that BR15 grew much faster than BR8 having started with very close PrestoBlue readings on day 1. Well occupancy was quite evenly distributed for BR8s (Figure 5-19B and C) while well-to-well variation within BR15 conditions massively outstripped all experimental variation; this was visible as a colour difference even by eye while performing the PrestoBlue assay and therefore definitely not arising from a problem with the plate reader. Higher readings come from wells originating from P0's well 1, while lower readings come from wells originating from P0's well 2, which is additionally evident in the images taken on day 5 (Figure 5-20). If the problem were the well seeding order this could be significantly improved in the future by pooling and mixing identical wells during passage before separating them out again to seed, ensuring any particularly high- or low-occupancy well is distributed among wells in subsequent passages. An alternative possibility is that the gels each well were separated into were very different; for example if one contained too much NaOH this might prohibit growth of cells. This is unlikely however, given relative consistency between previous gels seeded simultaneously and the fact that a batch of four gels were created

simultaneously for this experiment at a stage when gel production was incredibly consistent with all gels requiring 80µl NaOH.

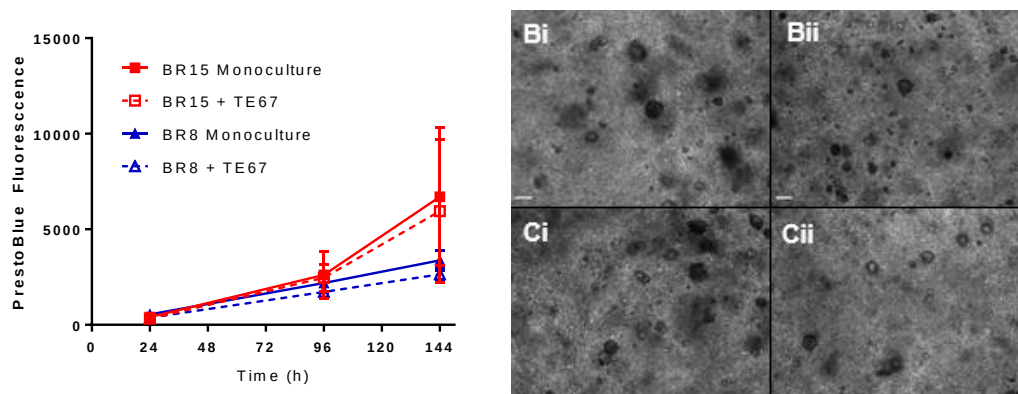


Figure 5-19: Co-culture of BR8 and BR15 PDXs with TE67 immortalised MSCs in peptide gel has no significant effect on growth rate relative to monoculture Growth of BR8 and BR15 PDX-derived cells grown in peptide gel after one passage as measured by PrestoBlue assay of four replicates per condition (A). Error bars show standard deviation from the mean. Red lines show BR15 while blue lines show BR8, solid lines show monoculture while dashed lines show co-culture. BR8 PDX-derived cells grown in co-culture with TE67 immortalised MSCs (B) or in monoculture (C), showing the first (i) and fourth (ii) pairs of gels seeded for these conditions.

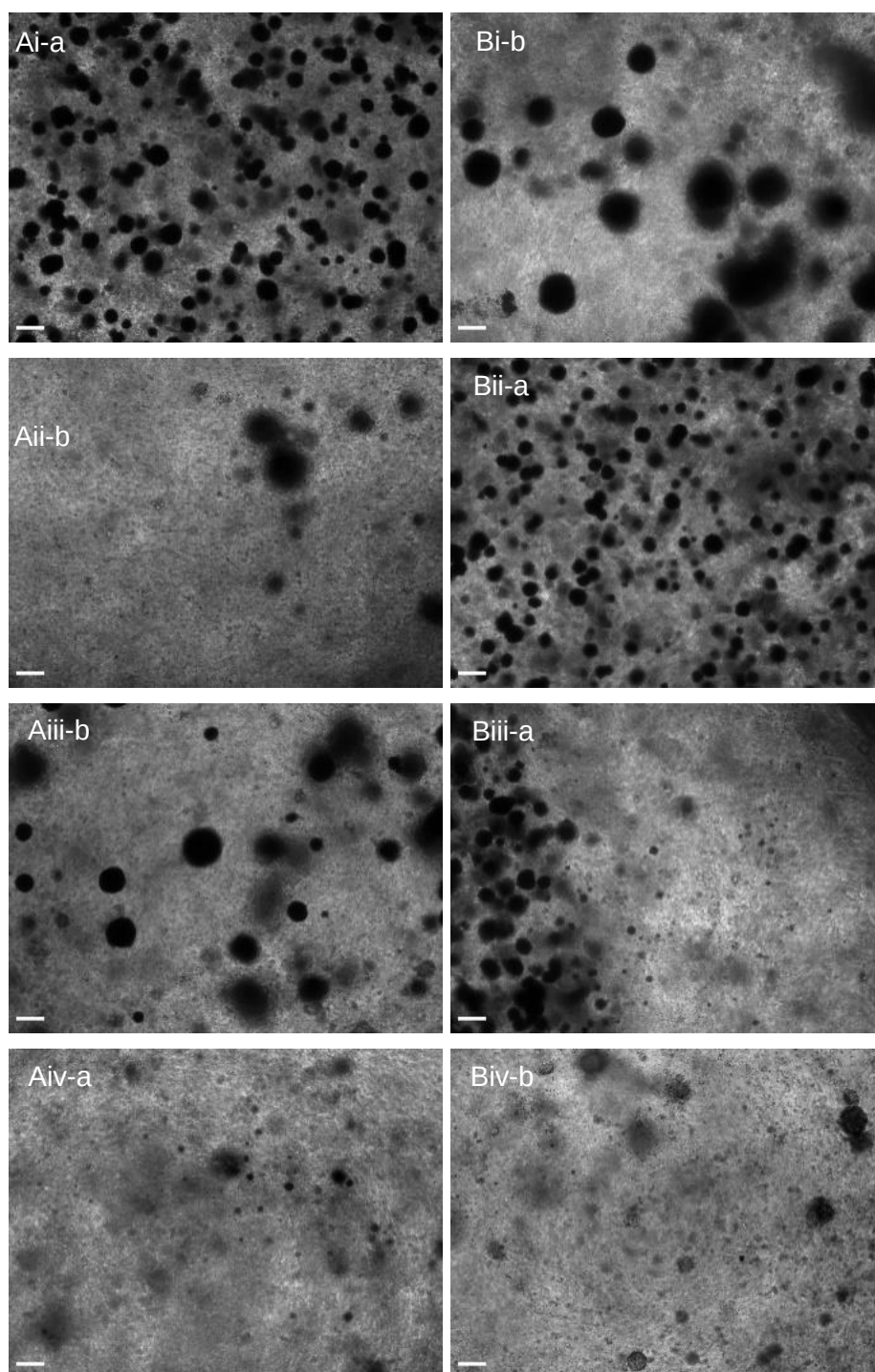


Figure 5-20: Co-culture of BR15 with TE67 MSCs in peptide gel shows variability between replicates. BR15 grown in co-culture with MSCs (**A**) or in monoculture (**B**) with well seeding order from top to bottom (**i to iv**). Wells originating from two different gels (incorporating cells taken from two different wells of the previous passage) are denoted with **(-a)** and **(-b)**. Images taken at 4x magnification, Scale bars are 200 μ m.

While inclusion of MSCs in PDX-derived cell culture is undoubtedly a step in the direction of increased realism and improved model complexity, an effect on cancer

cell proliferation was anticipated based on previous data from direct co-cultures. If the lack of effect proves consistent in repeated experiments, possibly the initiating passage stage before co-culture was introduced selected for cells which were not reliant on and therefore not affected by support from stromal cells. Perhaps prolonged exposure to a murine environment *in vivo* during PDX generation and passage has altered the cells' response to the MSCs. Alternatively direct co-culture may be required to generate an effect, which would be a major challenge for both assays of growth in optimisation and product purity in model use. There is also a possibility that something about the peptide gel itself either suppresses the growth-promoting effect or mimics it to a point that its addition has a negligible effect.

Having established these baseline behaviours and techniques for growth of PDXs in passage, it was next necessary to perform extended passage of a range of PDXs using consistent techniques. This would ensure any effects observed were not a result of the highly varied early passage approaches used during optimisation, and would allow early and late passages to be compared using additional assays.

5.2 Prolonged Passage of PDXs in Peptide Hydrogel

The optimisation process described in 5.1.1 involved prolonged passage, with changes at each passage to the approach used as the method was optimised. Having established during passage optimisation that repeated passage was possible, a series of experiments was planned to both test whether this applied to a wider range of PDXs and to obtain samples for comparison of PDX-derived cells from early in peptide gel culture and from late in peptide gel culture following multiple passages.

Following development of a passage protocol for cells in the peptide gels it was possible to passage a range of different PDXs and compare their condition before and after passage. Live/Dead stain served as one readout of cell health and growth, which could be used in both early and late passage, and a PrestoBlue based growth curve provided a readout of growth patterns once the PDX had been expanded. Intervening passages additionally provided samples for species testing and attempts at cryopreservation (Figure 5-21).

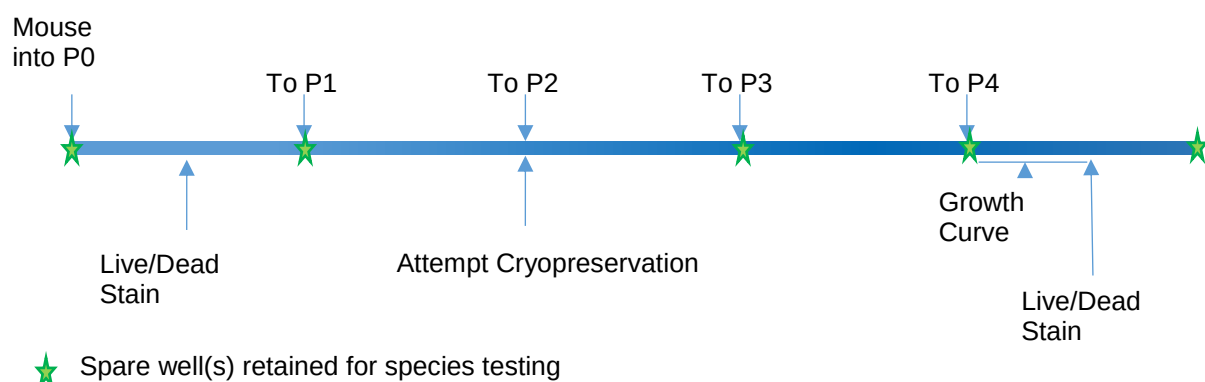


Figure 5-21: **Schematic of assays performed on passaged PDXs**

Four PDXs were selected on which to perform this series of passages and assays, with the aim of covering as much variation as possible between the PDXs. Two were hormone receptor positive while two were triple-negative, and two were BR-series PDXs while two were BB6RC-series PDXs (Table 5-2). BBR6RC84, marked with *, was not used however, due to time constraints and slow growth.

