Developing Three Dimensional Models of Breast Cancer

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MD Thesis

The University of Edinburgh

2014

Declaration

I, Alexander Leeper, confirm the research and data documented in this thesis to be my own. The contributions of other researchers/technical staff are acknowledged. This work has not been submitted for any other degree or professional qualification.

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Abbreviations

+	Positive
-	Negative
2D	Two dimensional
3D	Three dimensional
ADH	Atypical Ductal Hyperplasia
Akt	Protein Kinase B
ALDH	Aldehyde dehydrogenase
AI	Aromatase Inhibitor
AnCar	Anaplastic carcinoma
ANOVA	Analysis of Variance statistic
AP1	Activated protein 1 transcription factor
APC	Adenomatous polyposis coli
AQUA	Automated quantitative analysis
ASCO	American Society of Clinical Oncology
ATAC	Arimidex, Tamoxifen, Alone or in Combination
au	Arbitary units
BaB	Basal B
BABB	1:2 Benzyl alcohol, Benzyl benzoate
Bim	Bcl2-Interacting Protein
BMI	Body mass index
BRCA	Breast cancer susceptibility protein
CAF	Cancer associated fibroblast
CCASP3	Cleaved Caspase-3 antibody
CDC	Centers for Disease Control, United States
CI	confidence interval
CK	Cytokeratin
CSC	Cancer stem cell
CSF1	Colony-Stimulating Factor Type 1
DAB	3,3-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Oestrogen receptor
ERBB2+	See HER2
ERE	Oestrogen Response Elements
ERK	Extracellular signal-regulated kinases
ETOH	Ethanol
FEA	Flat Epithelial Atypia

FCS	Fetal calf serum
FFPE	Formalin fixed paraffin embedded
FGFR	Fibroblast growth factor receptor
FRR	familial relative risk
GSK3β	Glycogen synthase kinase 3 beta
H	Hydrocortisone
H&E	Haematoxylin and eosin
HER1	The Human Epidermal Growth Factor 1 gene
HER2	The Human Epidermal Growth Factor 2/neu gene (aka Erb-B2)
HMECs	Human mammary epithelial cells
HRP	horseradish peroxidase
HRT	Hormone replacement therapy
I	Insulin
ICD	International Classification of Disease
IDC	Invasive ductal carcinoma
IF	Immunofluorescence
IGFR	Insulin-like Growth Factor Receptor
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
ISD (Scotland):	Information and Statistics Division of the Scottish Executive
L	Litre
LBA	Ligand Binding Assay
LBX1	Ladybird homeobox1
LDL	Low-density lipoprotein
LN	Lymph node
m	Milli
MAPK	Mitogen-activate protein kinase
MEGM	Mammary Epithelial Growth Medium
MEOH	Methanol
MET	Mesenchymal to epithelial transition
miRNA	MicroRNA
MMP	Matrix metalloproteinase
ms	milliseconds
N	Normal
n	Sample size/population
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NHSBSP	NHS Breast Screening Programme
NICE	National Institute for Health and Care Excellence
NK	Not known
NST	No special type
p	Probability that null hypothesis is correct
PI3K	Phosphatidylinositol 3-kinase

Pap	Papillary
PARP	Poly ADP ribose polymerase
PBST	Phosphate Buffered Saline with Tween 20
PCR	Polymerase chain reaction
PIP	Phosphoinosites
PR	Progesterone receptor
qRT-PCR	Quantitative real time polymerase chain reaction
RR	Relative risk
RTK	Receptor Tyrosine Kinase
SERM	Selective Oestrogen Receptors Modulators
SPSS	Statistical Package for Social Sciences
TEB	Terminal end bud
TCF/LEF	T-cell-specific transcription factor/lymphoid enhancer binding factor
TDLU	Terminal duct lobular unit
TJ	Tight junction
TMA	Tissue micro-array
Tris	Tris(hydroxymethyl)aminomethane
UK	United Kingdom
US	United States of America
WB	Western Blot
WHO	World Health Organisation
Wnt	Wingless + integration site
WT1	Wilms' tumour protein
µ	Micro

Abstract

Background and Aims

Breast cancer is a leading cause of female mortality in the Western world. It is well established that the spread of breast cancer, first locally and later distally, is the major factor in patient prognosis. Experimental systems of breast cancer rely on cell lines usually derived from primary tumours or pleural effusions. Two major obstacles hinder this research: (i) Some known sub-types of breast cancers are not represented within current cell line collections; (ii) the influence of the tumour microenvironment is not usually taken into account.

Experimental Design

We developed a three-dimensional assay prepared from freshly harvested breast cancer tissue embedded in soft rat collagen I cushions. Invasive behaviour and tumour response to tamoxifen therapy was measured. Changes in proliferation, apoptosis and tumour volume in response to tamoxifen treatment were quantified using image analysis software and optical projection tomography. Further cell line based experiments and histopathological analysis of resection specimens were subsequently investigated to investigate the role of EGFR signalling pathways in driving invasion.

Results

We demonstrate a technique to culture primary breast cancer specimens of all sub-types. Within 2-3 weeks, individual and collective invasion of epithelial cells into the surrounding collagen I was observed using phase contrast light microscopy and histopathological methods. Addition of tamoxifen to preparations derived from ER+ tumours demonstrated a range of response as measured by proliferative and apoptotic markers and significant reduction in tumour volume not seen in ER- specimens. Changes in tumour volume allowed stratification into responsive and nonresponsive tumours. EGF within the culture medium appeared to drive a change in phenotype from ER+ to triple negative phenotype and acted as a driver for epithelial invasiveness.

Conclusion

Here, we developed an assay to culture human breast tumours without sub-type bias and to investigate and quantify the spread of these *ex vivo*. This method could be used to quantify drug sensitivity in primary cancers under conditions closer to real life. This may provide a more predictive model than currently used cell lines.

Chapter 1: Introduction

1.1 The Breast

1.1.1 Evolution and Development of the Normal Breast:

Evolutionary biologists hypothesise that lactation evolved prior to the divergence of monotremes (egg-laying mammals such as the platypus) and therian mammals (placental mammals and marsupials). They postulate that lactation provided a method of maintaining homeostasis within parchment-shelled eggs through the secretion of an evolutionary precursor to milk [1]. Since the emergence of mammals 220 million years ago, the initial rudimentary hypertrophic apocrine glands have evolved with the class. In humans, the primary function of the breast is to provide fluid, nutrition and transfer immunity from mother to infant.

The mammary gland is a complex secretory organ, consisting of epithelial and stromal components. Its unique developmental process spans the length of a human life and may be divided into three stages; embryonic, pubertal and adult. Tight hormonal control regulates these developmental stages [2].

Initiation of the first stage occurs midway through an embryo's gestative period. Two ridges of multi-layered ectoderm arise from the embryonic skin and run in an anterio-posterior line between forelimb and hindlimb. Ectodermal cells coalesce and form bulbs of epithelial cells that sink into the underlying mesenchyme. The mesenchymal cells surrounding the buds condense and form a mammary mesenchyme, which then facilitate invasion into the mammary fat pad to form a rudimentary ductal system. Multiple embryonic developmental pathways (including Hedgehog and Wnt signalling) along with growth factors play an essential role in driving cellular proliferation and invasion during the embryonic development of the mammary gland [3]. As gestation approaches term, the gland undergoes developmental arrest and the growth rate becomes commensurate with body growth, until the onset of the second developmental stage.

At puberty, in response to increasing levels of circulating oestrogen in females, terminal end buds (TEBs) appear at the tips of ducts and invade further into the surrounding fat pad. Proliferation within the TEBs leads to ductal elongation and bifurcation, while apoptosis of internal cells results in lumen formation. Growth factors such as EGF and CSF-1 play an essential role in branching morphogenesis. During a female's reproductive life, surging oestrogen levels during each oestrus cycle are associated with initiation of ductal side-branching. If pregnancy is not achieved, precipitous drops in serum oestrogen concentration lead to subsequent apoptosis.

In early pregnancy, circulating levels of progesterone rise leading to extensive side-branching and alveologenesis. As gestation come to term, levels of progesterone drop and rising prolactin stimulate alveoli maturation into the milk-synthesising units of the breast. Dramatic involution of breast tissue occurs on cessation of breast feeding and again at menopause as a result of hormone withdrawal.

1.1.2 Anatomy and Histology of the Normal Breast

In the adult female the breast is located in the superficial fascia of the anterior chest wall. Its superior and inferior borders are the 2nd and 7th rib and medial and lateral borders are the sternal edge and midaxillary line respectively [4]. The breast parenchyma is made up of 15-20 lobes, each based on a pyramidal branching ductal system. Dilatations at the apex of each lobe, lactiferous sinuses, converge behind the nipple and create a reservoir for milk. Behind the lactiferous sinus, branches of the lobular duct connect to terminal ductlobular units (TDLUs). The TDLU is the functional unit of the breast, comprising of succules (ductules/acini) branching from the intralobular ducts. The acini are small glandular spaces lined with cuboidal epithelial cells surrounded by a single outer layer of myoepithelial cells, derived from the embryonic mammary mesenchyme. The ducts have a similar histological appearance, with small cuboidal epithelial cells lying on a discontinuous myoepithelial layer [5]. The TDLU is supported by delicate intralobular connective tissue and stroma which contains a range of stromal cells including lymphocytes, macrophages and fibroblasts. Dense interlobular connective tissue and adipocytes help maintain the breast structure and function.

1.2 Breast Cancer

1.2.1 The development of breast cancer

In papers published by Wellings et al. in 1975, it was postulated that almost all breast cancer arose from the TDLU, using morphological and histological evidence gathered from systematic analysis of a large collection of primary breast cancer specimens [6]. In addition, they proposed a linear model of cancer progression; from normal breast epithelia through aytpia and hyperplastic changes to non-invasive carcinoma (carcinoma in situ) and finally invasive breast cancer [7]. Subsequent genomic and transcriptomic analysis of these distinct breast pathological classifications over the last decade have provided further evidence of this continuum. Furthermore low-grade and high-grade breast cancer appear to evolve down genetically distinct pathways with stepwise genomic alterations including 13q loss and high-level amplifications of 17q12 and 11q13 in high grade DCIS and IDC and loss of heterozygosity at chromosome 16q in FEA, and loss of 16q in ADH, low grade DCIS and IDC [8].

The aetiology of breast cancer is complex; an array of genetic traits, environmental and behavioural factors, as well as endogenous hormones are implicated [9].

The genetic component of the disease is reflected in its tendency to cluster in families. This may be calculated as familial relative risk (FRR), defined as the ratio of the risk of disease for a relative of an affected individual to that for the general population and varies dependent on the demographics of the affected relatives[10]. High penetrance *BRCA1* and *BRCA2* mutations together were estimated to explain 32% of breast cancer FRR for ER- breast cancer and 9.4% of FRR for ER+ disease[11]. In fact the major contributors of familial risk come from medium and low penetrance genetic variants, many of which have not been identified.

Changes in fertility patterns, BMI and use of alcohol in Western Society have correlated with an increase in the incidence of breast cancer. As modification of these environmental factors may

mitigate risk, intense research has been conducted to rationalise the relevance of these factors. Korenman et al. hypothesised that risk correlated with length of lifetime exposure to circulating oestrogen, "the oestrogen window"[12]. Lower parity, increasing BMI, alcohol consumption and prescription of exogenous oestrogen have all been shown to increase circulating endogenous oestrogen levels[13], in turn leading to oestrogen driven DNA synthesis and production of potentially carcinogenic metabolites of oestrogen [14].

1.2.2 Epidemiology of breast cancer

The impact of cancer on society is significant, cited as the primary cause in 29% of all recorded deaths in the UK in 2009 [15]. Over 85% of diagnosed malignancies arise from epithelial tissue, due to comparatively high mitotic indices and cell turnover. Breast cancer makes up a substantial proportion of these, for example accounting for 28.3% of all cancers diagnosed in Scottish females in 2008 and the highest ranking cancer diagnosis in the country.

Over time, the epidemiology of breast cancer appears to be changing. In 2010, Scotland had an estimated population of 5,222,100 [16]. In the same year 4,457 new cases of breast cancer in females were registered. When compared with 2000, this represents a 12.0% increase in annual breast cancer incidence, compared with a 1% increase in population over the last ten years[17].

Despite the rising incidence of breast cancer, the associated death rates are in decline, with a 18.3% reduction in breast cancer related mortality in 2010 compared with the previous decade [17]. Improvements in breast cancer survival over the last decade have arisen due to a combination of earlier diagnosis, better public awareness and the widespread introduction of effective systemic therapy following resection surgery.

Additionally, following its introduction in 1988, it is likely that the NHS Breast Screening Programme (NHSBSP) has increased the survival rates of breast cancer through earlier disease detection. Controversy has arisen as breast screening additionally increases the reported incidence of

asymptomatic breast cancer, detecting disease in patients that may otherwise have remained undetected until their death by another cause [18].

Along with changing epidemiology, there has been a shift in the underlying cellular biology of detected breast cancers over recent years. A recent retrospective study analysed changes in ER positivity between two cohorts of breast cancer pathology specimens, collected in the 1980's and 1990's respectively. They identified a statistically significant increase in ER positive disease between each cohort, with 64.2% of tumours demonstrating positivity in the 1980's and 71.5% positive in the 1990's ($p=0.042$) [19]. Both an increase in exposure to aetiological factors combined with a lengthening life expectancy are likely causal factors behind this increase.

Taken together, the combination of increasing breast cancer incidence combined with a disproportionate increase in ER+ disease indicates that the demand for more efficacious treatment of ER+ breast cancer will continue to grow at a global level.

1.3 Development of Breast Cancer Models

1.3.1 Modelling in Breast Cancer

In order to further our current understanding of the mechanisms by which breast cancer develops and discover new treatments to improve survival for patients, researchers increasingly rely on modelling disease in a laboratory environment. It is essential to remember that breast cancer is not a single disease but a diverse collection of neoplasms occurring in breast with distinct genomic and proteomic variations. Over many years multiple alternative models have been developed in order to recapitulate specific characteristics of a variety of breast cancer subtypes. The benefit of modelling is to simplify and reliably reproduce a complex biological system in a controlled environment. As a result each model is, to an extent, imperfect. However this can be mitigated through selection of an optimal model in order to answer a specific question whilst keeping its specific limitations in mind. Models can provide insight in to multiple facets of oncogenesis and help provide initial evidence as to the likely efficacy of a treatment strategy[20].

1.3.2 Breast Cancer Cell Lines

Cell lines remain the principal model for cellular biologists studying breast cancer. Easily propagated, cheap, reproducible and amenable to genetic manipulation, cell lines have provided a considerable proportion of our knowledge regarding cancer proliferation, apoptosis and migration.

To validate the relevance of cell lines, Neve et al. investigated a panel of 51 breast cancer cell lines and compared their biological and genomic properties against 145 primary breast tumours, concluding that the recurrent genomic and transcriptional characteristics are, to an extent, mirrored in the cell line model[21]. Sorlie et al. had previously described five common breast cancer subtypes (Luminal A, Luminal B, Basal-like, ERBB2+ over-expressing and Normal Breast-like), observed in hierarchical clustering of gene expression using DNA micro-arrays from primary breast cancer [22]. This was in part evident in Neve's panel of cell lines, though Basal A and Basal B subtypes were

identified and Luminal A and Luminal B subsets were not apparent. Furthermore other cancer subsets such as 1q/16q aberrations, a common mutation previously linked to high steroid receptor expression and low proliferative index in primary breast cancers[23] were absent in Neve's cell line panel.

The under-representation of indolent disease may be as a consequence of the majority of cell lines being derived from aggressive tumours and metastatic deposits such as pleural effusions. This relative bias towards more aggressive cell lines may be due to the difficulties of developing cell lines from primary breast cancer tissue. Success rates for the development of cell lines from primary samples are quoted to be as low as 10%[24] to 1%[25]. Furthermore, the process of repeated culture and passage exerts selection pressures on cells, positively selecting those cells with the highest mitotic rate. Cells that do not proliferate on plastic or are dependent on growth factors not present in the cell culture media will eventually be passaged out from culture.