Table 5-2: **Variation in PDXs Planned for Prolonged Passage**

Established by	Triple Negative	Hormone Receptor Positive
Anna Grabowska's Group	BR8	BR15
Rob Clarke's Group	BB6RC84*	BB6RC87

It was not possible to obtain or work with all four PDXs simultaneously, and so while passage of the PDXs could not be synchronized they were all subjected to the same assays on the same day of growth where possible. Two replicates of each series of passages and assays were planned for each PDX. Growth, monitored by microscopy, and live-dead staining are described later in Section 5.2, outcomes of resuscitation following cryopreservation in Section 5.3.1, page 153, and species testing in Section 4.3, page 92.

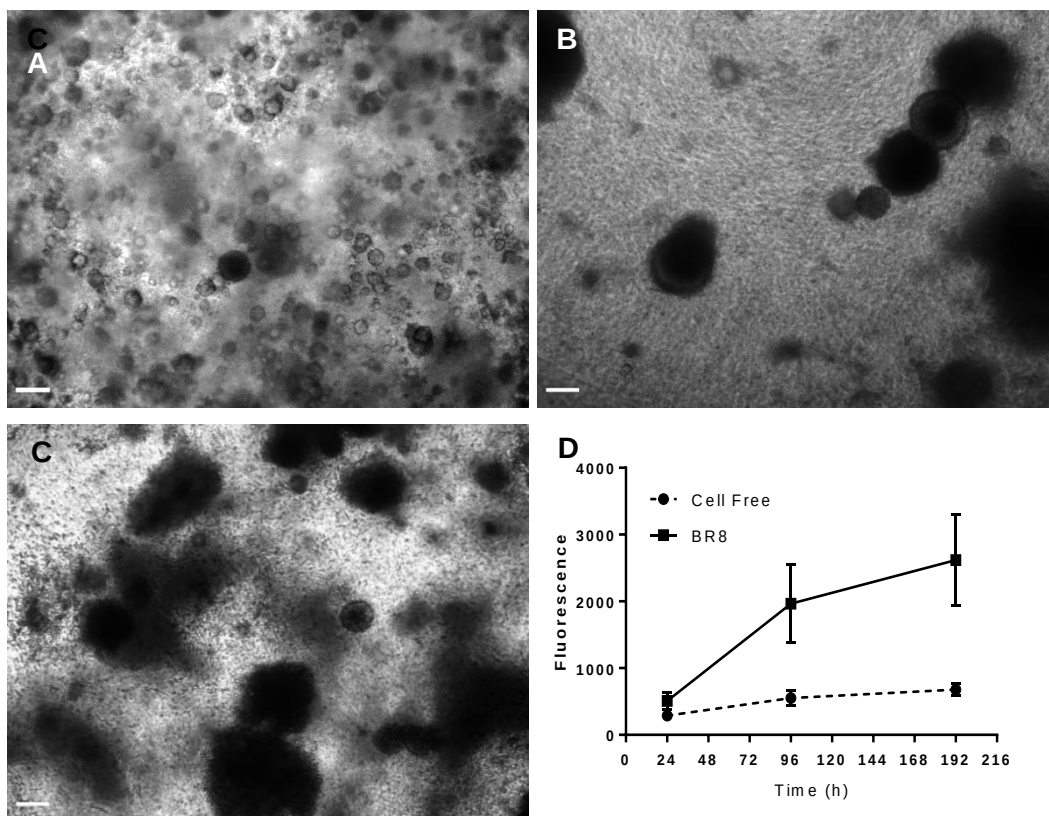


Figure 5-22: Consistent prolonged passage allows continued growth in subsequent passages
Representative images from 3 replicate wells of BR8 cells at P0 D7 (A), P2 day 9 (B) and P3 day 16 (C), taken in the insert without moving the cells or adding any reagents. A PrestoBlue growth curve showing the first 8 days of growth in P4 (D). Fluorescence readings are from 3 technical replicates each, error bars show standard deviation. Images taken using Nikon Eclipse microscope, scale bars are 200 μ m.

BR8 functioned in some ways as a positive control having successfully been passaged further than P4 previously. The BR8 PDX was successfully passaged to P4 (Figure 5-22), with Approach 7 (as described in section 5.1.1) used for the first passage and then approach 8 in subsequent passages due to concerns about excessive loss of cellular material between passages. Time between passages increased slightly over the course of passage, possibly due to the early 1:20 passages (Approach 7), to a peak of 20 days preceding P3. Nonetheless, by P3 it was clear that cells were still occupying a large proportion of the gel, and in passage four PrestoBlue assay showed considerable growth even in the first eight days compared to the cell free control in which minimal increase in fluorescence was observed (Figure 5-22D).

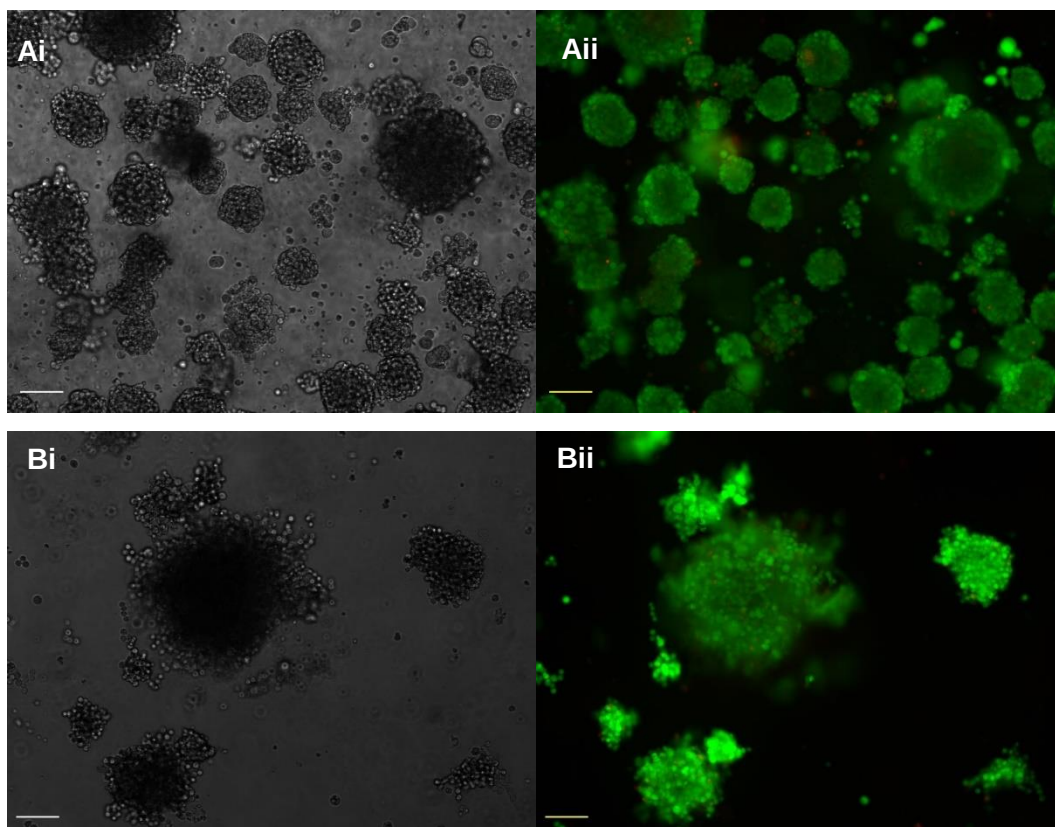


Figure 5-23: High ratios of live to dead cells are maintained throughout passage in BR8 passaged in peptide gel Live/Dead stained BR8 **(A)** before passage and **(B)** at P4 after 8 days of growth. Brightfield **(i)** and LiveDead (green/red) **(ii)**. Representative images from 2 replicate wells, taken using Nikon Eclipse microscope, scale bars are 100 μ m.

Live/dead staining at both P0 and P4 showed a very high percentage live cells compared to dead cells, and the majority of cells arranged in clusters, but a dramatic difference between the size and shape of the clusters is visible in the different passages (Figure 5-23), which reflects changes visible in brightfield in intermediate passages (Figure 5-22). While live/dead staining was performed at day eight of growth in both passages, in P4 the clusters of live cells are visibly larger, sparser and less circular than in P3. The most likely explanation for this is a combination of imperfect disaggregation and a high passage ratio in P1 resulting in loss of cell numbers not entirely recovered in longer later passages.