During the design of an experiment, the selection of a particular breast cancer cell line is often influenced by its ability to thrive in specific experimental conditions. As a result, the full spectrum of breast cancer cell lines is rarely utilised, with the majority of breast cancer research based on a small minority of cell lines. One study suggests that almost 70% of breast cancer cell biology research is restricted to just three breast cancer cell lines: MCF-7, MDA-MB-231 and T47-D[26].

Despite these short-comings, cell-lines have played an essential role in guiding novel drug discovery and development[27]. For instance, preclinical models demonstrating MCF-7 sensitivity to anti-oestrogen development led to the development of fulvestrant[28] and its introduction as a second line treatment in ER+ metastic breast cancer[29]. In addition PARP inhibitors have been developed to exploit DNA repair deficiencies in cancer[30]. Cell lines with BRCA1 and BRCA2 deficiency were used to demonstrate that synthetic lethality was an extremely effective strategy in targeting cells with BRCA mutations[31]. This provided evidence for the design of clinical trials that have gone on to support the use of PARP inhibitors as an effective adjunct in patients with BRCA mutations [32].

However, there are at least two major disadvantages in the use of cell lines grown on plastic to model breast cancer; the lack of three dimensional context and the absence of cellular heterogeneity found in primary breast cancer. In order to improve validity and more accurately reflect cellular biology *in vivo*, alternative assays have been developed.

1.3.3 Modelling in Three Dimensions

A well-recognised sequela of harvesting primary tissue and placing in 2D culture is a resultant change in cellular morphology and functional behaviour[33]. In 2D culture conditions mammary epithelial cells are unable to induce expression of certain tissue-specific genes[34]. Over the preceding three decades Bissell and colleagues have championed the use of three dimensional cell culture as a method of providing a physiologically relevant model. They argue that through these models, the dynamic and reciprocal signalling required to induce branching morphogenesis may be recapitulated and studied[35]. Cell-cell and cell-extracellular matrix interaction may result in the establishment and maintenance of underlying tissue specificity, overcoming the rapid de-differentiation induced even in non-malignant cell lines upon culture on two-dimensional plastic substrata[36].

In order to investigate the relevance of 3D culture, Kenny et al. compared changes in the gene expression profiles of cell lines grown in 2D and 3D cultures [37]. They identified 96 Affymetrix probes strongly up/downregulated consistently across the majority of the cell lines. Of these, 41 corresponded to genes with annotated functions. Using Gene Ontology annotations [38] they determined one classification– "signal transducer activity"– was significantly overrepresented in the set of genes which differ between 2D and 3D culture. Furthermore, these gene expression profiles correlated with distinct morphologies and invasive behaviour. This provides evidence at a genomic level that 3D cultures provide an alternative model of cell signalling pathways with which to investigate cancer morphology and invasion.

1.4 The Dynamics of Cancer

1.4.1 Tumour Microenvironment and the Extra Cellular Matrix

The importance of the extra-cellular matrix (ECM) in cell physiology and cancer pathology should not be underestimated. Not only does it provide environmental cues and contextual information to regulate cellular differentiation but also provides a structural scaffold on which epithelium may create complex tissue structures[39]. The ECM provides both a physical and chemical barrier to migrating cells. In itself, uncontrolled proliferation of cells following malignant transformation is not sufficient to lead to localised invasion into surrounding healthy tissue. Indeed, these cells will remain *in situ* until they acquire the ability to traverse the ECM. In order to move within and between tissues, cancer cells may invoke mechanisms of invasion used during physiological migration of non-neoplastic cells[40].

In-depth histopathological analysis of carcinoma reveals that tumours are not just disorderly collections of tumour cells but maladjusted living entities, composed of cancerous cells and additionally non-neoplastic tissue recruited to provide essential support for the growth of the neoplasm[41]. Until recently the changes occurring at the tumour-host interface were thought to be of secondary importance to the genomic mutations detected in tumour tissue by molecular biologists. However, work on peritumoural support mechanisms, such as the role of angiogenesis to aid tumour growth[42] allowed for the development of inhibitors[43] as therapeutic strategies that have subsequently become available for cancer treatment in combination with other chemotherapy regimens[44]. Along with endothelium, other cell populations within the stroma have critical roles in tumour growth, invasion and metastasis. The tacit support of the host's microenvironmental stroma is essential for the tumour's existence and growth.

In developmental biology, it is well recognised that interaction between mesenchyme and epithelium is essential for structural development of tissue to occur. These interactions continue through adult life in order for organ repair and maintenance[45]. In the initial stages of cancer,

changes in tissue adjacent to the site of neoplasia are pronounced. Experimentally induced carcinogenesis using irradiated skin demonstrates epidermal thickening and changes in collagen configuration, dermal infiltration by stromal cells from the subdermis and neovascularisation, followed by degradation of the basement membrane at the tumour-host interface[46].

Taken together, both the spatial context and interaction between cancer and its associated stroma play an essential role in carcinogenesis and tumour progression. The wider context of the changes occurring in surrounding host tissue must be taken into account when designing and interpreting cell-line based experiments. 2D monocultures of epithelial cells may provide an insight in to cell signalling pathways that epithelia are capable of utilising whilst in the *in vitro* setting. However it is misleading to extrapolate these findings as being physiologically relevant in the absence of corroborating *in vivo* evidence. Through application of three dimensional experimental systems that incorporate stromal elements the gap between the laboratory and clinic may to an extent be bridged.

1.4.2 Modes and Mechanisms of Migration

Movement is initiated within the motile cell through induction of a pseudopod at its leading edge, elongating the cell in a specific direction of travel. This may be induced by a chemotactic pathway, such as growth factor receptor stimulation, leading to activation of the phosphatidylinositol 3-kinase (PI3K) pathway. The activated PI3K pathway may generate phosphoinosites (PIPs), inserting in to the cell membrane and binding to actin filaments polymerising in the cytosol. The effect is to push the cell membrane in an outward direction to form lamellapoda, filopoda, pseudopods or invadopods depending on morphology and function[47].

Focal contact assemblies, often mediated through the integrin family, allow attachment between the pseudopod and the ECM such as $\alpha 2\beta 1$ integrin, through binding fibrillar collagen [48]. Subsequent actin shortening, mediated through myosin II, results in contraction of the cell's long axis with its unanchored tail gliding towards the attached focal contact assembly at the cell's leading

edge. Cells may either move as groups, termed collective migration[49] or individually, termed single-cell migration [50].

1.4.3 Collective Migration

Collective migration requires complex co-ordination of the invading cohort, with maintenance of some cell-cell junctions and simultaneous dissociation from non-motile cells, along with supracellular organisation of actin cytoskeleton and modification of stromal barriers[51]. The mode and morphology of collectively migrating cells vary, dependent on the context of the ECM. Along tissue surfaces cells may migrate as two dimensional sheets, or penetrate into the underlying stroma as poorly organised strands as seen in invasive cancer, or detach completely from their origin as distinct clusters. Collective migration is seen during normal breast development, during glandular and ductal formation and is termed branching morphogenesis [52]. Cells located at the leading edge, "path leading cells" produce traction through pseudopod activity[53]. Constraining ECM may be initially degraded by the tip cell extending an invadopod to vitiate fibrillar collagen using membrane bound proteases such as MT1-MMP[54]. The narrow channel may then be further enlarged to accommodate the following multicellular strands by additional ECM modification and deposition of basement membrane to smooth the path of following cells[55]. Alternatively, pre-existing defects and tracks through the ECM such as fascial planes between tissues and the lumen of lymph vessels, may be used by the tip cells and provide a quick method of collective cell translocation[56].

1.4.4 Single Cell Migration and Epithelial to Mesenchymal Transition

Anoikis is a process of programmed cell death that occurs in epithelium following disruption of contact between cell and basement membrane[57]. This physiological process, which prevents displaced epithelia from reattachment and dysplastic growth, is essential for homeostasis and is mediated through disruption of integrin/ ECM engagement[58]. Loss of integrin engagement inhibits ERK and PI3K phosphorylation of Bim, a pro-apoptotic protein, resulting in its accumulation and

ultimately caspase-3 activation with a terminal increase in mitochondrial permeabilization, substrate proteolysis and disruption of focal adhesion architecture[59].

An important hallmark of neoplastic transformation of epithelium is the acquisition of anchorage-independent growth[60]. In the non-malignant breast cell line MCF-10A, following cell detachment from extracellular matrix, EGFR expression and its downstream markers, Erk and Akt are downregulated. Anoikis and apoptosis ensue. However, in the same cells, stable overexpression of EGFR conferred resistance to anoikis, with downstream Erk activation maintained and Bim upregulation blocked[61], permitting anchorage independent growth. A substantial fraction of breast cancer cell lines overexpress EGFR [62], which may provide a survival mechanism by which cell lines may tolerate regular disruption and passage.

Independence of anchorage to the underlying extracellular matrix is a key step in the process known as epithelial to mesenchymal transition (EMT) [63]. The ability for a polarised epithelial cell to transdifferentiate into a motile cell of mesenchymal morphology is essential for normal embryonic development and is seen during the development of the trilaminar embryo in vertebrate gastrulation [64] (Type 1 EMT). Subsequent reversion back to an epithelial phenotype is also possible (Mesenchymal-epithelial transition - MET), such as during conversion of the metanephric mesenchyme to create epithelial nephric tubules[65]. Such behaviour demonstrates the plasticity of the embryonic epithelium. In the epithelial response to injury, the wound healing process demonstrates an intermediate phenotype (Type 2 EMT) with both epithelial and mesenchymal properties demonstrated by the involved epithelia. Loose contact between keratinocytes is maintained, with an increase in Snail2 expression at the leading edge, formation of lamellipodia and migration of the lead cells, with dragging of the trailing epithelial cells [66]. This is seen during menstruation as part of the post-ovulatory wound healing process and is induced by EGF [67].

Type 3 EMT or oncogenic EMT is a postulated mechanism of tumour invasion and metastasis, whereby epithelial cells may demonstrate a variety of EMT-traits, but complete epithelial to mesenchymal transition may not occur [68]. In turn, acquisition of these traits may facilitate anoikis

resistance, increased motility, invasiveness through basement membrane, intravasation and evasion of the immune system.

However, this intuitive explanation for cancer dissemination remains controversial. A proportion of histopathologists argue that EMT, whilst often seen in the *in vitro* setting, is rarely visualised in cancer resection specimens. Pathologists describe the invading edge of tumours not as comprising of single cells of mesenchymal phenotype, but instead of well-connected epithelial sheets invading locally and intravasating as glandular structures[69]. Single cells circulating within the vasculature may be found but these retain epithelial characteristics. Furthermore, the use of antibodies such as vimentin and FDP1 often used to demonstrate a mesenchymal transition are not markers expressed specifically by the mesenchyme but are also expressed by normal epithelial tissue[41]. For EMT to occur, a radical and fundamental change in function, developmental fate and character of a cell lineage is required through implemented whole-scale gene expression reprogramming to abandon one and adopt a totally different life-style[70]. Functionally, however, these changes may be unnecessary, as histopathological features of tumour shows that they can readily invade and metastasize when they are in epithelial configuration.

Attempts have been made to reconcile these polarised views by synthesising available evidence to provide an overarching definition of EMT. Cells may be defined as being "EMT-like" based on three functional criteria: i) state of cell polarization, ii) state of cell cohesiveness, and iii) intermediate filament protein expression[71]. This descriptive term does not require an active dedifferentiation process to occur but emphasizes an intermediary phenotype occurring through activation of "EMT pathways" such as tyrosine kinase receptors, integrins, Wnt, NF-κB, and TGF-β. These adaptive physiological responses may be employed by the cancer to improve cell survival in a hostile tumour microenvironment.

1.5 Tumour Phenotype and Treatment Strategies

1.5.1 The role of basal cell populations in breast cancer

An integral part of the pathological assessment of breast cancer is immunohistochemical quantification of markers that can predict a probable response to targeted treatment. Only ER and HER2 expression are approved for quantitative assessment in the current guidelines from NICE, using immunohistochemistry with a standardised and qualitatively assured methodology [72]. Tumours that lack expression of ER, PR or HER2 are termed triple negative breast cancers and have a worse prognosis in comparison to ER/HER2 expressing tumours[73].

Triple negative cancers are in fact a heterogeneous collection of tumours, comprised of different histological variants including infiltrating ductal, medullary, squamous and apocrine carcinomas [74]. A common assumption is that immunohistochemically triple negative cancer and genomically clustered basal-like tumours are the same entity – based on the observation that tumours with basal gene expression profiles as described by Perou et al. are usually triple negative tumours on immunohistochemical analysis[75].

The term basal originates from analyses of stratified cytokeratin expression in cells cultured in 2D gel. Cells located adjacent to the basement membrane in a basal position were found to express cytokeratin 14, cytokeratin 17 and cytokeratin 5 [76]. As a result these cytokeratins were subsequently labelled as basal markers. The term 'basal' became synonymous with expression of these basal keratins (CK5/CK14) rather than with a position adjacent to the basement membrane. *In vivo* validation of these cytokeratins in resection mammoplasties demonstrated that CK5, CK14 and CK17 are expressed in luminal cells in large ducts and in the terminal duct lobular unit (TDLU) complex and are not restricted to cells within the myoepithelial compartment [77-79]. Cells expressing these basal cytokeratins rarely express hormone receptors or HER2. However, unlike myoepithelial cells[80], the cells expressing basal cytokeratins also express CK8/18, a cytokeratin used to demonstrate cells of luminal lineage [81].

In order to provide improved stratification of breast cancer in to more accurate prognosis groups, Perou et al. proposed that cancers could be categorised using molecular phenotypes through gene expression analysis. Gene expression profiles identified a subset of tumours characteristic of basal epithelia using a gene expression cluster including keratin 5, keratin 17, integrin-β4 and laminin. This group contrasted with another gene expression cluster thought to be characteristic of luminal cells anchored by transcription factors that included ER. Immunohistochemical analysis of these tumours identified proteomic expression of basal epithelial markers using IHC[75]. Further work, analysing a larger number of tumours and correlating gene expression patterns to overall and relapse-free survival demonstrated the clinical relevance of dividing patients into molecular subsets[82].

On the basis that breast cancer specimens clustered according to expression profiling analysis is the gold standard for identification of tumours with basal gene expression, the basal group is enriched with triple-negative tumours[83]. But surprisingly the gene expression patterns and histopathological analysis are not 100% concordant. Among tumours clustered as basal-like, 5%–45% were reported to be ER+, and 14% were shown to be HER2+ on IHC[84]. This indicates that even in ER+ cells basal-like expression pathways can be active.

From this it may be concluded that at a morphological level, basal-like breast cancers appear to arise not from the myoepithelium but from luminal cells, most likely the TDLU. They can produce glandular structures and express markers associated with luminal origin such as mucin and either cytokeratin 8 or cytokeratin 18. Taken together this evidence appears to contradict the suggestion that triple negative tumours arise from myoepithelial cells or exclusively from a basal subpopulation[80].

It may be of interest to note that the poor prognosis associated with triple negative breast cancer is most likely due to the lack of effective targeted therapy, such as oestrogen antagonists or trastuzumab, rather than an intrinsically worse prognosis: ER+ and ER- cancers adjusted for grade and size appear to have similar overall rates of relapse in patients where hormone therapy is withheld, though ER- disease relapses earlier and is more likely to metastasize to brain than bone

[85, 86]. It is important to remember that ER- disease encompasses a range of different breast cancer subtypes with a range of prognoses.

1.5.2 The role of the oestrogen receptor

As discussed earlier, quantification of oestrogen receptor (ER) expression provides important therapeutic and prognostic information. As a result of intense research, advances have been made in the understanding of oestrogen's mechanism of action on cells. But despite this, the relevant pathways by which oestrogen stimulates cell growth remain both poorly understood and controversial [87].