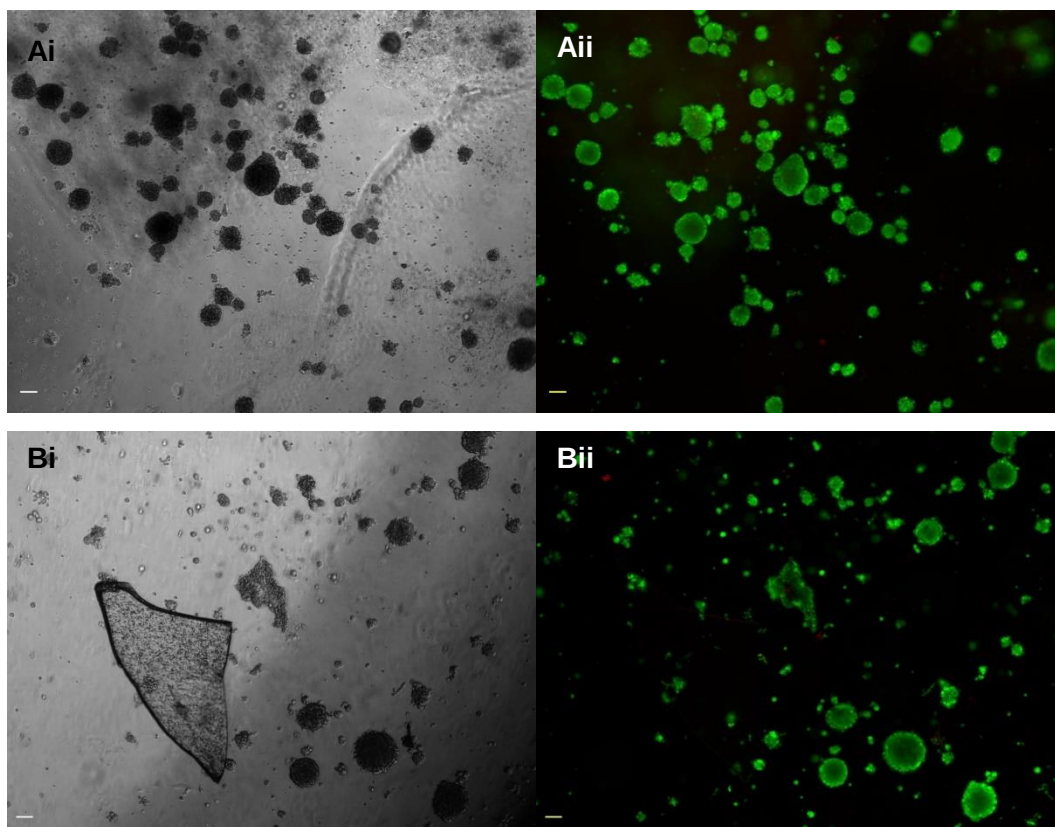


Figure 5-24: Consistent passage ratios throughout passage produce similar growth patterns to P0 following four passages of BR8 Second instance of Live/Dead stained BR8 **(A)** before passage and **(B)** at P4 after 8 days of growth. Brightfield **(i)** and LiveDead (green/red) **(ii)**. Representative images from 2 replicate wells taken using Nikon Eclipse microscope, scale bars are 100 μ m.

The full process was performed for another BR8 PDX tumour, this time with every passage using Approach 8. In the P4 live/dead staining images from this passage it appears that cluster size, number and shape is very similar to P0 growth (Figure 5-24) and no passages longer than 14 days were required to maintain good cell numbers.

It was observed during P2, as occasionally during other experiments involving cells seeded in peptide gel, that more cell clusters formed in wells seeded earlier from the same gel than those seeded later (Figure 5-25A). Identifying and eliminating the cause of this would likely be useful for replicability in future experiments. At P2 samples were again preserved at day 11 by three techniques in order to attempt cryopreservation for later restoration. Growth appeared similar in P3 (Figure 5-25B) to P0, and at P4 a growth curve was performed as before and showed similar patterns of growth (Figure 5-25C).

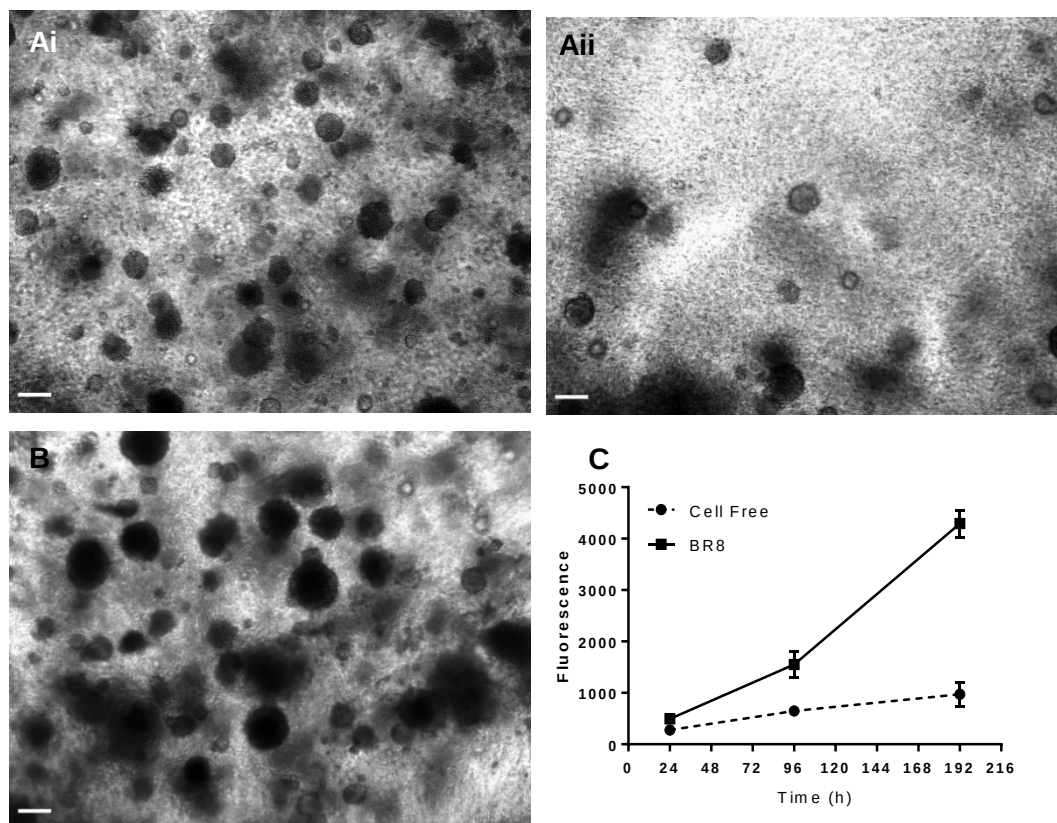


*Figure 5-25: **BR8 continues to grow over four passages in the peptide gel consistent with previous replicates** Representative images from 3 replicate wells, showing P2 day 10 (A) showing first (i) and final (ii) wells. P3 after 14 days of growth (B) and growth during the first 8 days of P4 (C).*

Time limitations prevented the BR15 PDX from being assayed exactly as for BR8; P4 growth assays were omitted and post-passage live/dead staining was performed at P3 instead of P4. Nonetheless it was evident that BR15 growth was consistent with that of BR8 (though possibly slightly slower) in that it passaged without difficulty as far as P4 and formed spherical cell clusters of live cells with very few dead cells (Figure 5-26).

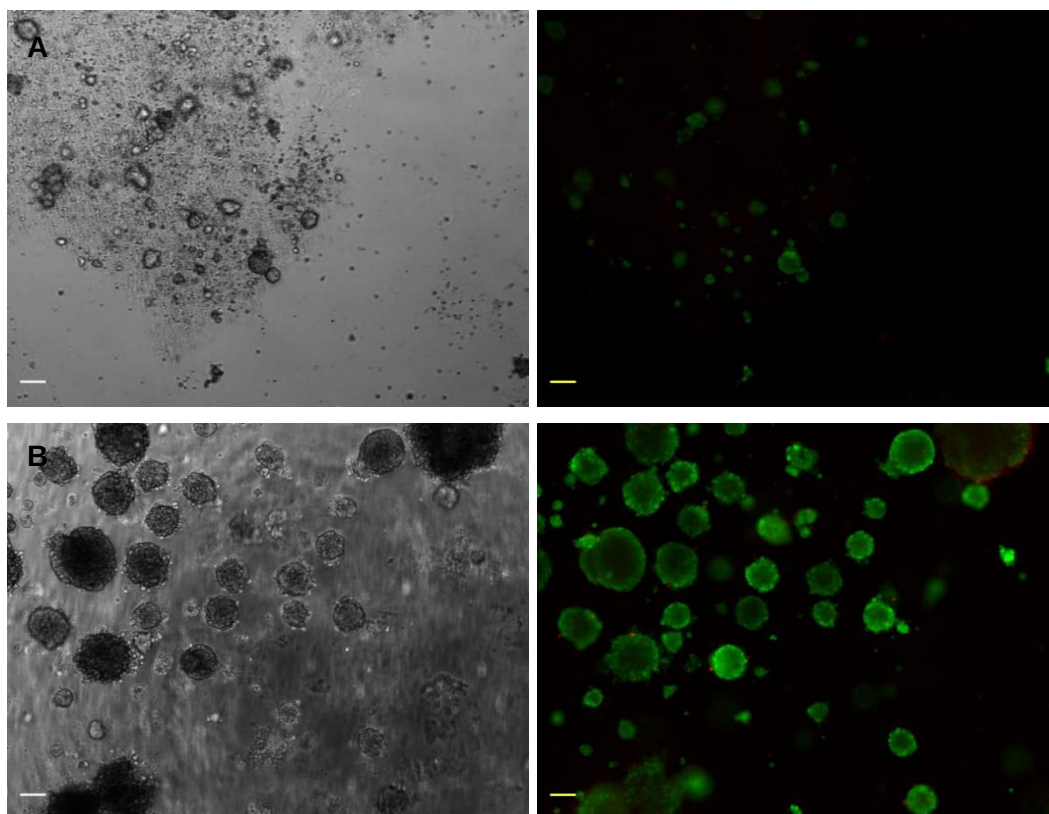


Figure 5-26: BR15 continues to grow as clusters of living cells after 3 passages Representative images from 2 replicate wells of Live/dead stain of BR15 at P0 (A) and at P3 (B) In both cases grown in 10mg/ml peptide gel in phenol red-free RPMI for 8 days before live/dead staining, scale bar 100 μ m 4x magnification images taken using Nikon Eclipse widefield microscope. Left shows brightfield and right shows red (dead) and green (live) stains.

The final PDX for which this process was attempted was BB6RC87. The results of live/dead staining at P0 clearly showed that BB6RC87, like BB6RC160 and BB6RC84, grows in a dispersed pattern within the gel and produces a limited number of living cells (Figure 5-27). Particularly, while the dead stains appear to correspond to real, dead cells, the live stain appears to resemble bubbles; if they are cells, they are either exceptionally abnormal or neither epithelial-like nor fibroblast-like, possibly immune cells or adipocytes.

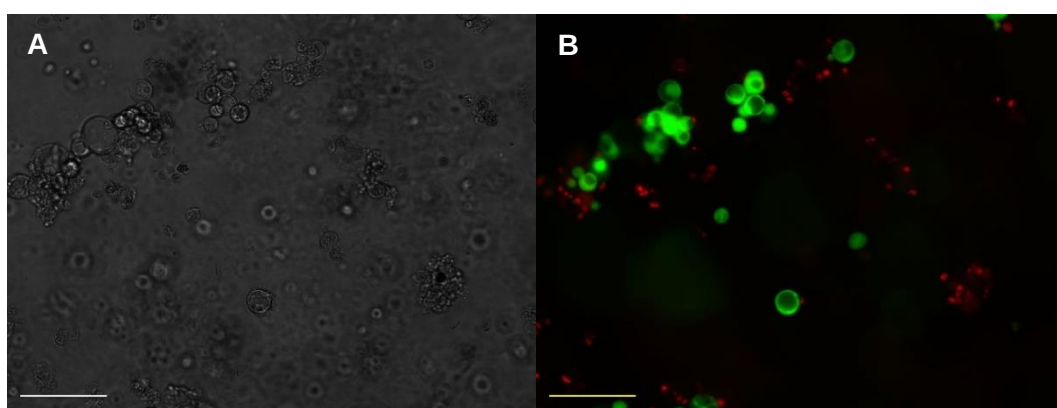


Figure 5-27: **BB6RC87 displays very limited growth in peptide gel.** Representative images from 2 replicate wells; Brightfield (A) and live/dead (green/red) (B) images. 20x magnification image taken using Nikon Eclipse widefield microscope, scale bar shows 100µm

The time required for BB6RC87 to reach a cell density that appeared likely to survive passage rapidly became unreasonable for a passage model, with P1 taking place after 21 days and not reaching an adequate cell number for passage to P2 after a full month of growth, at which point the experiment was halted (Figure 5-28).

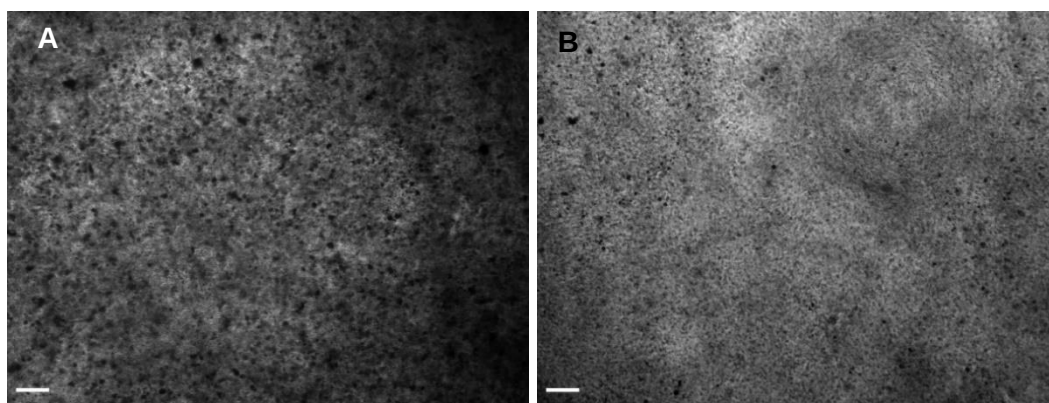


Figure 5-28: **BB6RC87 becomes progressively less able to reach confluence with passage** Representative images from 2 replicate wells of BB6RC87 P1 at D21 (A) and P2 at D28 (B) scale bar 200µm images taken at 4x magnification using Nikon Eclipse microscope

This failure of BB6RC87 to survive prolonged passage in the peptide gel is reflective of the behaviours of other BB6RC-series PDXs and represents a potential difficulty with passage of PDX-derived cells in 3D *in vitro* models. It would be of interest to find out how PDXs established in other groups grow in the peptide gel.

Overall, however, this series of experiments have demonstrated that some PDXs can be passaged over prolonged periods using the same passage technique, that growth appears normal in later passages and that live/dead staining shows similar distribution of live and dead cells between passages 0 and 4 when a full well is passaged into the gel of the next passage.

Time constraints and slow growth of some PDXs meant that the series of experiments was not completed for every intended passage in the intended number of replicates.

For those replicates for which prolonged passage could be achieved, species-specific qPCR was performed by Catherine Probert, with the results confirming preliminary results (Section 4.3, page 92) which suggested murine stromal cells were present in the initial seeded cells but not following growth in peptide gels (Figure 5-29).

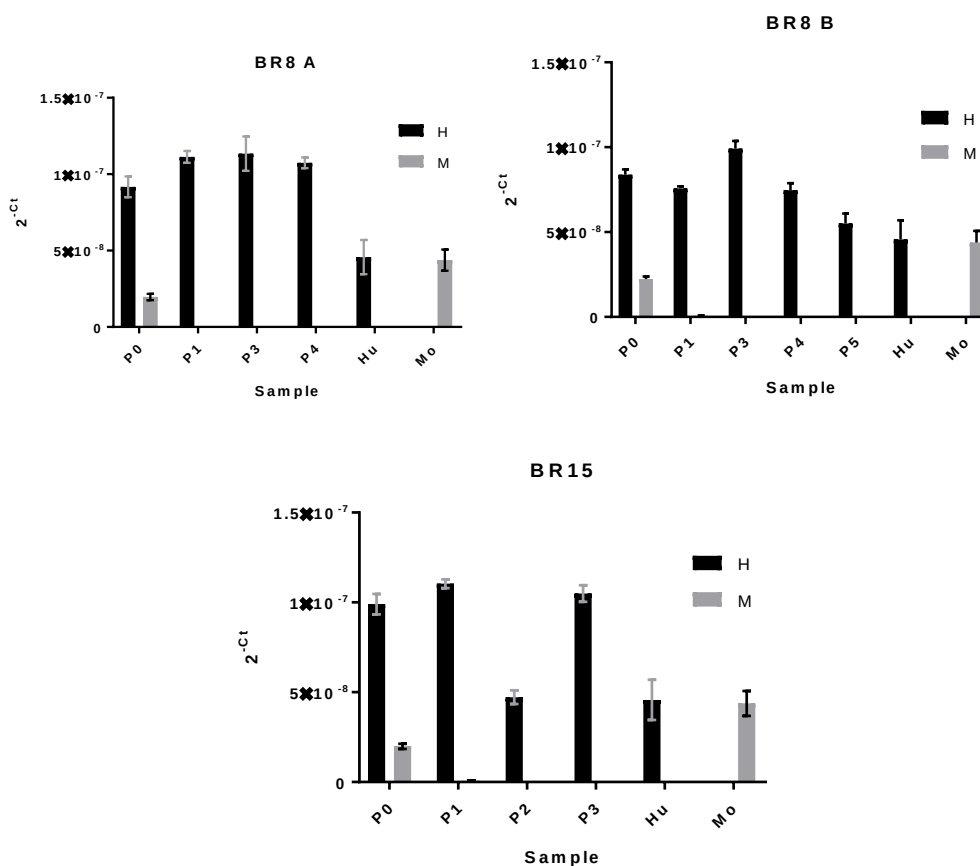


Figure 5-29: Murine cells are consistently lost during passage across different PDXs and biological replicates Species-specific qPCR results show that throughout the three series of PDX passages which continued beyond passage 2, murine contamination (grey) present in passage 0 is no longer present in subsequent passages. Error bars show standard deviation based on 3 (BR1, BR8 B) or 6 (BR8 A) technical replicates.

The cryopreserved cells generated by this experiment were particularly of interest as no cryopreservation had previously been attempted on these kinds of samples. The next aim relating to this series of experiments was therefore to determine whether they could be restored in good condition following storage at -80°C for several months.

5.3 Further Investigation of PDX-Derived Cells in the Peptide Gel model

Having established that the core goal of the project, to grow PDX-derived cells from passage to passage in the peptide gel, was achievable, it was of interest to probe the flexibility of the model. Particularly, how broadly compatible with normal laboratory practices and variations between labs it could be.

5.3.1 Freeze-Thaw Compatibility of PDX in Peptide Gel

The ability to cryopreserve tumour tissue from a model, rather than continuously passaging it, greatly increases its usability. By storing particularly primary material one can mitigate the risk of its complete loss, for example due to medium contamination, and gives flexibility around the timing of its use in experiment creating opportunities for collaborations and very thoughtful experimental design. Using cryopreservation protocols derived from those used for cell lines and fresh tissue, three techniques to freeze PDX-derived cells grown in peptide gel were used, and after several months resulting frozen samples were restored to determine whether the cryopreservation had been effective in maintaining a sufficient population of living cells to resume passage.

Three techniques for cryopreservation were attempted (Table 5-3). One involved no disaggregation of the cell clusters within the gel and at the freezing stage resembled the protocol used for tissue chunks. The second used the full disaggregation technique typically used for passage in the gels and a freezing stage identical to that used for cell lines disaggregated from 2D culture. The third involved a brief mechanical disaggregation and freezing as for 2D culture.

Table 5-3: Techniques used to attempt cryopreservation of PDX-derived cells grown in peptide gel

Technique Name	Full Disaggregation	Mechanical Disaggregation	No Disaggregation
Disaggregation	Medium removed, 100µl disaggregation fluid added, repeated pipetting, 20 min incubation at 37°C with pipetting at mid-point.	Repeated pipetting in insert	Single movement pipette from insert
Freezing Medium	Transwell insert contents added to 100µl DMSO plus 800µl completed RPMI		
Freezing Temperatures	Immediately into MR Frosty in -80°C at least 4h	Immediately into MR Frosty in -80°C at least 4h	Immediately into MR Frosty in -80°C 25m, then -20°C 10m, then -80° at least 4h

These techniques were attempted on multiple trios of wells containing PDX-derived cells grown in peptide gel (following two passages in the peptide gel), with growing conditions exactly as for passage. All three technique were always attempted simultaneously on wells seeded together.

After 5-6 months in storage at -80°C, attempts were made to restore the cryopreserved samples. In each case, a centrifuge-compatible tube of at least 10ml 37°C-warmed complete RPMI medium was prepared per vial restored. Each vial was rapidly defrosted in a 37°C water bath, its contents added to the pre-warmed medium and mixed thoroughly by inversion. The tubes were then centrifuged for 5 minutes at 300g to pellet the cells; the DMSO-containing supernatant was removed and the pellet re-suspended in 250µl completed RPMI to be immediately seeded in a 10mg/ml peptide gel.

In a first attempt only a BR8 Mechanical Disaggregation sample was restored to establish practical feasibility of the technique. It was possible to separate, identify and re-suspend a cell pellet from the sample after centrifugation and the gels into

which the pellet was seeded were retained for 17 days with refeeding every 2-3 days for observation of survival. At 17 days, a live/dead stain was performed to identify living versus dead cells visible in the clusters.