Understanding of the dual roles of oestrogen, both as an agent driving physiological growth and as a key agent in the pathological development and growth of breast cancer have been intertwined since the end of the nineteenth century. Oophorectomy as a treatment for breast cancer preceded the discovery of the ovarian hormone oestrogen in 1923 by almost 27 years [88]. By 1950, advances in radioisotope chemistry and detection techniques allowed Jensen et al. in a set of elegant experiments to demonstrate the binding and retention of tritium labelled oestrogen in specific tissues without metabolic alteration of the hormone itself. This led to the hypothesis that the proliferative effect of oestrogen was mediated through the presence of an oestrogen specific receptor protein in oestrogen sensitive tissues[89]. Isolation of the oestrogen receptor (ER) followed, along with demonstration of its conformational change, dimerization and coactivator binding on exposure to oestradiol[90]. Consequently, the use of nonsteroidal antioestrogens as competitive antagonists of oestrogen helped provide insight into the complexities of steroid endocrinology and opened the door to molecular targeting in the treatment and prevention of breast cancer.

The effect of oestrogen on target tissues is mediated mainly through its binding with oestrogen receptors (ER). As a steroidal hormone, oestrogen passes through the target cell's phospholipid membrane and binds with cytoplasmic free ER. Two ER isoforms (α and β) have been identified[91], members of a class of ligand-dependent transcription factors known as the nuclear receptor

superfamily which regulate the expression of genes that promote growth, differentiation and metabolism[92]. The ERα isoform has a pro-proliferative action and is antagonised by ERβ. In this thesis ER refers to the ERα isoform.

The classical (or "genomic") mechanism of ER action occurs as a consequence of oestrogen binding to the receptor's ligand-binding domain. Following ligand specific conformational change, the receptor protein translocates to the nucleus. Subsequent dimerization and binding of the complex to DNA occurs, at zinc finger-containing domains known as oestrogen response elements (ERE). Addition of co-activators or co-repressors to the ligand-receptor group creates multiprotein complexes. In turn, these recruit co-regulatory proteins (such as histone acetyl transferase or ubiquitin ligase) and may promote or inhibit gene transcription. As a consequence, proliferation within steroid-sensitive tissue may increase. EREs on genes controlled by other transcription factors such as NF-κβ and STAT5, allows complex regulation of gene transcription [93].

In addition, activated ER may exert rapid non-genomic effects through interaction with other transcription factors in the cytoplasm. As ERs have no kinase activity, its effect is mediated through tyrosine kinases such as c-Src and lead to activation of multiple signalling pathways such as MAPK, PI3K, EGFR and IGFR. These diverse effects on downstream signalling following ER activation lead to multiple alternative gene expression patterns in ER+ cells and consequently distinct biological patterns of behaviour in alternative steroid sensitive tissues.

1.5.3 The role of Epidermal Growth Factor Receptor Family

Epidermal growth factor receptor (EGFR) belongs to a family of receptor tyrosine kinases that also include HER2, Erb-B3 and Erb-B4, which play a pivotal role in both embryonic development and carcinogenesis. These transmembrane RTKs consist of a ligand binding extracellular domain (with the exception of HER2 - an orphan receptor) and an intracellular domain with kinase activity. Ligand

binding and activation leads to homo- or heterodimerisation with subsequent phosphorylation of downstream targets.

Despite their structural homology, HER receptors differ in their ligand specificities. Two ligand classes been identified: neuregulins (NRGs) which bind exclusively to HER3/HER4 and different EGF-related proteins. Binding of specific ligands is followed by conformational changes of the ECD, facilitating receptor dimerization and subsequent kinase domain activation with induction of intracellular signalling cascades, mediating cell growth and survival. EGFR and HER2 classically couple to Ras-Raf-MEK-mitogen-activated protein kinase (MAPK)-dependent pathway, whereas HER3 is a potent activator of phosphatidylinositol 3-kinase (PI3-K)-Akt. These pathways are associated with pro-growth and anti-apoptotic signalling and is activated in a wide range of cancers. In contrast to the other HER receptors, HER4 is characterized by antiproliferative and pro-apoptotic activity. The intracellular domain of HER4 is characterized by multiple, diverse biological activities and cellular responses including differentiation of mammary epithelial cells and lactation, activation of pro-apoptotic pathways and cell cycle arrest. Unlike the other members of the HER family, HER4 expression has been correlated with favourable prognostic factors and a more positive outcome in patients with breast cancer[94].

The activity of EGFR is mediated through major signalling pathways such as PI3kinase and MAPK which modify not only proliferation but also motility, invasion and angiogenesis, all of which are not only critical for development but also for cancer dissemination and metastasis.

In contrast to HER2 - a ligand orphan receptor constitutively active following homo- and heterodimerisation, activation of EGFR is ligand dependent [95]. Activation is restricted to a small number of ligands; EGF, TGF-α, amphiregulin and epiregulin [96]. Furthermore, unlike HER2, protein overexpression of EGFR is associated with gene amplification in only 37% cases [97] with EGFR expressing mutations extremely rare in breast cancer[98].

EGF acts as a chemoattractant for mammary cells both *in vivo* [99] and *in vitro* [100]. Macrophages are a known source of EGF and can be directly visualised co-migrating with mammary epithelium using *in vivo* invasion assays. Furthermore, using multiphoton imaging in these assays, perivascular macrophages can be visualised directly assisting intravasation of mammary tumour cells and occurs as a result of an EGF mediated paracrine loop between the two cell types[101].

Taken together, it appears that EGFR activation is not driven by genomic mutation and amplification but occurs as a result of ligand activation from the surrounding stroma. With the help of stromal elements the carcinoma may be able to appropriate pathways normally active in physiological processes to invade and metastasize.

1.5.4 Current treatment strategies in ER+ breast cancer

The continued improvement in breast cancer survival rates over the last decade have arisen due to a combination of earlier diagnosis through breast screening programmes, better public awareness and the widespread introduction of effective systemic therapy following resection surgery. The aim of systemic therapy is to treat any undetected local and distant disease that, if left untreated, may subsequently develop into life-threatening recurrence. In 1976 Bonadonna and colleagues demonstrated a significant improvement in disease free survival with the use of chemotherapy consisting of the alkylating agent cyclophosphamide in combination with two antimetabolites; methotrexate and fluorouracil (CMF)[102]. As a result CMF treatment became the standard adjuvant chemotherapy used in higher risk women with early stage breast cancer[103]. Subsequently anthracyclines were incorporated into adjuvant chemotherapy regimens and a subsequent meta-analysis has shown that anthracycline based regimens, such as cyclophosphamide, doxorubicin and fluorouracil (CAF), confer a moderate but highly significant advantage over CMF[104].

Tamoxifen is a selective oestrogen receptor modulator that binds to the oestrogen receptor and blocks the proliferative actions of oestrogen on mammary epithelium[105]. Numerous clinical trials

and meta-analyses have demonstrated that in patients with oestrogen receptor positive breast cancers, tamoxifen significantly reduces recurrence rates and improves survival[106]. For this reason it became the mainstay of treatment for ER positive breast cancer for many years. A large meta-analysis on adjuvant treatment reported only marginal benefit with the addition of chemotherapy in older women compared with tamoxifen alone. It also highlighted that no large trials had been performed of concurrent versus sequential chemoendocrine treatment[104].

Any advantages in reducing breast cancer recurrence conferred from combining hormonal and cytotoxic therapy must be balanced against the increased morbidity and mortality associated with these treatments: Tamoxifen has oestrogenic effects on uterine epithelium in post-menopausal women and is associated with increased rates of endometrial carcinoma[107]. Chemotherapy regimens which include anthracyclines are associated with myelodysplasia, neutropenic sepsis, cardiac damage and increased rates of leukaemia[108]. It has been postulated that the concurrent use of tamoxifen may attenuate the efficacy of anthracyclines: Tamoxifen reduces the proliferation rate of hormone sensitive breast cancer cells and in so doing may make them less sensitive to the cytotoxic activity of chemotherapy[109, 110].

Recently Albain et al. reported the results of long-term follow-up phase 3 trial of 1477 postmenopausal women with hormone-receptor positive, node positive operable breast cancer. They demonstrated that the addition of CAF chemotherapy to tamoxifen treatment significantly improves 10-year disease-free survival. Recurrence rates were even lower if the tamoxifen is given sequentially after chemotherapy rather than concurrently. Despite the increased morbidity and adverse events which could increase non breast cancer deaths, chemotherapy conferred a significant 10 year overall survival benefit [111].

With the introduction of the aromatase inhibitors, tamoxifen use in post-menopausal women is declining. Both BIG 1-98 and ATAC trials have shown superior disease-free survival in postmenopausal women with ER positive disease with aromatase inhibitors (AI's) compared with

tamoxifen [112, 113]. Aromatase inhibitors have become the mainstay of endocrine treatment in postmenopausal women particularly those who are node positive.

However, chemotherapy is a blunt tool that reduces mortality in a few and causes significant morbidity in many. Future development must focus on improving identification of patients at low risk of recurrence, who have little to gain from adjuvant systemic therapy. Within the hormone receptor positive lymph node positive population there will also be many women who will be cured by surgery and endocrine therapy alone.

Gene expression profiling of breast cancer (initially reported by Perou et al[75] and subsequently developed by Sorlie et al[82]), allowed for stratification of breast cancer into molecular subtypes. In turn these subgroups were demonstrated to correlate with significantly different patient prognosis. This technology has been further developed to stratify a patient's individual risk of recurrence and therefore guide treatment options.

Commercial 70 gene signature (Mammaprint) and 21 gene signature (Oncotype DX) tests are now available as a method of providing patient stratification into recurrence risk groups[114]. Using the 21 gene recurrence score, patients with low predicted recurrence scores get no additional benefit from receiving chemotherapy [115]. Patients with high recurrence scores had improved disease-free survival if they received additional chemotherapy compared with participants who received tamoxifen only. High risk women do have greater absolute benefits from chemotherapy but the majority of relapses are not prevented and identification of patients who do and do not benefit from chemotherapy is urgently needed. This would reduce the over-treatment and unnecessary exposure of patients to toxic therapy that is unlikely to alter disease course. Further stratification of breast cancer subtypes and genome analysis are required and bring the prospect of individually tailored therapy ever closer.

1.6 Thesis Aim

This thesis aims to develop a three dimensional model of breast cancer using primary tumour material from patients, thus overcoming the disadvantages associated with reliance upon cell lines or disaggregated biopsy tissue. As the relative proportions of epithelial and mesenchymal components vary both within and between tumours, behaviour may be variable even between assays created from the same patient. Moreover, using two-dimensional immunohistochemistry to quantify an asymmetrical three-dimensional culture will lead to variable results depending on the two-dimensional plane in which the assay is sampled.

We therefore aim to develop 2D and 3D methods of objectively quantifying the response of tumour samples to tamoxifen and compare the results with those seen in neo-adjuvant tamoxifen trials. The model must produce viable assays for all patients and all breast cancer subtypes. The underlying mechanisms driving invasion shall also be examined and their links to overall survival investigated. This could provide a method of individualising cancer therapy for a patient using an array of novel therapeutics and quantifying tumour response.

Chapter 2: Materials and Methods

2.1 Breast Cancer Cell Lines, Characteristics and Optimal Culture Conditions

Cell lines used in this thesis were obtained from stocks within the Breakthrough Laboratory and were of the following lineage and characteristics:

Cell Line	Gene cluster	ER	PR	EGFR	Tumour type	Source	Culture media	Culture conditions
SUM-159PT	Basal B	Neg	Neg	Pos	Anaplastic Carcinoma	Primary Breast	Ham's F12, 5% FCS Insulin (0.01 mg/ml); Hydrocortisone (500 ng/ml)	37 deg °C, 5% CO_2
MCF7	Luminal	Pos	Pos	Neg	IDC	Pleural Effusion	DMEM/ 10% FCS	37 deg °C, 5% CO_2

Taken from Neve et al.[21]

MCF-7 cell line obtained from ATCC (American Type Culture Collection; www.atcc.org)

SUM159PT obtained from Dr. A. Orimo, University of Manchester.

2.2 Extracting collagen from rat tails

Frozen fresh rat tails were placed in 70% ethanol to thaw. The overlying skin was degloved using a scalpel, exposing the underlying tendons. These tendons were stripped away from the tail and placed in 70% ethanol to sterilise. The collected tendons were weighed and transferred to acetic acid (1g tendon to 250ml 0.5M acetic acid). These were placed in the cold room and mixed for 48hr at 4°C. The tendon/acetic acid mix was centrifuged at 10,000g for 30min and the pellet discarded.

An equal volume of 10% (w/v) NaCl was added to the supernatant and the mix allowed to stand overnight at 4°C. The collagen-rich, insoluble bottom layer was collected and centrifugated for a further 30min at 10,000g. The collagen-rich material was resuspended in 0.25M acetic acid at 4°C and dialysed against 1:1000 acetic acid at 4°C for 3 days, changing the dialysis buffer twice daily. To further sterilise the collagen solution, a further centrifugation step (20,000g for 2hr) was performed and then stored at 4°C. The collagen was diluted as required with the addition of sterile 1:1000 acetic acid. We diluted the collagen to a stock concentration of 1mg/ml.

2.3 MEGM and components

In order to maintain proliferation and invasion of primary breast cancer tissue, media designed specifically to support mammary epithelium was used. Clonetics® MEGM® (Lonza CC-3150) is a mammary epithelial Cell Growth Medium, optimized for the growth of Mammary Epithelial cells. It is specially designed to support the growth of human and animal primary derived cells. MEGM® Medium is serum-free and consists of a basal medium (MEBM) with additional growth factor supplements (BulletKit®).Unfortunately, as the formulations of the media are proprietary Lonza could not provide us with exact concentrations of the growth factors. However, on further questioning they were able to supply us with approximations of the ranges used for the additional factors. These are all the final concentrations in the media.

Supplement	Approximation of final concentrations
hEGF	10 ng/ml
BPE	50 µg/ml
Insulin	5 µg/ml
Hydrocortisone	0.5 µg/ml
Gentamycin	30 µg/ml
Amphotericin	15 ng/ml

2.4 Creation of β-oestradiol and tamoxifen supplemented media

For some experiments MEGM Complete media was supplemented with β-oestradiol to create a concentration of 3.2 $x10^{-10}$M. β-oestradiol was purchased in powder form (Sigma; E8875) and stored in a cytotoxic cabinet at room temperature. The molecular weight of β-oestradiol is 272.4kDa. β-oestradiol was made up as a $1x10^{-7}$ M stock solution and stored at -20°C with a 3 month expiry:

To make an initial concentration of $1x10^{-7}$ M, 27.2mg of β-oestradiol was weighed out into a sterile glass vial and 10ml of absolute Ethanol added. This required thorough mixing to make sure that the powder had gone into solution. Protect from light by covering in tin-foil. Serial dilutions were made:

$1x10^{-5}$ M = 1:1000 dilution of $1x10^{-2}$ M stock (10µl/10ml) in ETOH.

$1x10^{-7}$ M = 1:100 dilution of $1x10^{-5}$ M solution (100µl/10ml) in ETOH.

In order to use at a final concentration of $3.2x10^{-10}$ M:

$3.2x10^{-10}$ M = 1:312.5 dilution of $1x10^{-7}$ M solution (3.2µl/10ml) in MEGM.

Tamoxifen was purchased in powder form (Sigma; T5648) and kept in a cytotoxic cabinet at 4°C. The molecular weight of Tamoxifen is 371.5kDa. Tamoxifen was made up as a $2x10^{-3}$M stock solution and subsequently stored at -20°C. 7.43 mg of Tamoxifen to 10ml of absolute Ethanol was weighed out into a sterile glass vial. Mixed thoroughly. Stock solution of tamoxifen was added to fresh β-oestradiol supplemented MEGM to establish a concentration of $4x10^{-6}$M.

2.5 Creation of 3D invasion assay using Primary Breast Tissue

The use of tissue from invasive breast cancer treated at the Edinburgh Breast Unit at the Western General Hospital was approved by the Lothian Research Ethics Committee (08/S1101/41).

Multiple core biopsies were harvested from consenting patients at the time of curative surgical resection for invasive breast cancer. Cores were divided by eye using a scalpel into $1mm^3$ fragments. Macroscopically distinct fat was trimmed and discarded. Tumour samples were then immersed in MEGM Complete.

To create the collagen gel, 1 mg/ml rat tail collagen, 1:1000 filtered acetic acid, 10x DMEM and 0.22M NaOH were mixed on ice at concentrations of 30% collagen, 50% acetic acid, 10% DMEM and 10% NaOH to create a final collagen concentration of 0.3 mg/ml. 1.2 ml of collagen mixture was put into individual wells of a 24 well plate and placed into an incubator at 37°C for 10 min to initiate gelling. After 10 min, tumour samples were placed on to the gelling collagen and pushed in to the centre using a pipette tip. The 24 well plate was then returned to the incubator. After 1 h, using MEGM Complete supplemented with β-oestradiol to a conc. of 3.2 $x10^{-10}$M, 1.2 ml of the supplemented media was carefully introduced into each well.

The final β-oestradiol concentration within the assay equilibrated to 1.6 x 10^{-10} M to recapitulate physiological oestrogen levels found in breast tissue [31]. A pipette tip was run around the inner edge of each well, releasing each gel circumferentially, thus allowing each gel to float freely within the media. Supplemented media was then exchanged twice weekly until termination of the experiment using formalin.

2.6 Creation of 3D invasion assay using Cell Lines

In order to recreate the 3D invasion assays using cultured cell line, plugs were created by mixing cells in to 1mg/ml collagen to create a final cell concentration of $2x10^6$ cells/ml. 200µL of cell/collagen mix was pipetted into multiple wells of U-shaped 96-well plates. Following overnight incubation, the gelatinous cell plugs were carefully transferred from the 96-well plate and embedded into the centre of 1 ml of rat collagen I (1 mg/ml) in a 24-well plate. These cultures were incubated for 1 hr and then carefully freed from the inner edge of each well and supplemented with appropriate media, as above. Media was changed every 3-5 days.

2.7 Sectioning of Samples for 2D Analysis

Formalin fixed preparations were embedded in paraffin and 3 µm sections were cut and mounted on to glass slides (Cell Path, positively charged adhesion slides, cat. no. MDB-0102-54A).

2.8 Immunohistochemical (IHC) protocol

Dewax sections in xylene and take down to water through graded alcohols. Perform antigen retrieval on slides by treating them with 0.1M sodium citrate/0.1M citric acid pH6 or Tris/EDTA pH9.0 or commercially available solution for 5 min in microwave in pressure cooker. Allow 20 min for slides to cool. Wash slides in PBST 2 x 5 min. Treat sections in H_2O_2/H_2O for 10 min. Wash slides in PBST 2 x 5 min. Load sections onto the sequenza. Wash slides with PBST. Block with Dako Total Protein block for 10 min. Incubate section in primary antibody diluted as required for 1hr. Wash with PBST. Incubate section with Dako Envision labelled polymer for 30 minutes. Wash slides with PBST. Add DAB to visualise. Wash sections in water. Counterstain and mount.

2.9 Immunofluorescence Protocol For Mouse Primary Antibody

Dewax in xylene twice for 5min each. Rehydrate using sequential immersion for 2min in 99%, 99%, 80%, 50% alcohol then running tap water. Place in Tris-EDTA pH9.0 or NaCitrate pH6.0-pressure cooker for 5min. Allow to cool down for 20min. Rinse in 0.05% PBST for 5min using coplin jar on the rocker. Treat sections in 3% H_2O_2 for 10 min in a coplin jar. Rinse in 0.05% PBST for 5min. Add the sections to sequenza and wash once more. Treat sections in Dako Serum-free protein block for 10 min. Incubate Primary antibody diluted with optimal dilution in Second Primary antibodies (Rabbit Anti-cytokeratin, Dako, #Z0622, 1:150 and Rabbit anti-pan Cadherin, Cell signalling, #4068, 1:50) diluted in Dako antibody diluent either for 1 hr at room temperature or for overnight at 4°C. Rinse in 0.05% PBST 3 x 5min. Prepare a 1:25 dilution of the goat anti-rabbit Alexa555 Ab (Invitrogen, #A21428) in the pre-diluted Envision goat-mouse HRP antibody solution(Dako, #K4001) - or HistoRx tube D. Incubate slides in the dark for 1.5 hr at room temperature. Rinse in 0.05% PBST 3 x 5min. Transfer the slides from the sequenza to the humidity chamber. Combine Target signal amplification diluent and the Cy5 Tyramide at 1:50 concentration (use HistoRx tube F and E). Vortex to mix thoroughly. Incubate slides in the dark for 10 min at room temperature. Rinse in 0.05% PBST 3 x 5min. Dehydrate them in 80% IMS for 1 minute. Air dry in the dark. Apply 45µl Prolong Gold anti-fade reagent with DAPI (Invitrogen, P36931), nuclear visualisation media, on the coverslip (22x40mm) and place the coverslip over the tissue. (For the smaller coverslips [22x26mm], apply 30µl). Let the mounted slide dry overnight in the dark. After slides are completely dried, seal the coverslips with nail polish.

2.10 Immunofluorescence Protocol For Rabbit Primary Antibody

Steps as for Mouse Primary Antibody (see 2.7) except for the following steps:

Primary Antibody and anti-cytokeratin (Tumour mask) incubation

Incubate Primary antibody in Dako antibody diluent at optimal dilution for 1 hr at room temperature or overnight at 4°C. Rinse in 0.05% PBST 3 x 5min. Incubate Second Primary antibody (Mouse Anti-cytokeratin, Invitrogen, #18-0132) in Dako antibody diluent with 1:25 dilution overnight. Rinse in 0.05% PBST 3 x 5min. Prepare a 1:25 dilution of the goat anti-mouse Alexa555 Ab (Invitrogen, #A21422) in the pre-diluted Envision goat-rabbit HRP antibody solution (Dako, #K4003). Incubate slides in the dark for 1.5 hr at room temperature. Rinse in 0.05% PBST 3 x 5min.

2.11 Antibodies used for IHC and IF

Antibody name	Company	Catalogue	Species	Antigen retrieval	IHC dilution	Primary Ab incubation condition	IF Dilution
Cleaved Caspase-3	Cellsignaling	9661	Rabbit	NaCitrate pH6.0	1 in 200	Overnight at 4ºC	1 in 200
Pan Cadherin	Cellsignaling	4068	Rabbit	NaCitrate pH6.0		1hr at room temperature	1 in 50
Anti-cytokeratin	Dako	M3515	Mouse	NaCitrate pH6.0	1 in 50	1hr at room temperature	1 in 25
Anti-cytokeratin	Dako	Z0622	Rabbit	NaCitrate pH6.0	1 in 500	1hr at room temperature	1 in 150
CK 5/6	Dako	M7237	Mouse	TE buffer pH9.0	1 in 50	1hr at room temperature	1 in 50
ki67	Dako	M7240	Mouse	TE buffer pH9.0	1 in 50	1hr at room temperature	1 in 50
Anti-cytokeratin	Invitrogen	18-0132	Mouse	NaCitrate pH6.0	1 in 100	1hr at room temperature	1 in 25
EGFR	Invitrogen	28-0005	Mouse	Trypsin Digest 10min at 37deg *	1 in 50	1hr at room temperature	1 in 50
ER	Neomarkers	RM-9101-S1	Rabbit	NaCitrate pH6.0		1hr at room temperature	1 in 100
CK 8/18	Progen	GP11	Guinea pig	NaCitrate pH6.0	1 in 50	1hr at room temperature	1 in 50
ER alpha	Vector	VP_E613	Mouse	NaCitrate pH6.0	1 in 50	1hr at room temperature	1 in 25

*To make 0.1% trypsin in 0.01% $CaCl_2$: Place 100micrograms of trypsin in 100ml of 0.01% $CaCl_2$

2.12 Image analysis using Definiens software

Whole slides (one per patient per treatment) were scanned using an automated fluorescence microscope (HistoRx PM-2000) at 40x magnification, creating multiple images. The target channel exposure time was fixed at 50 ms. The epithelial mask and DAPI channel exposure times were variable to optimise visualisation. Images (6-26 per preparation, totalling >700 cells) were exported as TIFF files and imported into Definiens Tissue Studio 2.0 (http://www.tissuestudio.com). Data were automatically generated detailing the number of cells counted from the imported series of TIFF files and the number that were classified as positive or negative. We used previously a similar automated cell delineation programme to observe morphological changes during cancer cell invasion[116].

Parameters were set as follows: regions of interest were automatically selected based on intensity of the epithelial mask channel. Nuclei were detected based on an intensity threshold of >0.5 arbitrary units (a.u.) and average nucleus size of 60 mm^2. Cell boundaries were identified using "Membranes and Cells" function, using the epithelial mask channel. For nuclear staining, cell classification was determined by the target channel intensity with nuclei expressing ER or Ki67 at intensity >70 a.u. were classified as positive. Nuclei that demonstrated partial or low levels of target were classified as negative. For cytoplasmic or membrane staining, cells were determined to be positive if average target intensity (CCasp3 or HER2, respectively) expressed throughout the cell as >64 a.u. Cells with an average cytokeratin intensity of <10 a.u. were excluded, as were cells with no nucleus.

2.13 Staining protocol for Optical Projection Tomography specimen preparation

Formalin fixed preparations were washed initially for 30 min in PBS and the assay dehydrated stepwise in methanol with PBS diluent: (33%, 66%, and 100% for 15 min at each step). Preparations were incubated in freshly prepared MeOH: DMSO: 30%H_2O_2 at a ratio of 2:1:3 (i.e. 15% H_2O_2) at room temperature for 24 h to quench the auto-fluorescence. Preparations were then washed twice for 30 min in methanol. The preparations were frozen to 80°C five times for at least 1 h each time and back to room temperature (RT) to ensure that antigens in the deeper parts of the tissue were rendered accessible. Preparations were rehydrated back to PBST with methanol as diluent (33%, 66% and 100% for 15 min at each step). Preparations were blocked in blocking solution (10% serum in PBST) for 24 h. Preparations were then incubated with primary antibody to identify the epithelial content using rabbit pan-cytokeratin (Cell Signalling, #4068, 1:200), diluted in 1.5 ml of blocking solution containing 5% DMSO for 48 h. Preparations were washed with PBST four times for 30 min each. The tissue was incubated with fluorescent secondary antibody (Invitrogen, #A21422, 1:100), diluted in 1.5 ml blocking solution containing 5% DMSO for 48 h. Preparations were washed again with PBST four times for 30 min.

2.14 Optical Projection Tomography

Optical projection tomography is a microscopy technique previously developed to produce high-resolution 3D images of fluorescent biological specimens with a width up to 15mm[117].

The tissue was mounted in 1% low melting agarose and dehydrated in 100% Methanol for 24 h. The specimen was cleared in BABB solution (1:2 Benzyl alcohol, Benzyl benzoate) for 24 h and scanned using the Optical Projection Tomograph (Bioptonics 3001M). Both target and autofluorescence were captured using the Cy3 filter set (exciter 545nm/30 nm, emitter 610/75 nm) and GFP1 filter set (exciter 425nm/40 nm, emitter LP475 nm) respectively.

2.15 Reconstruction of images into a 3D visualisation using NRecon

The set of angular projections acquired at OPT were reconstructed to create a series of cross-section slices through the invasion assay (Nrecon, SkyScan). Misalignment compensation was performed manually for each channel to reduce image blurring. Dynamic image range minimal and maximal values were fixed at 0 and 0.5 respectively. This software is available to download for free at http://www.skyscan.be/products/downloads.htm.

2.16 Volocity

Quantification of tumour volume using Volocity software has been validated previously using z-stack data obtained with confocal microscopy[118]. The reconstructed data was then analysed using Volocity software (PerkinElmer). An initial training set of 2D cross-sections was used to identify a threshold of signal intensity that provided an accurate mask of invading epithelial shapes and excluding background signal. From this voxels with an intensity >9000 a.u. were considered to be positive for cytokeratin and therefore represent the epithelial compartment. Epithelium in continuity with the original core (demarcated in the auto-fluorescence channel) was considered to be epithelial outgrowth and the volume of the structure measured.

2.17 Statistical Methods

Data collected from the study was analysed using SPSS and Minitab 16 statistics software package under the guidance of a senior statistician. Normally distributed data was analysed using parametric Student's T-test and Analysis of Variance (ANOVA). Non-parametric data was analysed using Mann-Whitney and Fisher's Exact Test. Correlation was analysed using Spearman's Regression Analysis and Poisson Regression Analysis.

2.18 Tumour grading, ER and EGFR scoring

Formal histopathological grading and scoring was performed by a senior breast pathologist:

The method for grading used was as described by Elston and Ellis[119] and involves the assessment of three components of tumour morphology: tubule/acinar/glandular formation, nuclear atypia/pleomorphism and frequency of mitoses. Each is scored from 1 to 3. Adding the scores gave the overall histological grade.

For ER receptor status, only nuclear staining was considered, and the entire invasive component assessed. In order to ensure uniformity, the quick (Allred) scoring system was used [120]. This is based on assessment of the proportion and intensity of staining:

Score for proportion	***Score for intensity***
0 = no staining	0 = no staining
1 = < 1% nuclei staining	1 = weak staining
2 = 1–10% nuclei staining	2 = moderate staining
3 = 11–33% nuclei staining	3 = strong staining
4 = 34–66% nuclei staining	
5 = 67–100% nuclei staining	

The scores are summed to give a maximum of 8.

For EGFR expression, a score was calculated based on the intensity of membrane staining. 0=no membrane staining, 1=incomplete membrane staining, 2=weak or moderate complete staining, 3=strong complete staining.

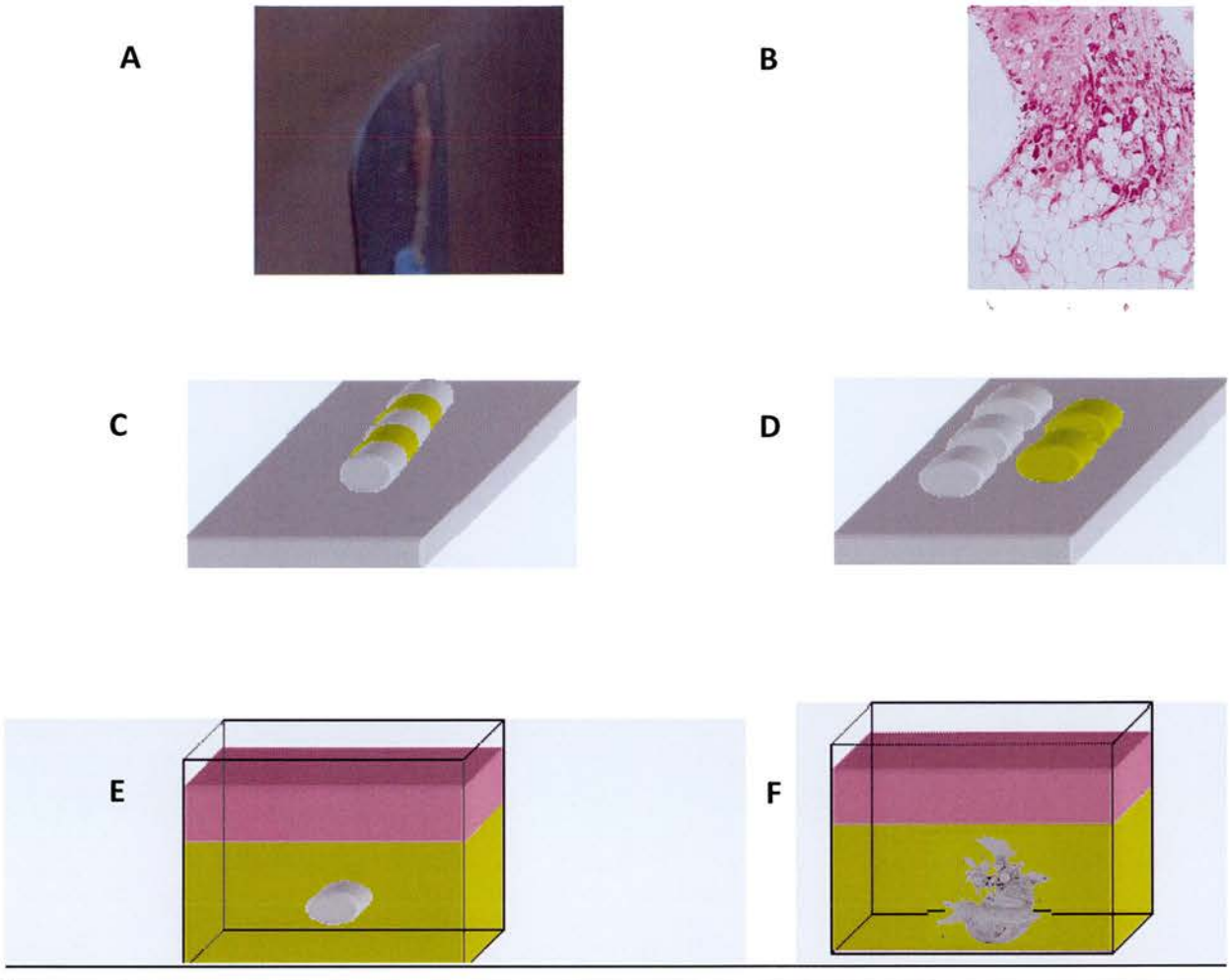


Fig 3.1 [A] Core biopsies from breast cancers were obtained at the time of surgery and transferred directly to the laboratory. [B] Fixation and histopathological examination revealed marked heterogeneity within each core, with areas high in adipocytes and areas with marked tumoural invasion. [C+D] In order to enrich the tumoural content of samples, biopsy material was trimmed and processed into $1mm^3$ fragments (white). [E] These fat depleted fragments were embedded in to the centre of Type I Collagen (yellow) in a 24 well plate. Mammary Epithelial Growth Media supplemented with β-oestradiol was added on top after a further hr of gelling (pink). [F] After one week evidence of invasion may be seen at the tumour/gel interface. The assay was subsequently fixed in formalin at three weeks.