While some live cells were clearly identifiable, both individually and in clusters, the majority of cells appeared to be dead. This suggests that this Mechanical Disaggregation technique was likely not successful at preserving the majority of cells and that even if passage could be resumed from this sample it would likely be with considerable loss of any heterogeneity present in the PDX-derived cells. While clusters of cells are visible they are far less numerous than would be expected of BR8 in peptide gel even after far less than 17 days (Figure 5-30); it is probable that they are not a result of significant growth after restoration but rather clusters from before cryopreservation that remained at least partially intact through the mechanical disaggregation.

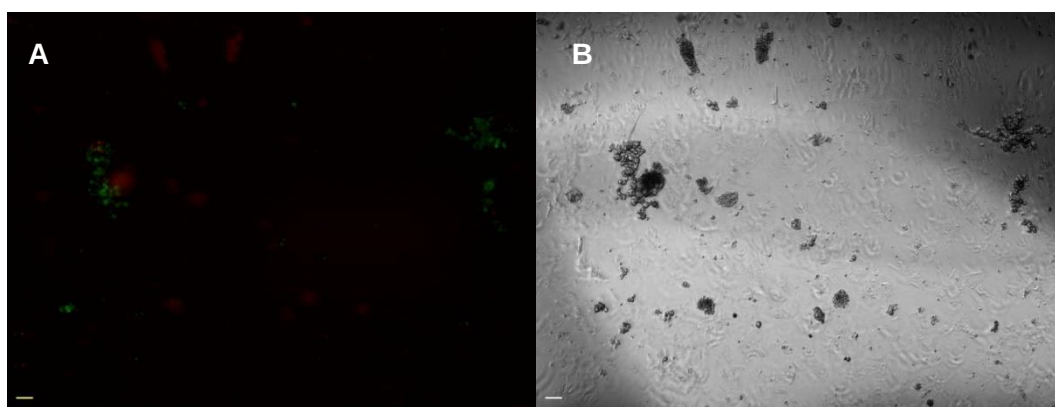


*Figure 5-30: **Preservation by the Mechanical Disaggregation technique restores some cells.** Live (green) and dead (red) stains (A) and brightfield (B) images of restored BR8 after 17 days of growth in peptide gels. Scale bar is 100 μ m. Representative images from 2 replicate wells using Nikon Eclipse widefield microscope.*

Following this attempt, a further restoration was performed with three simultaneously frozen samples representing the three techniques. As before, the cryopreserved samples were rapidly diluted into pre-warmed medium and the DMSO-containing cryopreservation medium centrifuged off before seeding into 10mg/ml peptide gels. The cells were grown for 7 days before staining for live/dead cells, at which point a visual estimate based on images would suggest approximately the same number of live as dead cells remained, or possibly more live cells (Figure 5-31). This did not seem to vary greatly between techniques used for freezing, and would likely still be a concern in terms of maintenance of heterogeneity given that some of these live cells may originate from growth of those

that survived preservation and therefore suggests fewer survived than died. However, the fact that a very significant growth lag is sometimes observed following *in vivo* restoration of PDX cells from cryo-preservation in comparison with passage suggests it is possible that the live fraction of cells observed at this time-point are reflective of those successfully restored without major growth, which would be more likely to allow heterogeneity to be maintained. Use of MSC co-culture to support the restored cells, as has been used by the EVPC group *in vivo*, might be a further step towards improving this process [97].

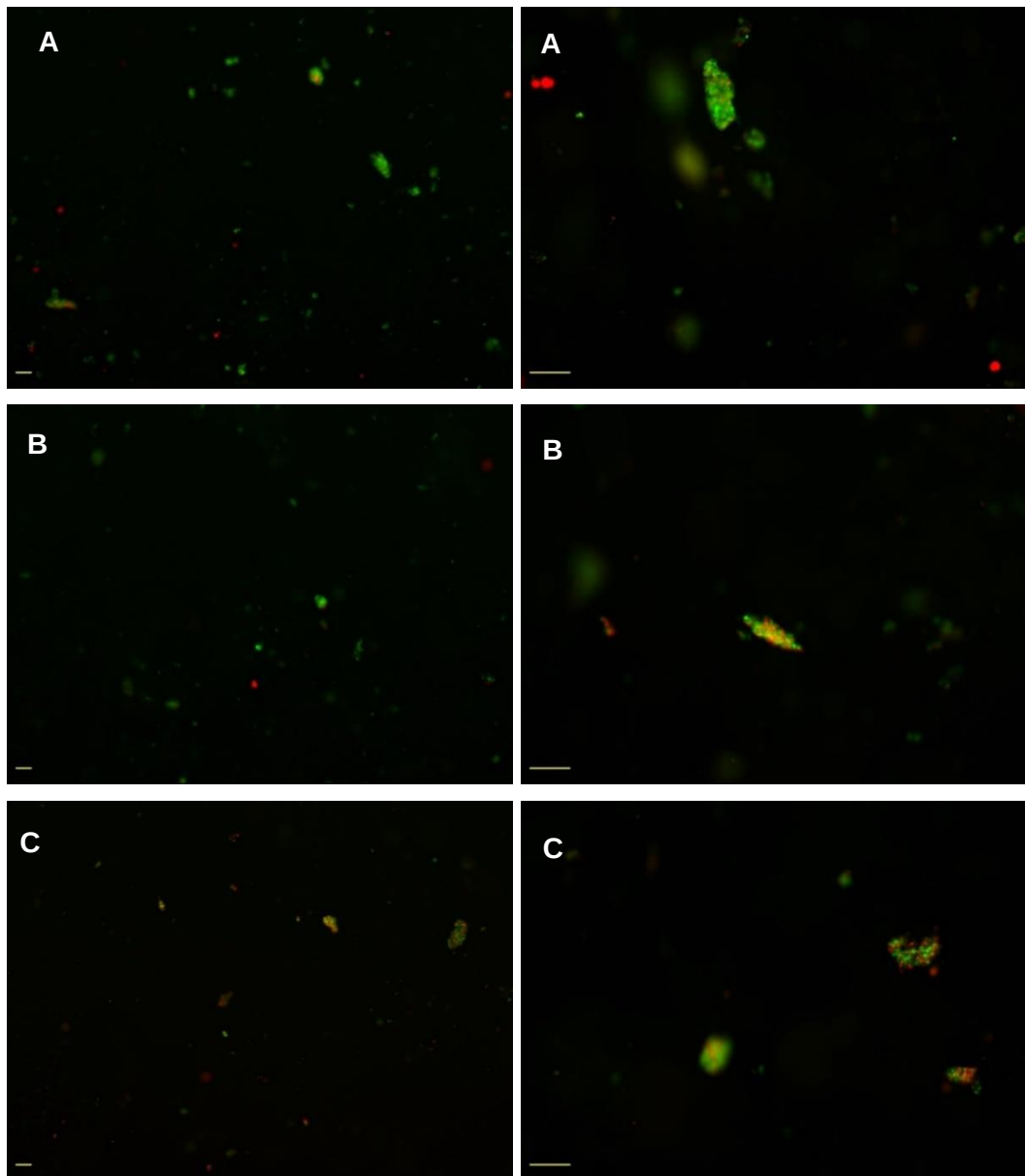


Figure 5-31: **Restoration of living cells from cryopreservation appears approximately similar for the three techniques attempted** Live (green) and dead (red) stains appear to show a fairly similar ratios of live to dead cells in samples restored from preservation using full (A), mechanical (B) or no (C) disaggregation. Scale bar is 100 μ m. Representative images from 3 replicate wells using Nikon Eclipse widefield microscope.

An attempt to compare the conditions quantitatively was made. Due to the large number of out-of-focus cells present in the 3D format, the typical method of counting live versus dead cells was not possible. Instead, mean pixel intensity was measured using ImageJ for the red and green channels of each of the six images per condition taken with the same exposures. Six green:red ratios (or live:dead ratios) were therefore produced for each condition and could be compared between conditions. They did not differ significantly between conditions (Figure 5-32); it would be interesting to compare this method with the more typical counting method using images (such as those taken by confocal microscopy) which are amenable to both.

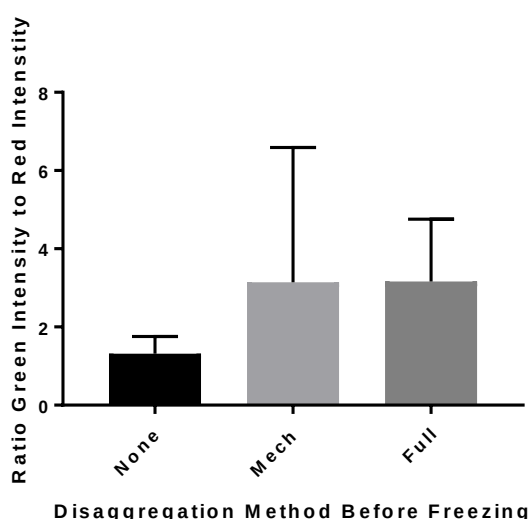


Figure 5-32: **Quantitative evaluation does not show significant differences between disaggregation methods** Based on two images per well from three wells per condition, error bars show standard deviation.

To conclude, the following information was gained from this attempt at restoring these PDX-derived cells from cryopreservation:

- All three methods were able to restore some living cells (Figure 5-31)
- Additional time may be required for restored cells to begin growing compared to in passage
- Quantitative evaluation of 3D Live/dead staining appears to agree with visual inspection that the three methods are not significantly different in their ability to preserve cells

As all three techniques seemed similarly imperfect but somewhat effective, it is likely that if cryopreservation can be achieved it will require alteration of a

parameter not varied in this experiment. One such parameter might be the freezing medium, as cell lines may respond better to being cryopreserved in FBS +10% DMSO than complete medium +10% DMSO as was used here. Specialised freezing medium recipes for sensitive cells might also be attempted.