Chapter 3: Development and validation of a three dimensional assay

3.1 Expansion of tumour materials *ex vivo*

We set out to develop a three-dimensional assay utilising breast biopsy material in order to provide individualised *in vitro* cultures for breast cancer patients. For the 3D culture to provide physiological relevance, both epithelial and stromal components of breast cancer material should remain viable with proliferation and invasion of epithelium maintained for the period of the experiment.

In order to maintain the appropriate cellular heterogeneity found in primary breast cancer, multiple biopsies were obtained at the time of curative surgical resection for histologically-proven invasive breast cancer[Fig 3.1A-B]. Macroscopically distinct fat was trimmed and discarded from the biopsy samples and the remaining tissue was dissected into $1mm^3$ fragments, preserving the original tissue architecture [Fig 3.1C-D]. In a 24 well plate, fragments were embedded into the centre of a type I collagen gel and mammary epithelial growth media added [Fig 3.1E]. Over a three week experimental time-course invasion of cells from the original fragment into the surrounding collagen was noted [Fig 3.1F].

Assay behaviour was observed in real-time, using brightfield microscopy at 10x magnification. In comparison to day 0 [Fig 3.2A], initial invasion of mesenchymal cells from the tumour edge into the surrounding collagen in the first week was observed using light microscopy [Fig 3.2B]. This was followed by collective invasion of epithelial structures into the surrounding collagen and associated collagen organisation and contraction [Fig 3.2C-E]. Invasion occurred in all dimensions and most frequently in the horizontal plane. This facilitated visualisation of progression using live capture microscopy. Experiments were terminated at day 20, at which time the tumour cells had invaded the collagen gel [Fig 3.2F].

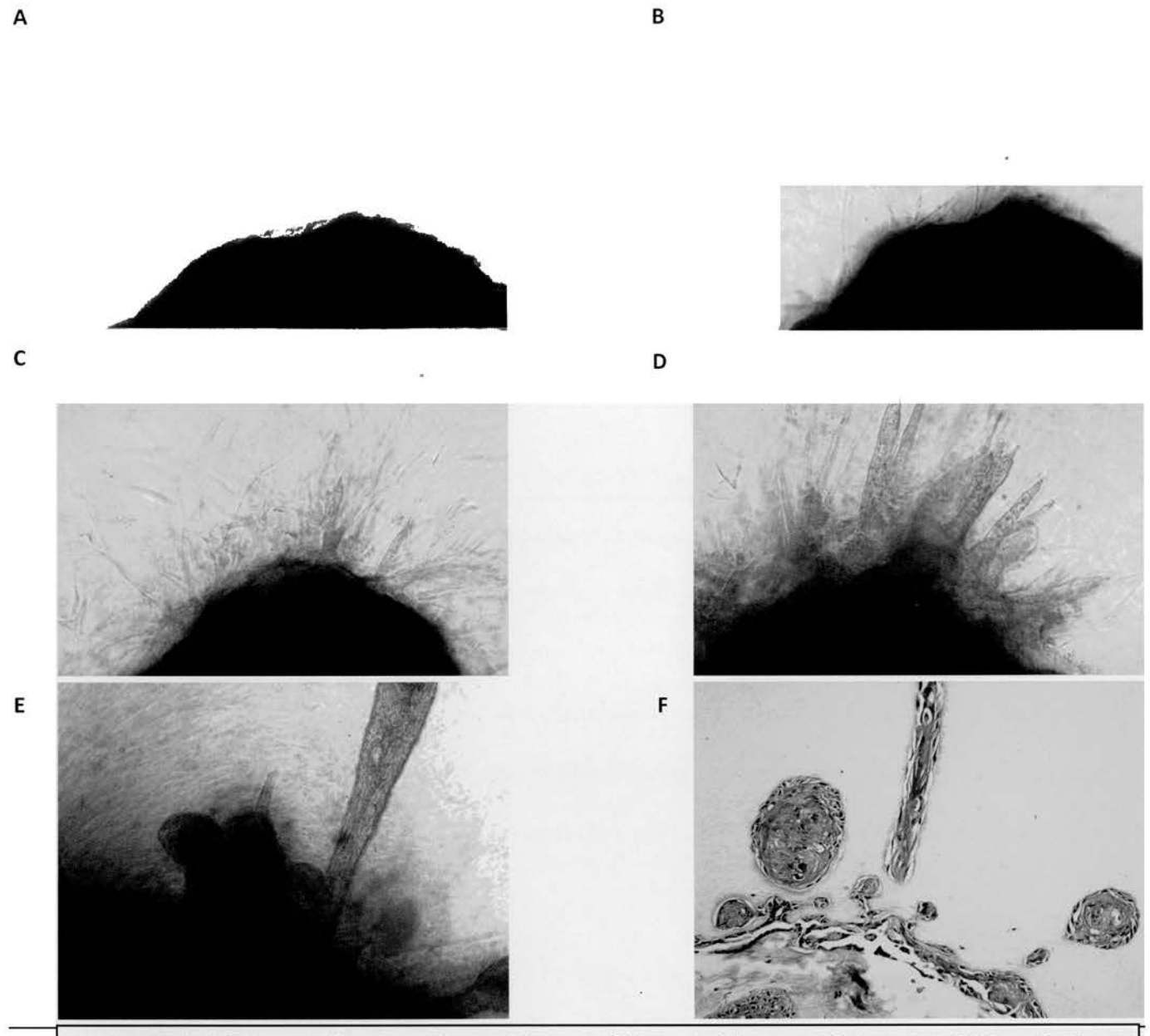


Fig 3.2 Brightfield image of tumour edge at [A] Day 0, [B] Day 8, [C] Day 12, [D] Day 14, [E] Day 19. These demonstrate an initial phase of mesenchymal cells invading in to the surrounding collagen followed by collective invasion of small organised structures. These structures extend and mature over the time course. [F] Representative section of assay following fixation and HE staining.

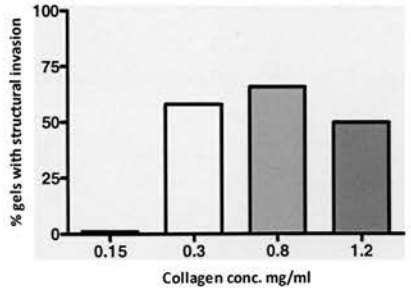


Fig 3.3 To investigate the effect of increasing collagen concentration, a total of 48 gels were created with 12 in each group with varying concentrations of collagen. Higher concentrations did not significantly increase invasiveness. At levels at less than 0.3mg/ml fragments did not remain within the matrix.

3.2 Types of tumour expansion *ex vivo*

A typical biopsy sample, comprising of 4 cores, would be processed and divided to produce a battery of up to 24 *ex vivo* preparations. Typically, at least 80% of preparations within a battery displayed variable degrees of invasion into the surrounding collagen by day 20. Within each battery, 17%-50% of preparations demonstrated evidence of epithelial structures invading into the surrounding collagen (hereon termed structural invasion), as illustrated in Fig 3.2D. Other preparations displayed more abundant single cell invasion (Fig 3.2B) but did not progress on to structural invasion. If structures penetrated through the collagen gel edge, ongoing cell proliferation and migration was evident on the surface of the gel, leading to additional 2D expansion of the epithelial culture.

Total experimental failure, where no viable assays were identified at day 20 was low. Over a two year period 61 patient biopsy samples were utilised, with six classed as total assay failures (90% success rate). Factors associated with assay failure included the quality and cellularity of the original cancer material and bacterial infection leading to experiment abandonment.

3.3 Development and Optimisation of Assay

In order to improve yield and reproducibility within the assay, multiple variables were tested. Previously published work using cell lines and collagen assays identified that both an increase in collagen density[121] and also the addition of fibroblasts[122] enhanced invasive behaviour of cells.

To assess the impact of increasing collagen density on invasion, assays with variable collagen concentrations were compared head-to-head using biopsy material from the same tumour sample. At very low concentrations (0.15mg/ml) collagen did not polymerise sufficiently to allow tumour fragments to remain embedded, leading to detachment from the gel and assay failure. At higher concentrations no trend was evident between increasing collagen concentration and improved structural invasion. To minimise consumption of reagents, collagen was therefore used at 0.3mg/ml [Fig 3.3].

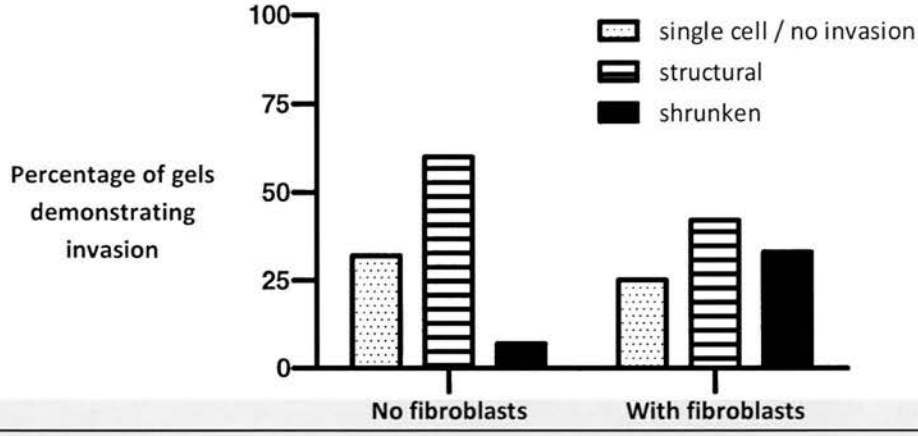
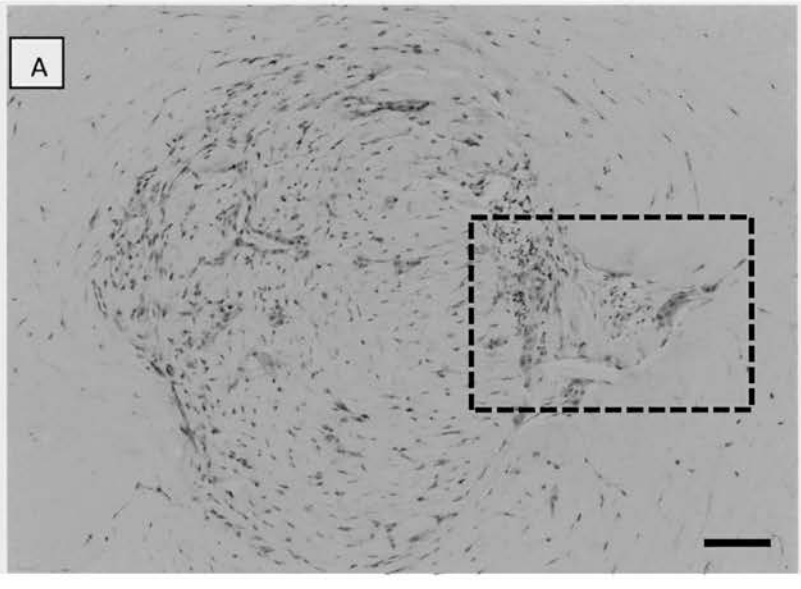


Fig 3.4 To investigate the effect of additional fibroblasts, 64 gels were created. 28 had no additional fibroblasts and 36 had mammary fibroblasts mixed in to the polymerising collagen. There was no evidence of enhanced structural invasion in the presence of additional fibroblasts, with a higher proportion of gels demonstrating excessive contraction.



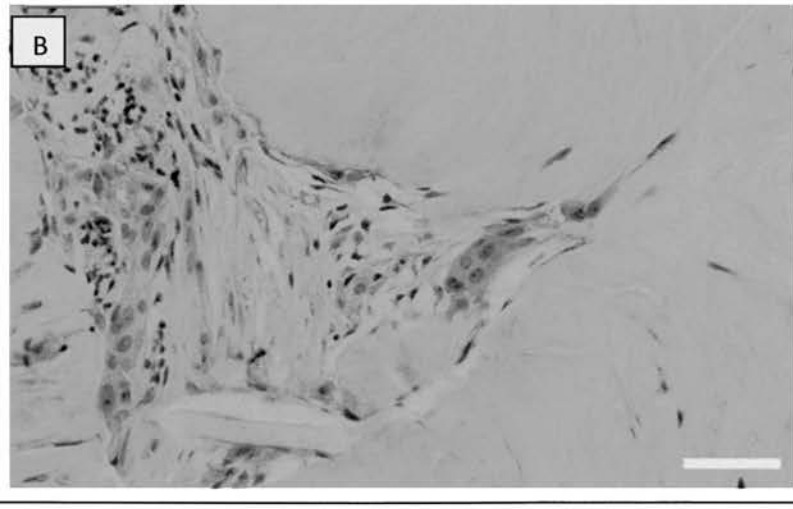


Fig 3.5 Fibroblasts isolated from reduction mammoplasties and passaged regularly on 2D plastic were mixed with polymerising collagen to create fibroblast cell plugs, which were inserted into the centre of collagen cushions as seen in Fig 3.1E. [A] At two weeks these assays were formalin fixed and assessed using H/E. [B] In addition to viable fibroblasts, clusters of cells with epithelial morphology were also evident. Black bar = 100μm, white bar = 50μm

Next, addition of fibroblasts as a method of improving invasive behaviour was assessed. Fibroblasts isolated from normal breast tissue and perpetuated in long-term culture were mixed into the collagen prior to breast cancer biopsy fragment insertion. A total of 64 viable gels were created from four individual tumour samples, 36 with fibroblasts and 28 without. On day 20, assays were assessed using light microscopy and categorised in to three groups: (1) gels with evidence of organised structures invading into the collagen, (2) gels in which the collagen had contracted around the fragment to a degree that assessment by light microscopy was not possible, (3) gels in which either no evidence of cellular invasion had occurred or only mesenchymal single cells were visible. Group (2) occurred as a result of excessive collagen remodelling and typically had shrunk by >90% as measured by ((Total initial assay volume – Media removed on day 20)/1.2)*100. The addition of fibroblasts to assays meant that it was not possible to determine whether single cells, seen at day 20 within the gel, had originated from the tumour fragment or not. Therefore it was not possible to determine whether addition of fibroblasts increased single cell invasion from the tumour fragment.

Addition of normal fibroblasts to the assay was not necessary to promote invasion of organised epithelial structures into the surrounding collagen [Fig 3.4]. Moreover, excessive matrix remodelling occurred in a greater proportion of assays supplemented with fibroblasts. Furthermore, assessment of single cell invasion was not possible with the addition of fibroblasts.

Currently there is no immunocytochemical marker which exclusively identifies fibroblasts. Instead fibroblast isolation is performed using stratified centrifugation and subsequent multiple passages of the isolated mesenchymal cells on plastic. Concerns were raised over this method's ability to isolate populations of cells purely of fibroblastic origin. Therefore using the standard method of fibroblast isolation and following multiple passages, mesenchymal cells were mixed into collagen to create multiple 200μl cell plugs. These cell plugs were embedded into the centre of collagen cushions in the same manner as primary tumour fragments and experiments were run for two weeks. Following termination, gels were assessed histologically [Fig 3.5A].

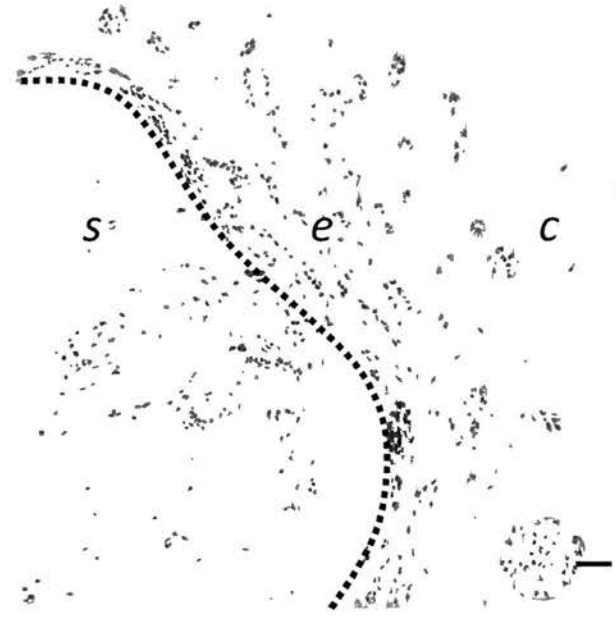


Fig 3.6 Representative H&E of 3D cultures, fixed on day 20. Histopathological assessment demonstrates original core biopsy ([s], border marked with dotted line) and neoplastic breast epithelium [e] invading in to the collagen [c]. A thick layer of viable epithelial tissue is demonstrated at the biopsy/collagen interface with evidence of structural invasion. Black bar = 100μm

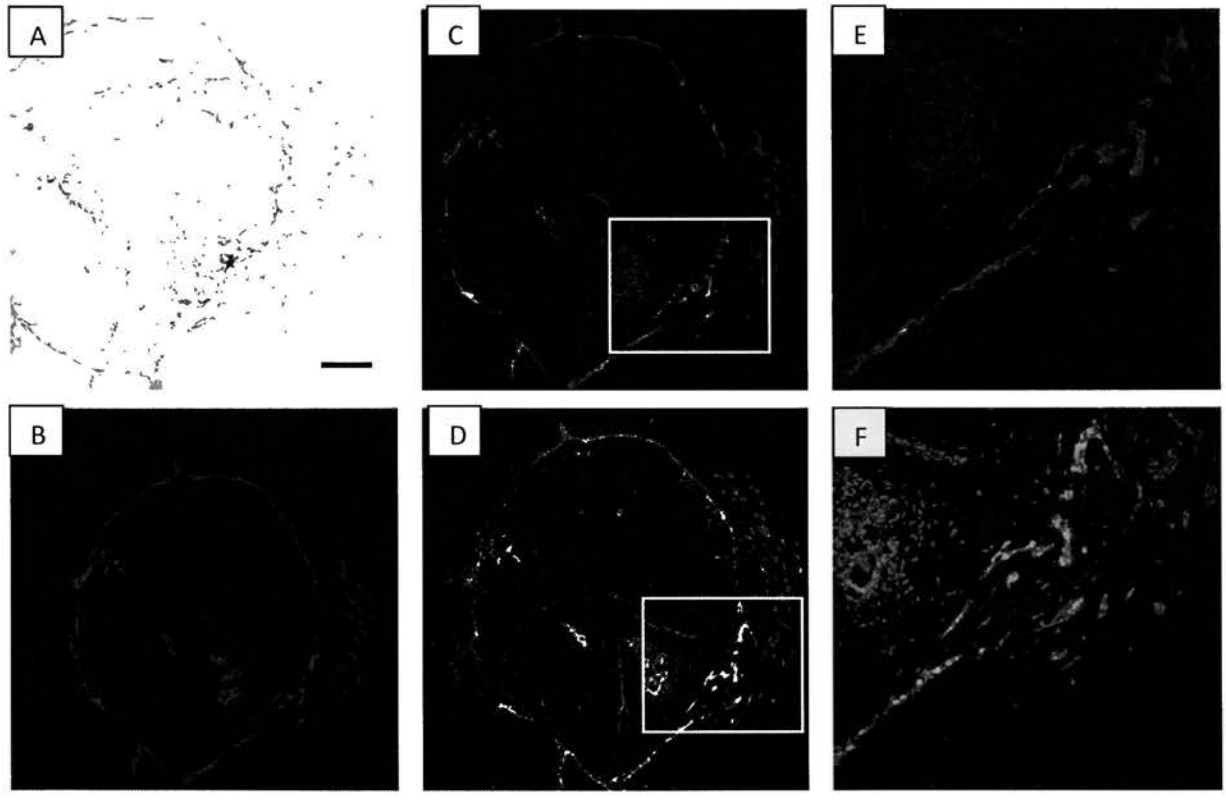


Fig 3.7 Immunofluorescence Analysis of invasion assay from an ER+, HER2+ Grade 2 lobular cancer. [A] H/E and [B] pancytokeratin stained serial sections, demonstrating epithelial cells invading into the surrounding collagen. Further sections were stained for [C] HER 2 and [D] ER alpha (target shown in red). HER 2 and ER positivity was demonstrated, with cells at the invading edge showing reduced levels [E+F]. Black bar = 200μm

As expected, cells of mesenchymal phenotype were evident both within the plug and invading into the collagen. Importantly, collective groups of cells with epithelial morphology were seen at the plug/collagen interface [Fig 3.5B], indicating that the fibroblast cultures were contaminated with epithelial cells. Therefore it was concluded that current fibroblast isolation techniques would not exclude anoikis-resistant epithelial cells of mesenchymal phenotype when grown on 2D plastic. Once in three dimensional culture these may then revert to an epithelial phenotype. Given that the additional fibroblasts were not necessary to induce epithelial invasion and that experiments may be confounded by addition of cells of unknown origin mixed into the surrounding collagen on the day of experiment initiation, it was decided that additional fibroblasts should not be part of the assay design.

3.4 Viability of tumour subcomponents within 3D *ex vivo* preparations

For an assay to display behaviour similar to that of a tumour *in vivo*, all epithelial subtypes and stromal components from a sample must retain viability. To assess this, formalin fixed paraffin embedded (FFPE) samples were stained with haematoxylin and eosin (H&E) after 20 days of culture. Viable epithelial cells and mesenchymal cells within the original biopsy material were indeed evident on histological examination [Fig 3.6]. Malignant epithelial cells with squamoid morphology were demonstrated invading into the surrounding collagen. Nuclear pleomorphism was evident within these cells, along with mitotic figures and apoptotic bodies.

In order to further characterize the invading cells, preparations derived from ER+/HER2+ biopsy samples were formalin fixed and paraffin embedded at day 20 [Fig 3.7A]. Serial sections were cut and stained for cytokeratin, HER2 and ER using immunofluorescence techniques. Cells staining positively for cytokeratin (and therefore of epithelial origin) were evident as a crust at the interface between the original core and collagen, with CK+ cells invading in to the surrounding collagen gel [Fig 3.7B]. ER and HER2 expression was evident within these cells, with cells at the invasive edge demonstrating ER/HER2-low phenotype [Fig 3.7C-D].

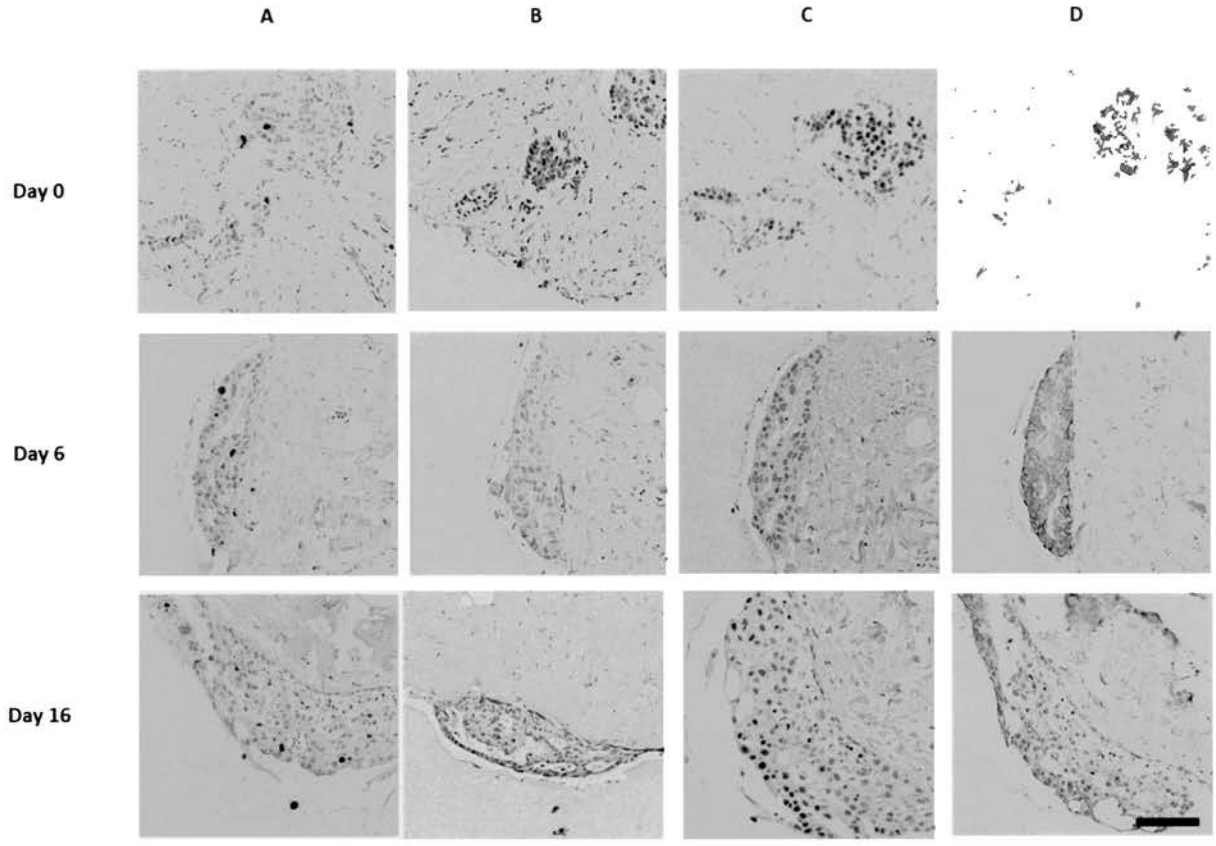


Fig 3.8 Immunohistochemical staining and qualitative changes in invasion assay over time. Representative 20x images taken from assays fixed at multiple points over a sixteen day experimental time course. [A] Ki67 expression was maintained and [B] CCasp3 remained at a low level. Interestingly [C] ER and [D] HER2, whilst strongly expressed at the beginning of the experiment became weakly expressed by the time of experimental termination. Black bar = 100μm

In order to evaluate changes in apoptosis and proliferation over the time course of the experiment, biopsy samples from two cancers were used to create multiple assays. Assays were selected at random over the experimental time course; on day0, day3, day6, day9, day13 and day16. Following formalin fixation and sectioning, antibodies to Ki67 and CCasp3, markers of proliferation and apoptosis respectively, were applied. ER and HER2 were also assessed over the time course. Multiple slides from each time point were stained and assessed qualitatively. Between samples and over the time course, Ki67 levels fluctuated. However, overall, the levels of Ki67 were maintained [Fig 3.8A]. Cytoplasmic levels of CCasp3, a marker of apoptosis, appeared moderately increased over time [Fig 3.8B]. ER status appeared moderately reduced over the time course of the experiment [Fig 3.8C], along with HER2 expression [Fig 3.8D].

3.5 Origin of invading epithelium

Following assessment of the time course it was noted that cellularity within the original core decreased over time, with a concomitant increase in cellularity at the core/gel interface. This manifested as a thick outer layer of epithelium surrounding the core. The shift in cellularity could be due to a combination of apoptosis in the central core and proliferation at the core edge. Alternatively, the redistribution of cellularity is secondary to translocation of malignant epithelium towards the periphery, potentially following a media dependent chemotactic gradient. In order to assess these hypotheses further, multiple assays from ten tumour samples were formalin fixed at day 20 and stained using Ki67 and CCasp3. Qualitative analysis of these slides demonstrated that a range of Ki67 expression was evident [data not shown]. Even in samples where Ki67 expression was low, significant crust accumulation and collective invasion into the surrounding collagen was evident [data not shown]. CCasp3 expression remained a rare event. Based on circumstantial evidence it was deduced that expansion of epithelium into the surrounding matrix occurred as a result of both epithelial migration from within the original biopsy material and sustained epithelial proliferation.

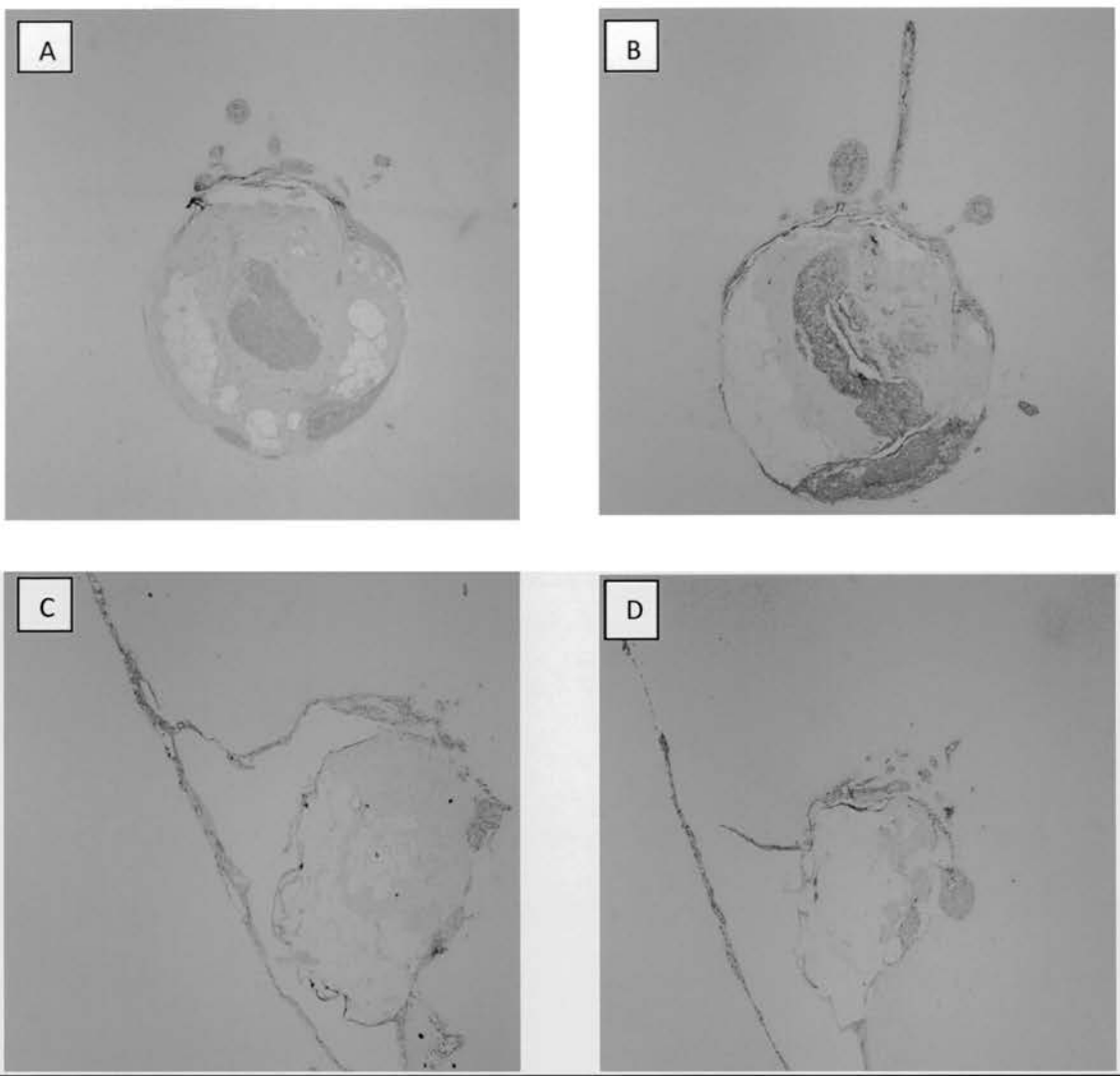


Fig 3.9 Serial sections cut through the same assay [A] 30μm [B] 60μm [C] 90μm [D] 120μm. A variety of different overall morphologies can be seen from the same assay cut at different levels, from large structures invading alongside high levels of centralised epithelium in [A] and [B] to acellular central core and smaller and individually invading cells in [C] and [D]. This highlights that the limitation of 2D sectioning to provide an overall morphological representation, which can be misleading depending on the level at which the specimen is sectioned.