5.3.2 Effect of Host Mouse Strain on PDX-derived Cells Properties

BB6RC-series PDXs had consistently failed to thrive in the peptide gel model, growing slower than BR-series PDXs and often failing to grow at all by P3 or earlier. At initial disaggregation BB6RC-series PDXs were consistently very difficult to run through a 70µm filter, flowing slowly and requiring additional filters for each PDX as the first and sometimes second became blocked, while BR-series PDX-containing disaggregation fluid would run rapidly through the filter. While cell counting at seeding revealed similar ratios of live cells to debris and dead cells, even very shortly after seeding in peptide gels the two PDXs series were very visually distinct; in brightfield microscopy regardless of molecular subtype of the origin tumour, BR-series PDXs would form spherical clusters of healthy-looking cells, with debris being comparatively insignificant. BB6RC-series PDXs on the other hand would not form large clusters, even when growing well the cells would be more evenly distributed in the gel, and debris would be a very significant proportion of what was visible by brightfield microscopy (Figure 5-33).

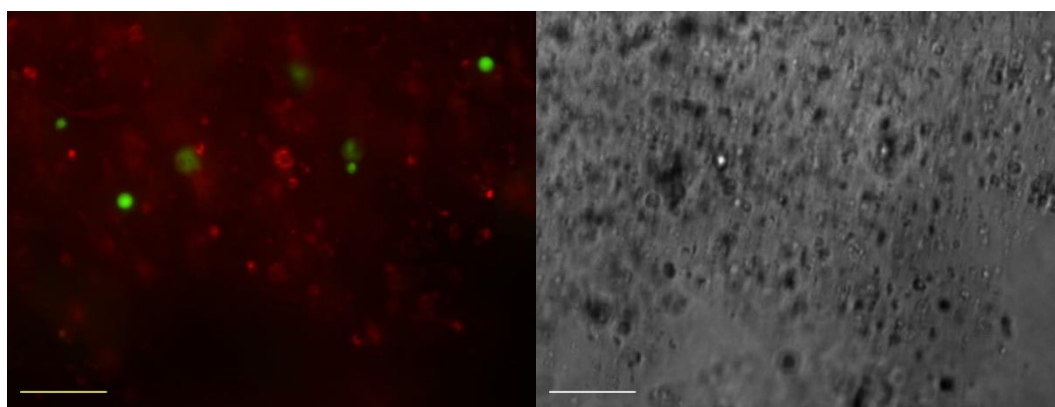


*Figure 5-33: **BB6RC160 cells grow in a dispersed arrangement in the gel, and include a very large percentage dead cells** Passage 0 BB6RC160 cells with live-dead stain (live stain green, dead stain red) grown in peptide gel demonstrating dispersed cells and low survival often seen from BB6RC-series PDXs in peptide gel. Scale bar shows 100µm. Representative images from 2 replicate wells using Nikon Eclipse widefield microscope.*

Given these significant differences, and particularly the fact that there was a large difference in compatibility with long-term growth in the peptide gel, an experiment was performed to identify the reason for these differences. While the two PDX series were generated by different labs they were also passaged *in vivo* in different mouse strains. One series was therefore grown in both mouse strains, to compare the features that typically vary between PDX series and identify whether the mouse strain used is likely to be the cause of the differences observed.

BR8 and BR15 PDXs, typically grown in Rag mice, were restored from cryopreserved samples into one Rag and one NSG mouse each by the *in vivo* team. Following one further *in vivo* passage each, maintaining the distinction between the mouse strains (*i.e.* each PDXs was passaged from NSG to NSG and from Rag to Rag) the four resulting tumours (BR15 in Rag, BR15 in NSG, BR8 in Rag and BR8 in Rag) were seeded in 10mg/ml peptide gels at 5×10^5 cells/ml.

Both PDXs from both mouse strains could be filtered without difficulty, as is typical for the series, suggesting that the difficulty in filtering the BB6RC series PDXs is due to a difference in how the PDXs were generated and not the mouse strain. Growth was typical in all four conditions of BR-series PDXs, with many spherical clusters of cells forming and growing rapidly, impeding visibility by microscope and compromising the integrity of the gel before even two weeks of growth (Figure 5-34). There was slightly more difference between BR8s grown in the two mouse strains when stained for live and dead cells (Figure 5-35) possibly indicating that there was some effect from the different mouse strains. BR8 grown in NSG mice nonetheless has none of the features of BB6RC series PDXs, suggesting that the differences which cause difficulty growing BB6RC series PDXs in peptide gels are not a result of the mouse strain used to passage them.

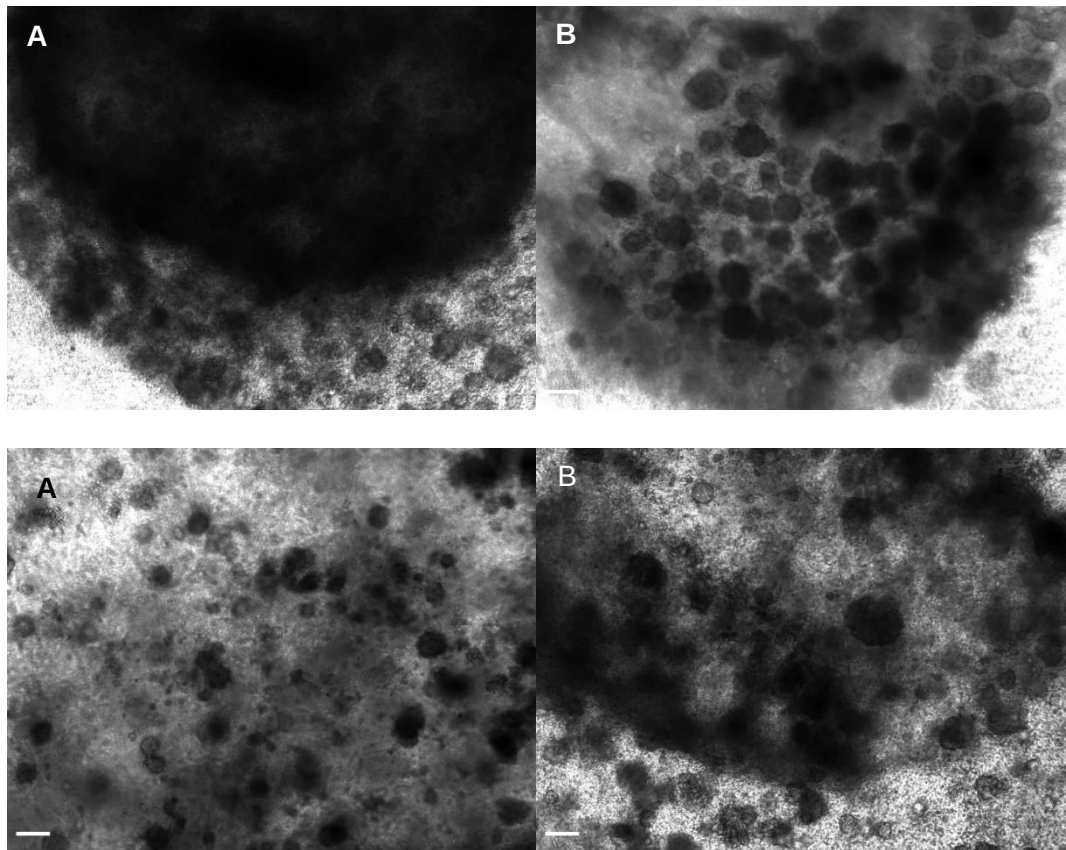


Figure 5-34: **BR15 grows in a consistent pattern regardless of mouse strain used for passage** BR15 grown for 9 days in peptide gel after passage in NSG (A) or Rag (B) mice. Scale bars show 200 μ m. Representative images from 3 replicate wells using Nikon Eclipse widefield microscope.

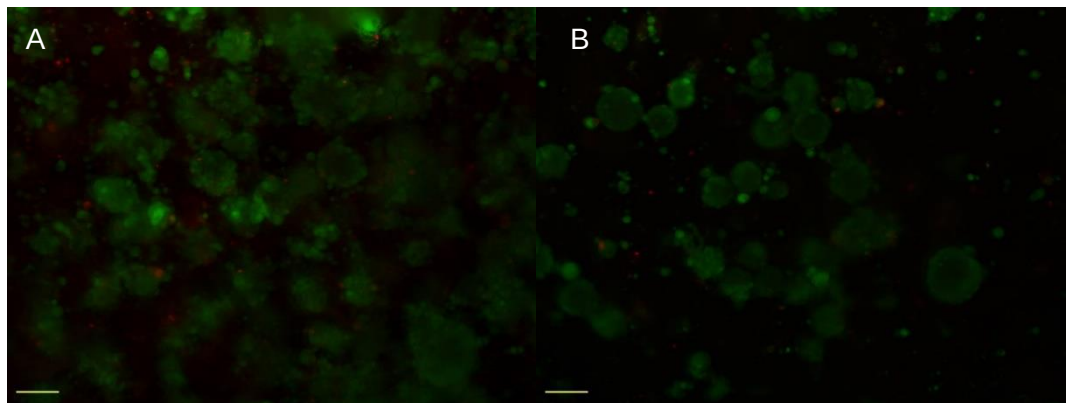


Figure 5-35: **BR8 grows in a consistent pattern regardless of mouse strain used for passage** BR8 grown for 11 days in peptide gel after passage in NSG (A) or Rag (B) mice. Scale bars show 100 μ m, green shows live cells while red shows dead cells. Representative images from 3 replicate wells using Nikon Eclipse widefield microscope.

5.3.3 Compatibility of gels with Embedding, sectioning and histology

The ability to perform histological staining on cells grown within the peptide gels would be an extremely useful tool to not only judge maintenance of heterogeneity passage in high detail for a great range of proteins, but also to make the model more useful in experimental contexts. These techniques allow samples to be preserved for a long time and stained for a large number of markers in a single gel providing spatial protein expression information.

It was possible, by pre-embedding in agarose, to paraffin embed PDXs in peptide gels for sectioning and histological staining. Integrity of the gel was not fully maintained despite embedding earlier than passage would typically be necessary, but individual clusters of cells could be clearly seen and stained. Figure shows Haemotoxylin and Eosin (H&E) staining, giving a simple overview of tissue structure, of PDX-derived cell clusters grown in peptide gels at different passages. P0 samples were grown by Sal Jones and sectioned and stained by Mireia Sueca-Comes, while P4 samples were grown by Jennifer Ashworth and sectioned and stained by Sal Jones. Importantly, differences between PDX line behaviours in P0 (most noticeably the looser packing of BR15 clusters than BR8 clusters) are still observable at P4, indicating retention of key characteristics. More importantly this experiment demonstrates the possibility that immunohistochemical staining for relevant proteins is likely to be compatible with the peptide gels.