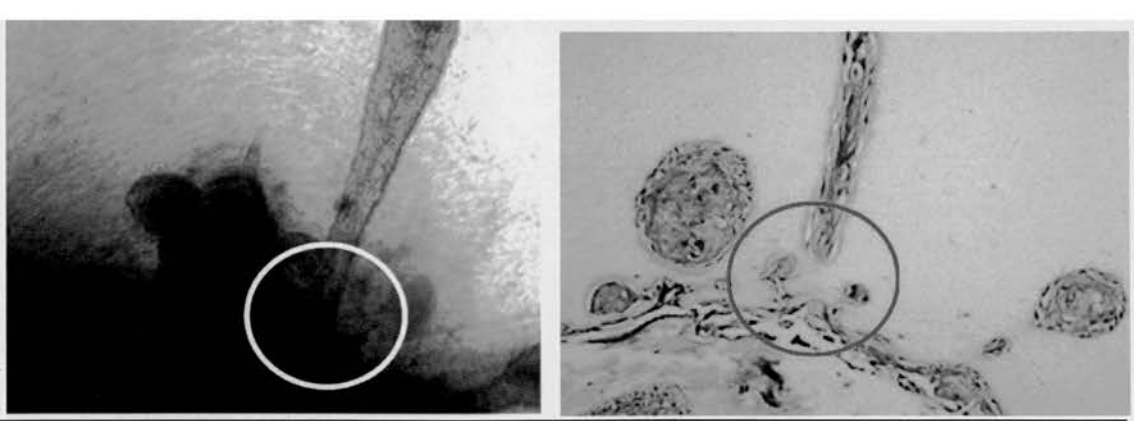


Fig 3.10 Further limitations of 2D assessment of 3D object. Objects which are adjoining when assessed as a 3D object may appear to be detached on 2D analysis. Subsequent 2D classification of modes of invasion in a 3D environment (into single cell /small cohort /cluster/ multicellular strands /sheets) may as a result be misleading.

3.6 Three dimensional assessment of assay

Multiple cross-sections taken at a range of depths through an individual assay demonstrated a range of overall assay morphology [Fig 3.9]. Whilst a single 2D slide provided a good method of assessing levels of ER, HER2, proliferation and apoptosis across a cross-section of cells (typically 800-3000 cells per slide), it did not provide sufficient data to assess overall shape, structure and volume of the assay as a whole. Moreover, the interpretation of a gel during the time of the experiment using live microscopy and subsequent interpretation of the 2D H&E slides often did not correlate. Invading structures that appeared continuous with the original core on live microscopy appeared as small discrete groups of cells, independent from their origin invading into the surrounding matrix [Fig. 3.10]. In order to reduce the sampling error associated with 2D sections taken from 3D objects, a three dimensional scanning technique was adapted to assess the assays; Optical Projection Tomography (OPT).

In order to visualise the epithelial component of the invading tumour in to the surrounding collagen matrix, cytokeratin antibodies were incubated with each sample followed by a fluorescent secondary antibody that fluoresced in the Cy3 range, appropriate to the OPT filter set. In order to visualise the connective tissue-rich original tumour fragment, an additional scan of each sample was performed in the GFP1 channel. The high levels of autofluorescence emitted from the original fragment in this channel allowed for image capture of the original core. The optical projection tomograph scanner acquired an image for every 0.9 degree the sample was rotated, producing 400 images during a 360 degree rotation [Fig 3.11A,B]. Using NRecon software, these rotational images were converted in to virtual sections, independently reconstructed using a back-projection algorithm similar to those used for Computerised Tomography [Fig 3.11C,D]. Using a further software package, Volocity, the two channels were combined [Fig 3.11E]. Stacking multiple sections results in a three dimensional reconstruction of the data [Fig 3.11F]. The result was a 3D reconstruction providing an overall representation of an assay, without the loss of data and misleading sampling errors associated with cross-sectional analysis.

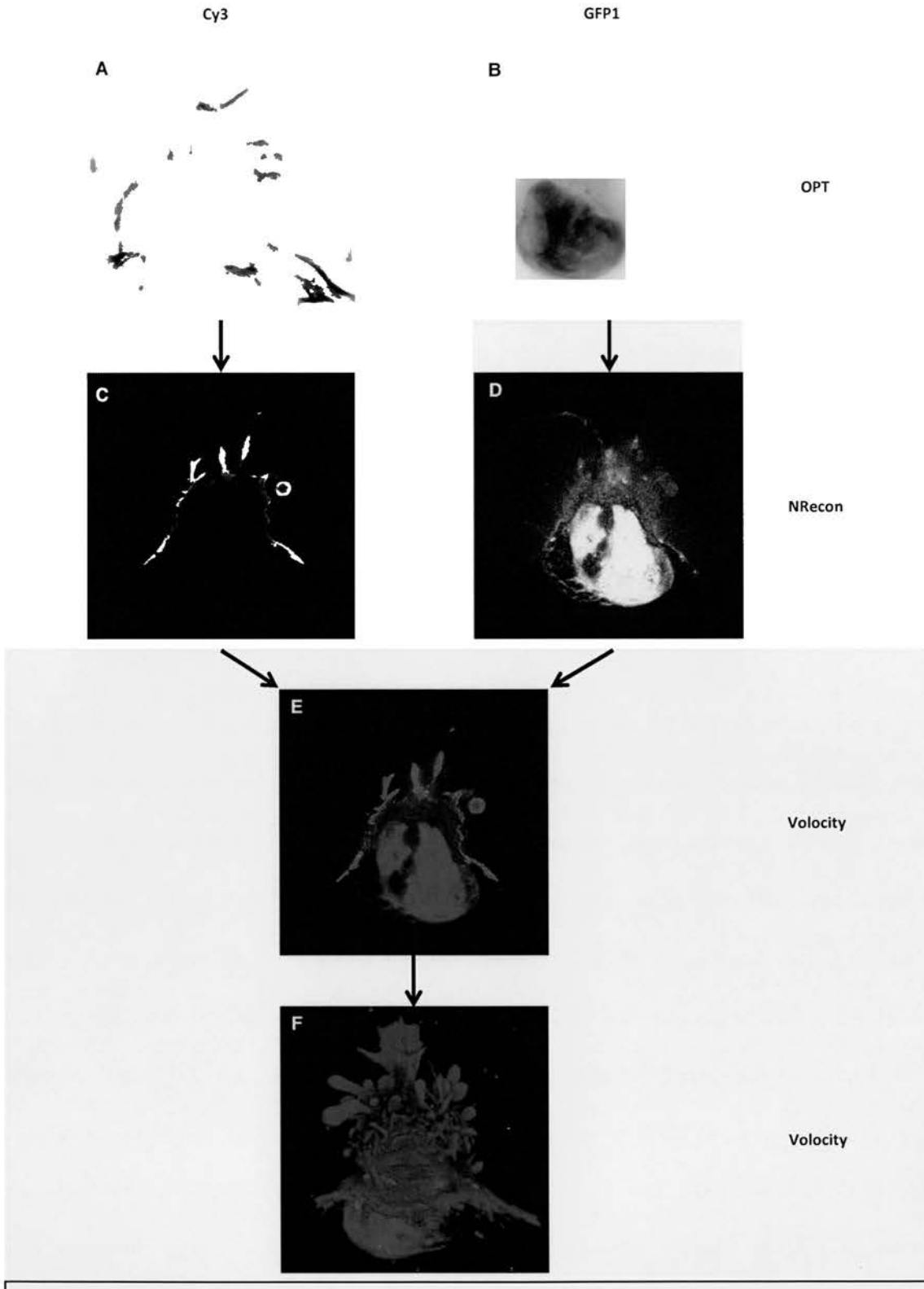


Fig 3.11 Following incubation of samples with cytokeratin antibodies and fluorescent-tagged secondary antibodies, Optical Projection Tomography was performed in [A] Cy3 to identify the fluorescently labelled epithelium and [B] GFP1 to identify autofluorescence from the connective stroma. Virtual sections [C-D] were generated and combined [E]. Using 3D reconstruction software these virtual sections allowed for a reconstruction of the original sample to be created.

3.7 Conclusion

Collagen-based assays to study the behaviour of cancer cell lines have been widely reported [123-125]. To a degree these assays have not reflected in full the complexity of the tumour and its environment. We have developed a 3D cell culture which demonstrates that human breast cancer materials can be used in a similar manner, *ex vivo*. Not only does this assay appear to maintain both epithelial and mesenchymal components found in breast cancer (as assessed visually), it also supports cellular proliferation and invasion. In contrast to other studies we found that inclusion of additional fibroblasts was not necessary to promote invasion and increasing the concentration of collagen resulted in no change to the phenotype of invasion[121, 122].

Two dimensional analysis of the assay using histopathological techniques provided a method of analysing cellular morphology and protein expression at a cellular level. However, the asymmetry and heterogeneity seen within the assay limited the utility of two dimensional assessment to evaluate overall shape, size and invasive morphology of the assay. Therefore we developed OPT as a useful tool to characterise the 3D expansion of breast cancer in this assay.

We found that the main limitation of this technique is the availability of tumour materials (a single core biopsy is sufficient for 4-6 assays). Additionally the heterogeneity in epithelial content found in primary breast cancer samples variably reduced assay viability. In order to minimise assay failure, macroscopic adipose tissue was dissected out. Structural invasion occurred in 40%-60% of assays and therefore multiple assays were required per patient to ensure assay success. Total assay failure did occur, but this was in a minority of cases (<10%).

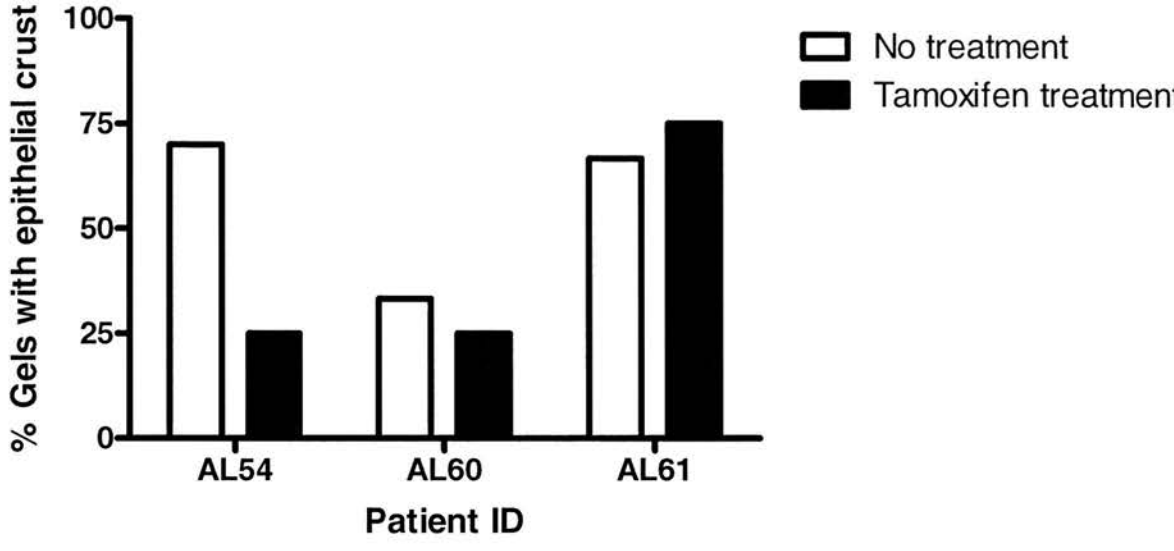


Fig 4.1 Assay response to tamoxifen. Samples from 3 ER+ patients (AL54, AL60 and AL61) were used to create multiple assays and randomised to a control group or receive tamoxifen. After 18 days gels were formalin fixed and cross-sections analysed using H/E. Response to tamoxifen was quantified by presence of epithelial crust (defined as a layer of epithelium >2 cells deep covering >10% of the original core/collagen gel interface).

AL54 - 71% (10/14) control assays and 25% (3/12) tamoxifen treated samples demonstrated epithelial crust.

AL60 - 36% (5/14) control assays and 25% (3/12) tamoxifen treated samples demonstrated epithelial crust.

AL61 - 69% (11/16) control assays and 75% (9/12) tamoxifen treated samples demonstrated epithelial crust.

Chapter 4: Determining tamoxifen sensitivity of primary breast cancer tissue in culture

Using the assay developed in Chapter 3, we aimed to design an *in vitro* method of quantifying an individual's response to drug therapy and measure their sensitivity. In order to develop the assay further, tamoxifen was chosen and applied to samples derived from ER+ breast cancer. Using a range of quantitative techniques, subsequent analysis of these specimens with presumed tamoxifen sensitivity would elucidate which method of quantification provided consistent and statistically significant results.

4.1 Morphological Analysis of Assay Response to Tamoxifen

To assess the effect of tamoxifen on ER+ breast cancer tissue *ex vivo,* breast tumour preparations were exposed to tamoxifen on the day of assay creation. A total of eighty gels were generated using biopsy samples obtained from 3 ER+ patients undergoing curative resection for invasive breast cancer. 36 gels (12 per patient) were immersed in MEGM supplemented with tamoxifen and oestrogen. Remaining biopsy material was used to make 44 control gels, immersed in tamoxifen-free, oestrogen supplemented MEGM. On day 18 the gels were formalin fixed and paraffin embedded. Histology sections were cut at random from each gel and H&E stained and semi-quantitative analysis of each slide was performed.

Epithelial crust, defined as a layer of epithelium >2 cells deep covering >10% of the original core/collagen gel interface, were found in both untreated and tamoxifen treated cohorts (between 25%-75% patient assays). Surprisingly, a similar proportion of gels generated epithelial crust despite tamoxifen treatment [Fig 4.1]. Gels derived from biopsies with a high proportion of adipocytes and low epithelial density were less likely to form epithelial crust at the core/gel interface independent of drug exposure[AL60 vs AL61]. However, qualitative analysis of H&Es demonstrated the morphology of epithelium between tamoxifen-exposed and untreated gels was markedly different.