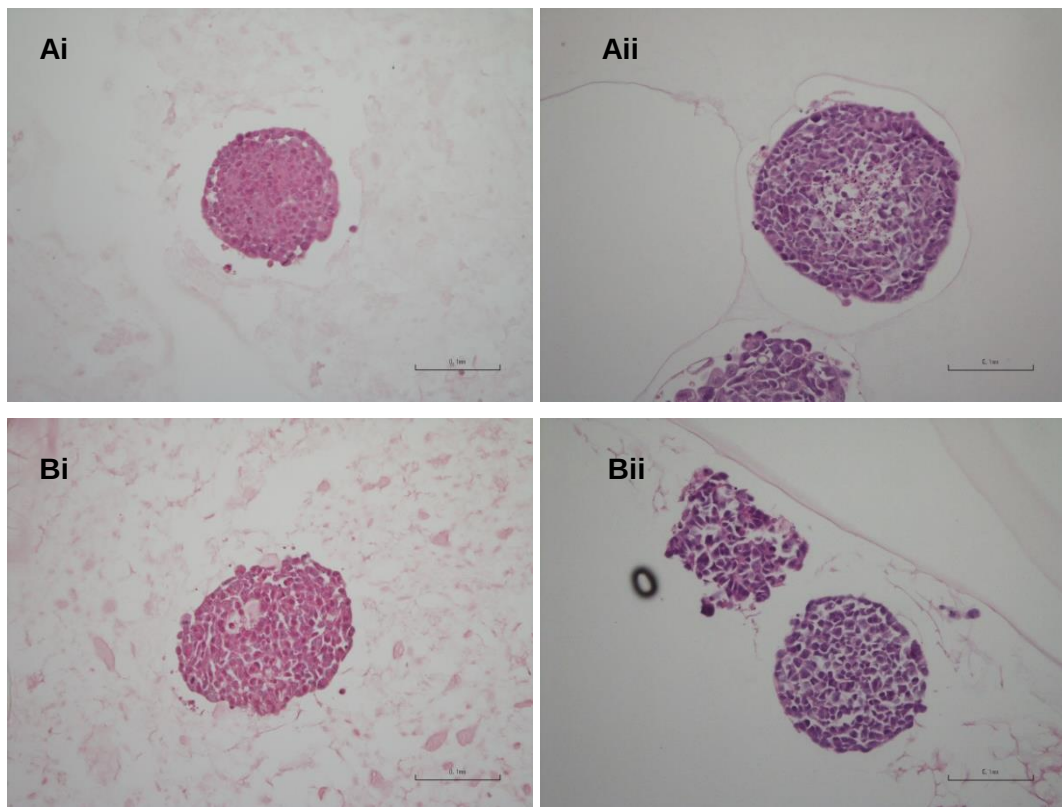


Figure 5-36: Peptide gels can be embedded for sectioning and subsequently stained for histological features H&E staining of BR8 (A) and BR15 (B) PDXs at P0 (i) and P4 (ii) Scale bars 0.1mm

5.4 Conclusion

The work presented in this chapter has answered a number of key questions regarding the proposed peptide gel model, in addition to uncovering some aspects that will require further investigation. Passage of PDXs in the peptide gel proved remarkably easy for some PDXs and far more challenging for others, a division that appears to be based on the lab in which the PDXs were established rather than on the molecular subtype of the tumour as might have been expected to be more relevant. The model proved to be highly compatible with Live/Dead staining, which will be a valuable tool for further optimisations. Preliminary results were also promising regarding the ability of cells grown in the gel to be restored following cryopreservation, though considerable further optimisation outside of the scope of this project is likely to be necessary to ensure no loss of intra-tumoural heterogeneity occurs during such a process.

6 Discussion

Overall this project achieved its key goal, to generate a peptide gel-based model in which PDX-derived cells could be grown and passaged. Through metabolite-based assays, Live/Dead staining and qPCR-based species testing it was possible to confirm survival and growth of almost exclusively human tumour cells in the model. PDX-derived cells grown in these models proved compatible with a range of techniques to investigate their molecular features of the cells, a key requirement for a model intended to produce cells for research. Initial attempts at cryopreservation were very promising, suggesting that this model could allow cells to be stored with at least the same degree of practicality as the *in vivo* models they would replace. Additionally, it was possible to replace serum-containing medium in this model with a xeno-free alternative, demonstrating potential for a completely animal-free model.

PDX-derived cells grow in peptide gel

It was possible, on the first attempt, to grow PDX-derived cells of both triple-negative and hormone receptor-positive tumours in the peptide gel without supplementation beyond that provided by the same RPMI (with bovine serum and l-glutamine) used for cell line culture. It would usually be expected that the cancer cells would require anti-apoptotic signals via integrin binding domains from basement membrane proteins [58]. Possibly these signals were provided initially by proteins or murine stromal cells in the disaggregated PDX, and then later by molecules secreted by the cancer cells themselves [95]. Incomplete disaggregation may also have aided in early survival in the peptide gel by providing cell-cell interactions to prevent anoikis.

PDX-derived cells and cell lines which grew in the peptide gel did so in the form of primarily spherical clusters; Figure 3-10 (page 52) shows 6 days of MCF7 growth in the 3D-TE model while Figure 3-26 shows 7 days of growth of the same cells in the peptide gel. In the 3D-TE, in spite of the 3D arrangement, the cells are dispersed have not achieved a tumour-like structure. In the 3D-TE only cell-matrix and not cell-cell interactions are present to improve the format's relevance over 2D [159]. Human, cell-derived matrix molecules are likely present in the peptide gel [95] to provide a far more patient-tumour-relevant environment for the encapsulated cells. Additionally, when growing cells in the peptide gel the cells remained fully suspended in the peptide gel unless cell growth was allowed to progress to a point where cell growth compromised gel integrity – this is in contrast

to the 3D-TE model where sinking of cells was evident from day one and, for some cell types, extensive enough to call into question whether the majority of cells were in 3D. Overall, the cluster formation and improved suspension in the peptide gel suggests that this model more completely mimics 3D elements of patient tumours than the 3D-TE model.

Some variation was observed between different PDXs in terms of their ability to grow and proliferate in the peptide gel. While ER positive PDXs might have been expected to require oestrogen supplementation which was not provided, these observed differences did not seem to have any relation to the molecular subtypes of the PDX-derived cells and work by others has not always found correlations between molecular subtype and compatibility with 3D *in vivo* expansion [165]. In an ideal case scenario it would have been possible to attempt to passage cells from a greater range of PDXs in the peptide gel, particularly for prolonged, consistent passage following optimisation, as different PDXs can vary considerably in how they respond to passage even in basic growth parameters [186]. PDXs from a greater range of laboratories would have been particularly interesting given the difficulties experienced exclusively with those established outside of EVPC – this would be a priority were more time available, as the different behaviours of the PDXs established by different groups is particularly relevant to concerns surrounding model relevance.

PDX-derived cells can be passaged in peptide gel

The ability to passage PDX-derived cells, in addition to being a successful key outcome for project, was a great practical benefit within the project, making it easier to work around limited PDX tissue availability in a strong reflection of the potential value of the model.

Testing by qPCR confirmed that the passaged cells were almost entirely human (section 4.3, page 92). 20-40% contamination by mouse cells was found in the initial disaggregated PDX tissue, consistent with descriptions in the literature of replacement of human stroma in PDXs with murine host stroma [118, 187], and yet this contamination was apparently lost on passage. Given the excellent overall growth in the model for PDXs which proved compatible, even survival without growth could have resulted in very rapid loss of this murine component. While it was necessary to test this, it is not surprising that it is the case given that what particularly distinguishes cancer cells from non-cancer cells is unrestrained

proliferation, which will allow them to rapidly outcompete stromal cells regardless of whether the stromal cells survive in the peptide gel.

PDX-derived cells remain highly viable after passage in peptide gel

PrestoBlue was able to assay proliferation of cells grown in the peptide gel with less variation between technical replicates than AlamarBlue (section 4.1, page 77). Without knowing exactly how PrestoBlue differs from AlamarBlue it is hard to form a hypothesis as to why this may be the case, however this does not seem to have been the experience of researchers working in 2D culture [172]. Possibly this is an effect which only exists in the peptide gel – altered reagent or buffer formulation might improve diffusion through the gel or penetration into the 3D cell clusters, neither of which is a barrier to assay progress in 2D. In general many proliferation assays were effective and informative in the peptide gel, and the major limitation was not imposed by the gel itself – without modifying the cancer cells and likely losing heterogeneity in the process, it would be a challenge to separate signals from cancer cells and any added stromal cells.

Live/dead staining performed in passage 0 compared with passage 3 or 4 gels showed remarkably similar cell cluster structures (section 5.2, page 144), which is a promising sign in terms of avoiding changes to the passaged cells in the gels. Staining itself was easy, clear and practical, though quantitative analysis of live/dead staining in 3D proved to be an interesting challenge – these images contain a high proportion of out-of-focus cells which cannot be properly thresholded for counting and which produce disproportionately large areas of fluorescence preventing calculation by area. An option in some cases would be to use confocal imaging, such as that in the Opera Phenix imaging system, to reduce out-of-focus signal, as confocal imaging has already been shown to work well with the peptide gel [95, 188], or including a DAPI stain to allow cell counting [189].

This Live/Dead staining also showed that an extremely large percentage of cells grown within the model were living for those PDXs that could be passaged. Given that the intent of the model is to preserve any intra-tumoural heterogeneity found in the PDX, this is a particularly important outcome. The lack of cell death implies a supportive environment, potentially maintaining a greater degree of intra-tumoural heterogeneity, now understood to be present in many tumour types and a likely driver of recurrence in patients not reflected by studies in cell lines [9, 11,

190]. Further experiments would be required to confirm maintenance of heterogeneity, but this is a very promising outcome at this stage.

Passaged Cells are Compatible with Heterogeneity assays

A number of assays with potential to test for intra-tumoural heterogeneity were performed on PDX-derived cells before and after passage in the peptide gel. Use of these kinds of assays will allow testing for retention of genetic variations between sub-clones of a given tumour. These variations may affect growth patterns and treatment outcomes in ways that cannot be explained by the findings of bulk assays, a situation thoroughly reviewed in 2016 [191].

High resolution melting proved technically highly compatible with the cells produced by passage in the peptide gel, and demonstrated that the original PDX did not have any heterogeneity in the small chromosomal regions for which primers were available (section 4.4.1, page 97). While this precluded actual heterogeneity testing on the PDXs available during the course of the project, were a large panel of target primers to be optimised it could be applied to PDX-derived cells before and after passage in the peptide gel to test for and changes in bulk DNA-level heterogeneity, even where that heterogeneity only exists at extremely low levels [177]. This would be a great improvement over the current situation in which, likely due to the lack of a convenient panel such as this, some papers describing culture of patient-derived tissue acknowledge that loss of intra-tumoural heterogeneity is a concern but have not addressed it in their own work [161], and even in the best cases only a small number of markers may be assessed [119].

Immunostaining of cells fixed in the peptide gel was achievable and could be imaged using a widefield microscope (section 4.4.2, page 100), confirming a major element of the model's capacity to be adopted for use within new laboratory spaces and using different equipment. Importantly, immunostaining allows spatial, single-cell, protein-level heterogeneity assessment, meaning the information provided is likely to be more reflective of phenotype than the DNA-level information of HRM and more able to distinguish changing sub-populations than HRM's bulk approach from pooled cells. A panel of relevant antibodies would have to be assembled, but while novel antibody generation would be more expensive than novel HRM PCR primer generation, antibodies against human cellular proteins are very broadly useful for other, related techniques and subsequently already exist for many markers, particularly those relevant in cancer [192]. Staining of paraffin-embedded fixed sections rather than whole gels

would give potentially greater ability to apply a large panel of antibodies to a single gel. Additionally, for certain pathways and processes, commercial antibody or RNA probe panels such as those by NanoString Technologies are already available [193]. Some of these are suitable for multiplex staining of intact samples, which would make them an incredibly powerful tool for heterogeneity monitoring.

Imaging Flow Cytometry, previously used by others to assess heterogeneity in cancer stem cell populations [194], proved compatible with the PDX-derived cells and showed potential to be optimised in future work for detection of a range of heterogeneity measures with cell-by-cell data generation (section 4.4.3, page 103). An experiment was planned to identify whether this was still true for cells post-passage in the peptide gel but could not be completed. If further experiments demonstrate that this is also possible following passage it could be incredibly powerful to optimise fluorescent antibodies for surface proteins such as hormone receptors [195, 196] to then identify and compare heterogeneity in this aspect of the tumour before or after passage in the gel, to assess whether expression of these treatment-relevant markers is altered by the *in vitro* environment. This is particularly valuable as it reflects protein expression rather than DNA or RNA changes which might be counterbalanced by other changes before having any effect on cellular phenotype and subsequent impact on patient outcomes.

Peptide Gel Model Demonstrated Further Features for Practical Usability

It was possible to grow and passage PDX-derived cells in the peptide gel in an entirely animal-free environment using xeno-free medium (section 5.1.3, page 132). While more prolonged experiments would be required to confirm the duration and other effects of this medium alteration and optimise for better post-passage growth, in comparison to the well-known and well-used alternative 3D *in vitro* expansion option of organoids which relies on animal-derived components including basement membrane extract [197] this is significant step towards a more ethical and relevant model.

A preliminary experiment found that cells grown in the gel and then frozen for several months could be restored with a reasonable fraction of living cells in new gels (section 5.3.1, page 153), and initial observations were that a lag-time before growth began seemed likely. This is consistent with observations sometimes made when restoring the same PDXs in mice within the group's animal unit. This is a key

practical requirement for any model used for long-term passage, and appeared to work similarly well using cryopreservation protocols adapted from that used for tissue chunk and that used for cell lines. Like passage, cryopreservation has potential to select particular sub-clones of a heterogeneous tumour sample, and therefore comes with a risk of reducing heterogeneity and subsequent similarity to patient tissue. Additionally, cryopreservation by different methods has been shown to have different effects on expression of genes with important roles in cancer [198]. This means that while these initial results are promising, heterogeneity tests applied before and after passage should also be applied before and after cryopreservation – if results are not promising alternative cryoprotectants to the DMSO used within the project may be more effective [199], or further anti-apoptotic additions may be required [200].

Given that MSCs are one cell type considered to be precursors to or otherwise closely related to cancer associated fibroblasts [201, 202] they were the subject of several experiments within this project. Within the course of this project an RNAseq experiment was performed to compare immortalised and non-immortalised bone marrow-derived MSCs, and it was observed that the major changes related, as expected, to cell cycle and particularly the intentional TERT expression change [183]. Conditioning with cancer cell line-conditioned medium was included to relate the investigation more closely to co-culture potential in a cancer model, however any effects of tumour-conditioning were insignificant compared to the effects of the immortalisation process on the MSCs. It is possible that both this and the lack of durable co-culture effect from MSCs in peptide gel experiments is due to the 2D arrangement of the MSCs in both cases, and that by ensuring MSCs are in 3D culture (and possibly with comparison of conditioning versus indirect co-culture versus direct co-culture, which allow single-direction paracrine signalling, bi-directional paracrine signalling and complete interaction respectively) an effect more consistent with other 3D experiments (including by other labs [171]) and patient outcomes [203, 204] can be obtained.

Practical Comparison of Peptide Gel and *in vivo* Passage

Table 6-1: *Comparison of practical aspects of in vivo and in vitro passage of PDXs*

	PDX <i>in vivo</i>	PDX in Peptide Gel
Time to Passage	Usually around 3 weeks for PDXs which grew successfully in gel	9-14 days for PDXs which grew successfully in gel
Cluster/Tumour Size	~10mm	<1mm
Approximate Cost	£180/mouse	£3-4/well
Imaging Options	Luciferin imaging can give information about the size and location of growing tumours if cells are modified to express luciferase, otherwise medical-type imaging methods such as X-rays or MRI are required for non-endpoint internal visual data.	Many brightfield microscopes can image in the transwell insert format without genetic modification, though visibility may be poorer than 2D models at high magnifications.
Maintenance	Every day	Every second or third day

Table compares practical considerations of passage of PDXs or PDX-derived cells *in vivo* versus in the peptide gel. Notably, cost per volume to use the peptide gel would decrease very significantly were the gel to be adopted as a wide-spread method allowing bulk production, but one well is not directly equivalent to one mouse. Several wells on the current scale would be needed for an equivalent cell number, but this granularity could be beneficial in avoiding waste once optimised for the context. Greater cluster sizes in gels were not attempted might reasonably be feasible, and would be a point of exploration for scale-up optimisation particularly.

Points for Future Users to Consider

It is important to consider tissue-specific features that will be relevant when developing a complex model. Different tissues of the body can vary greatly in ECM composition, and this can influence how cells behave within them [205, 206], as well as whether they can host metastases from particular tumours. For example breast tumours metastasise mostly to liver, lung and bone with bone being the most common [207] while neuroendocrine tumours, which have primary sites in various tissues, will usually metastasise to the liver if metastases form, additionally

metastasising fairly commonly to bone, lung and other intra-abdominal sites [208]. Comparison between the two types therefore shows some similarities in metastatic sites in spite of different primary sites as well some differences in metastatic sites, in line with the seed and soil hypothesis as initially described over 100 years ago by Paget [209] and more recently reviewed by Langley and Fidler [210]. In this case beginning with a single well-characterised cancer has provided a solid foundation for future adaptation of the model to other tissues based on their specific TME characteristics. The model described within this project has been generated with great care to reflect breast tissue particularly, and while the peptide gel is theoretically suitable to be used with any tissue except possibly blood and bone, optimisation would be required on a per-tissue basis and may involve inclusion of different ECM components and/or cell types.

Modification of the peptide gel by non-covalent [95] or covalent addition of ECM molecules has potential to be an extremely powerful improvement to the relevance of the model without greatly decreasing its practical usability – by assessing the composition of patient tumour extracellular matrix, key proteins or glycans could be identified to tailor the peptide gel model to particular tumour types. Incorporating such proteins or glycans in isolation and in combinations may identify a practical-but-relevant ideal for a given cancer model centred around the peptide gel, and a balance of molecule loss (of non-covalently-incorporated additions with media changes) over time versus possible changes to the physical properties of the gel or binding properties of the matrix molecules due to covalent attachment might define whether covalent or non-covalent incorporation is preferred.

When assessing assays for compatibility with the peptide gel, it appeared that the reagents reduced viability of cells slightly in wells that were re-used. This meant that while a large number of assays that could provide end-point survival and growth information were identified during the project, real-time growth information could only be obtained by a small number of approaches, each with some limitations. For example, while the luciferase assay was informative and has been used in other 3D models with cell lines by the group, it requires genetically modified and selected cells which by definition reduces heterogeneity. Further exploration of options for real-time growth monitoring might therefore be useful. In 2D formats extremely highly automated proliferation assessment is possible using systems such as Incucyte® Live-Cell Analysis System [211], however extending this approach to multiple cell clusters per well in 3D appears to be a challenge which many devices like these and their software are unable to tackle, though some such

as the Opera Phenix are compatible with the format [188]. Metabolite-based assays are therefore the only clear options for this, and with them the need for careful optimisation to cope with 3D-specific requirements relating to diffusion and possible toxicity relating to retention of reagents by the gel.

There is also potential for application of scale-up and automation of the peptide gel model. An arrangement that could be seeded and automatically provided with a flow of medium from a large reservoir for two weeks without further researcher involvement, to then be harvested for passage or experimentation using the generated cells, would be a time and effort investment that would pay off considerably in comparison to the cost and time input required to maintain PDXs *in vivo*. Commercial devices for such automated culture medium flows exist already. On a scale slightly larger than was typically used within this project are products such as Kirkstall's Quasi Vivo® series in which early experiments could be performed to establish relevant flow rates [212]. This would require identification of maximum automation durations and other optimisations such as whether covalent bonding of any incorporated ECM molecules would be required to prevent them from being washed out, before further scaling up where necessary.

If the peptide gel model is found to maintain heterogeneity found in PDX-derived cells, it would potentially be valuable to attempt to grow tissues taken directly from patients in the model, alongside appropriate matrix molecules and stromal cells. This would have benefits both in terms of further reduction of animal (and animal-derived product) usage and in generating human-relevant cells at a speed more conducive to personalised medicine approaches. This might be considered a key long-term goal following on from this project in terms of providing an animal-replacement model to expand patient-derived tissue efficiently in such a way as to ensure the generated cells are as similar to those of the tumour they originated from as possible.

7 References

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