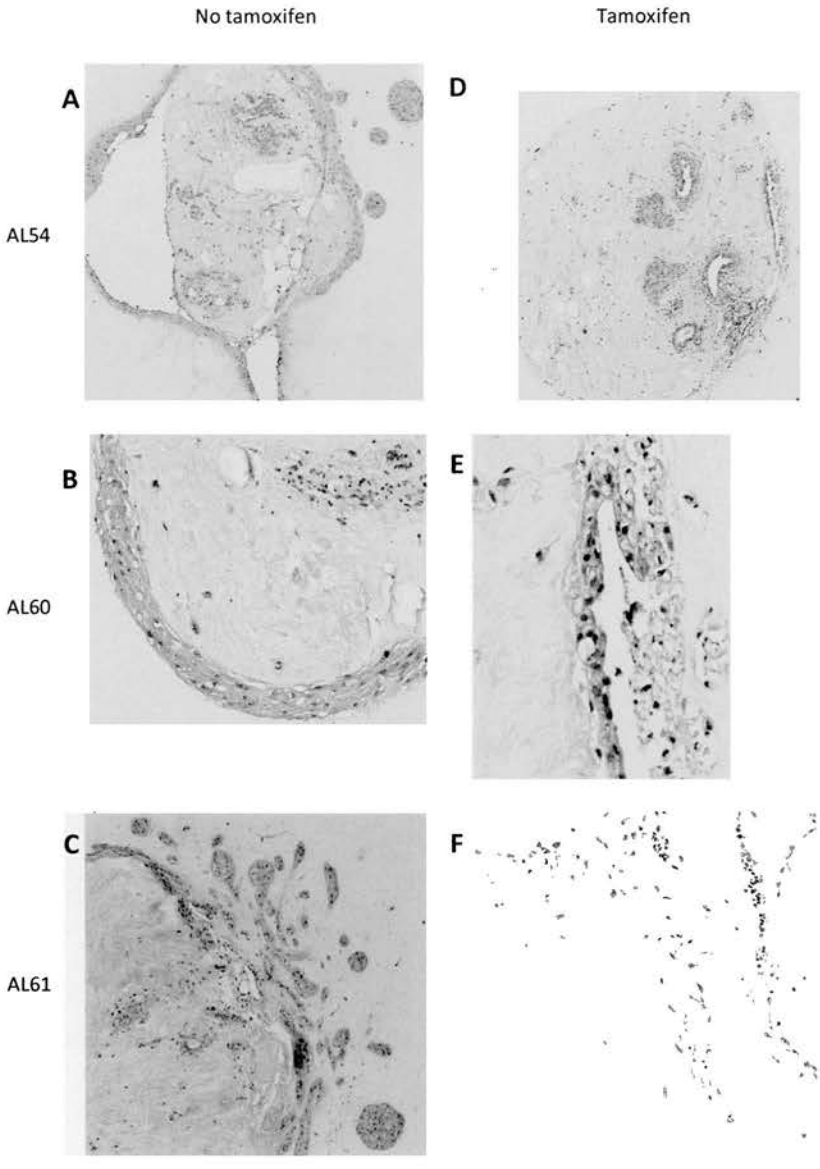


Fig 4.2 Histological analysis of assays following tamoxifen exposure. Biopsy samples were obtained from 3 patients with ER+ breast cancer and multiple assays created. 12 assays from each patient were exposed to tamoxifen supplemented media over the experiment al time course. Remaining tissue from each patient was used to created untreated controls. Untreated assays demonstrated epithelial margination and invasion in to the surrounding collagen [A-C]. Tamoxifen exposed samples also demonstrated epithelial margination and invasion, though with higher levels of apoptosis or mesenchymal phenotype [D-F].

In untreated gels the crust demonstrated a thick band of viable epithelium running parallel to the interface with large cohorts of cells invading into the surrounding collagen as bud-like or elongated structures [Fig 4.2A-C].

In contrast, in tamoxifen treated gels the epithelial crust was much less substantial, with a range of morphological response. In samples derived from AL54, treated gels exposed to tamoxifen appeared to demonstrate increased apoptosis [Fig 4.2D]. In tamoxifen exposed samples from AL60 [Fig 4.2E] and AL61 [Fig 4.2F], cells of predominantly mesenchymal morphology, consistent with myoepithelium, were evident at the interface but luminal cells were not. Rudimentary invasive structures were also apparent but appeared smaller and fragmented.

Taken together, tamoxifen had not inhibited the translocation of cells to the core/gel interface and invasion into the surrounding collagen, but rather had variably induced apoptosis or inhibited proliferation of luminal epithelium leading to a crust with reduced volume and viability.

These initial experiments identified two confounders. Firstly, between patients, the proportion of malignant epithelium within cores varied. As a result, a variable proportion of assays would demonstrate viable structural outgrowths. Secondly, a range of response to tamoxifen was seen, with some samples demonstrating a pro-apoptotic response and some showing an anti-proliferative response. From this, it was concluded that the appearance of an external epithelial layer could in itself not be reproducibly used as a surrogate marker of gauging individual tamoxifen sensitivity.

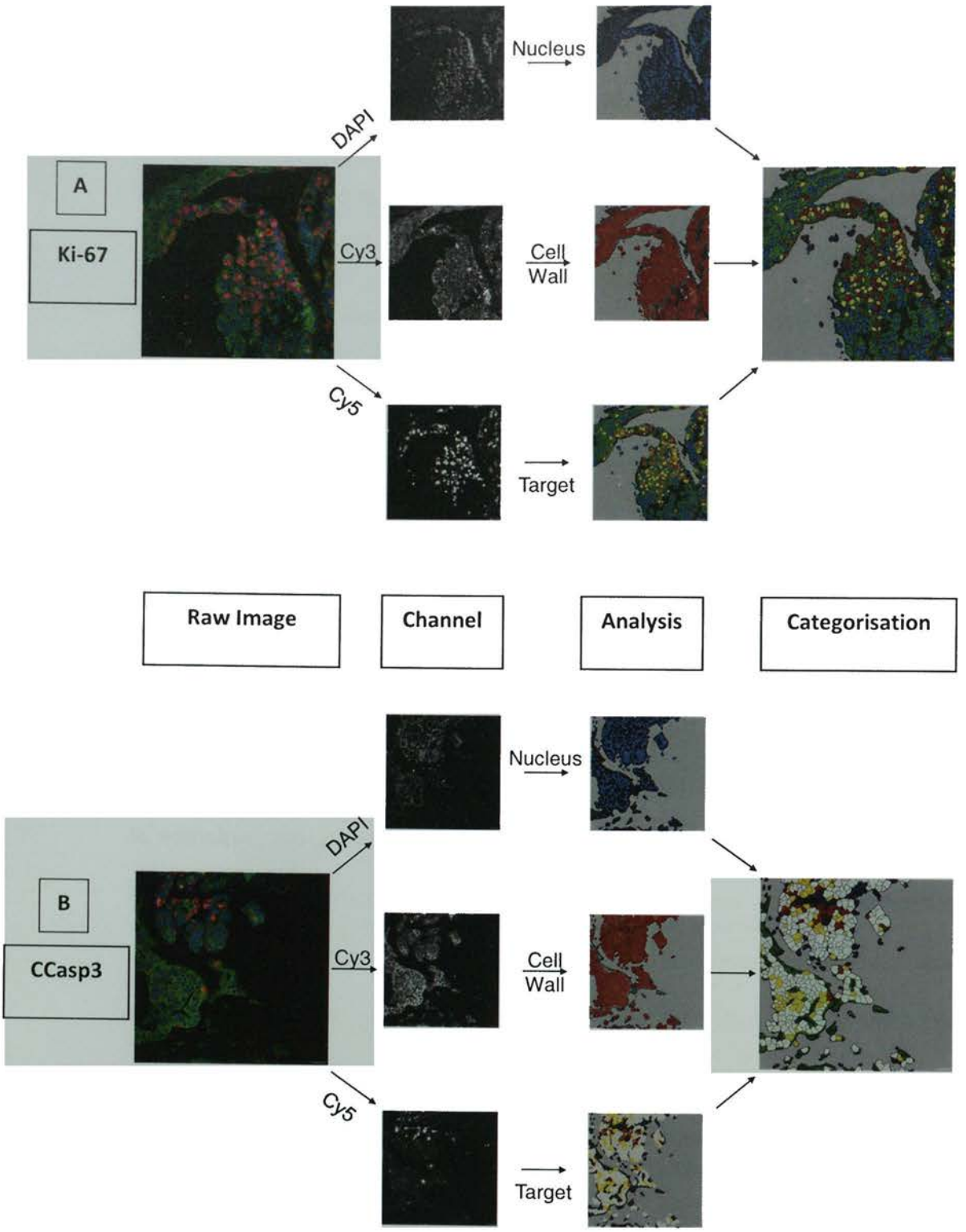


Fig 4.3 Objective analysis of IF slides using Tissue Studio. Sections through invasion assays exposed to tamoxifen and their matched controls were IF labelled for Ki-67 [A] and CCasp3 [B]. In conjunction with software developers at Definiens, an algorithm was developed that accurately identified individual cells based on nucleus and cell wall fluorescence. The intensity of target fluorescence within each cell (either in the nuclear compartment [A] or cytoplasmic compartment [B]) was calculated and each cell was stratified in to negative , low or high expressing cells. Category was colour coded: In Ki-67 negative nuclei = blue, low expression = yellow, and high expression (defining positive cells) = red. In CCasp3 negative cells = white, low expression = yellow, and high expression (defining positive cells) = red.

4.2 Quantification of changes in proliferation and apoptosis following tamoxifen treatment *ex vivo*

In order to provide a quantifiable method of evaluating patients' assay response to tamoxifen exposure, we investigated the validity of 2D immunofluorescence analysis of protein expression levels for markers of proliferation and apoptosis.

It had been previously noted that within core biopsies there may be marked intra-tumoural heterogeneity, with cores containing both foci of high epithelial content and areas of acellularity. In order to minimise the impact of acellular biopsy fragments, only preparations demonstrating invading epithelial structures were selected to enter the tamoxifen sensitivity study. Gel recruitment into the study was therefore deferred until eight days after assay creation. On day 8, gels which demonstrated structural growth were included and gels with no growth were discarded. This facilitated the exclusion of acellular preparations.

We investigated whether 2D immunofluorescence assessment could provide a consistent parameter by which sensitivity to tamoxifen may be gauged. Tumour samples from eight ER+ patients were obtained and divided to create multiple preparations for each patient. In order to quantify changes in proliferation and apoptosis, tamoxifen-treated and untreated preparations from each patient were stained for Ki-67 and CCasp3, using immunofluorescence (IF) techniques. In order to obtain an objective measure of protein expression at a cellular level, Definiens Tissue Studio pathology image analysis was used. This novel software programme provides automatic cell counting and categorisation across a large number of images. Multiple 40x magnification IF images were obtained for a cross-section from each sample and the percentage of cells expressing Ki-67 [Fig 4.3A] or CCasp3 [Fig 4.3B] was calculated.

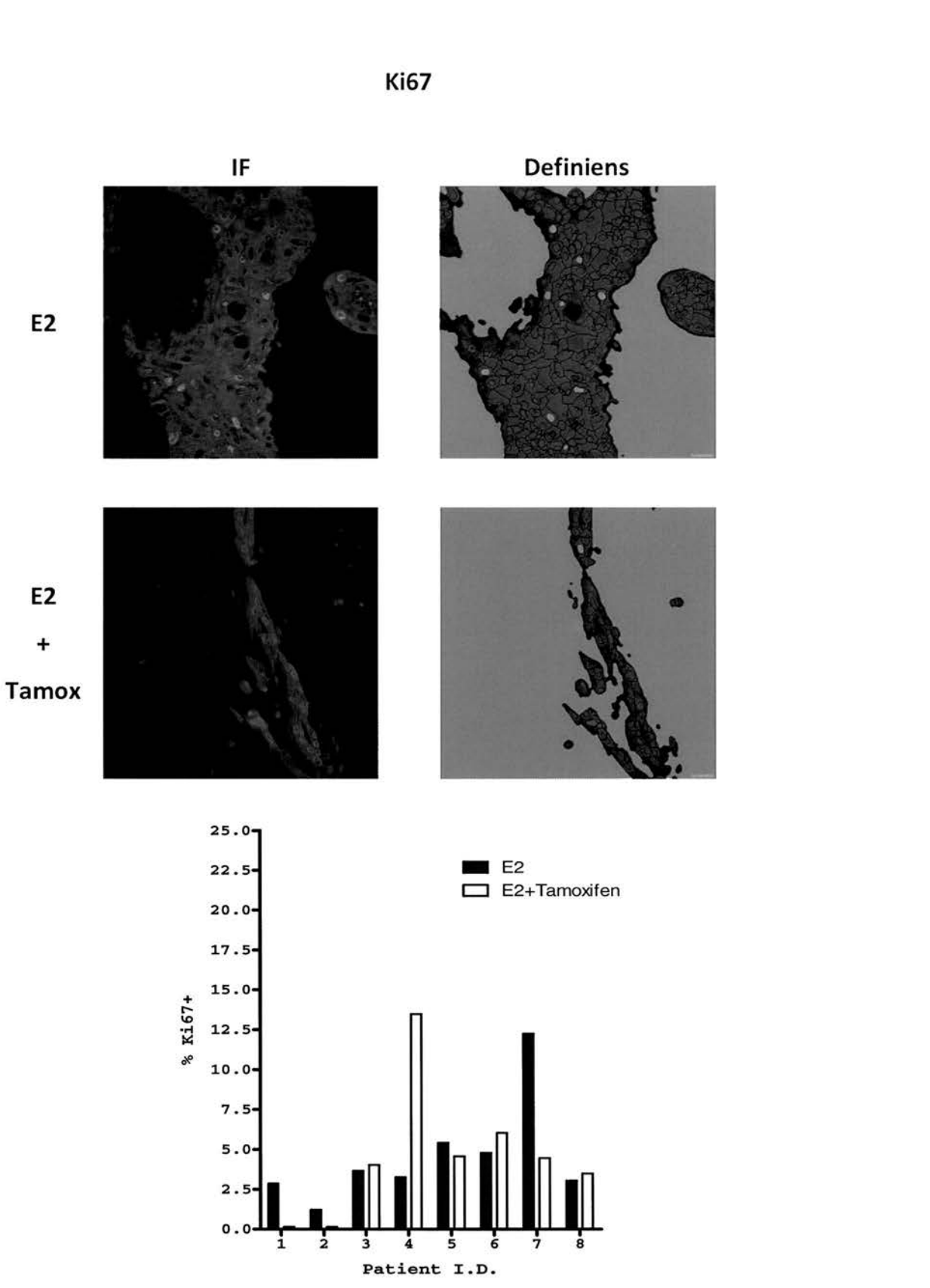


Fig 4.4 Two dimensional quantification of proliferation in response to tamoxifen *ex vivo*. Representative images following immunofluorescence staining for Ki-67 from same tumour preparations treated with oestrogen or with oestrogen and tamoxifen. Original 40x fields of view are shown in the left panels, with target (Ki-67) in red, cytokeratin in green, nuclei in blue. In the right panels, Definiens Tissue Studio analysis is shown.

The percentage of highly positive cells for Ki-67 is calculated for each treated and untreated preparation across multiple 40x images and analysed, by patient.

4.3 2D Quantification of Changes in Ki-67 Expression

Ki-67 is an antigen to a non-histone protein with an important function in cell division. Involved in the early steps of polymerase I-dependent ribosomal RNA synthesis, its exact role is obscure. Levels of expression vary throughout cell cycle, with increased expression during G1, S, G2, and M phases but not during the resting phase G0. Ki-67 levels are low in the G1 and S phases and rise to their peak level in mitosis[126]. Expression of Ki-67 correlates with mitotic figure count and therefore is commonly used as a method of identifying levels of proliferation [127].

A wide range of Ki-67 expression was seen between samples. Surprisingly, however, addition of tamoxifen did not significantly alter cellular proliferation as measured by the percentage of Ki-67 positivity [Fig 4.4]: On average 4.56% (range 1.21-12.25%) of cells in the control samples demonstrated Ki-67 positivity, whereas 4.54% of cells in the tamoxifen-treated samples (range 0.16-13.47%) were positive. 1 patient demonstrated an increase in proliferation following tamoxifen exposure 3 patients demonstrated a decrease in proliferation. 4 patients demonstrated no significant change in Ki67 expression. Poisson regression analysis confirmed that the number of Ki-67 positive cells in untreated samples was 0.97 times that of tamoxifen treated samples (95% CI 0.44 to 2.15, p=0.95).

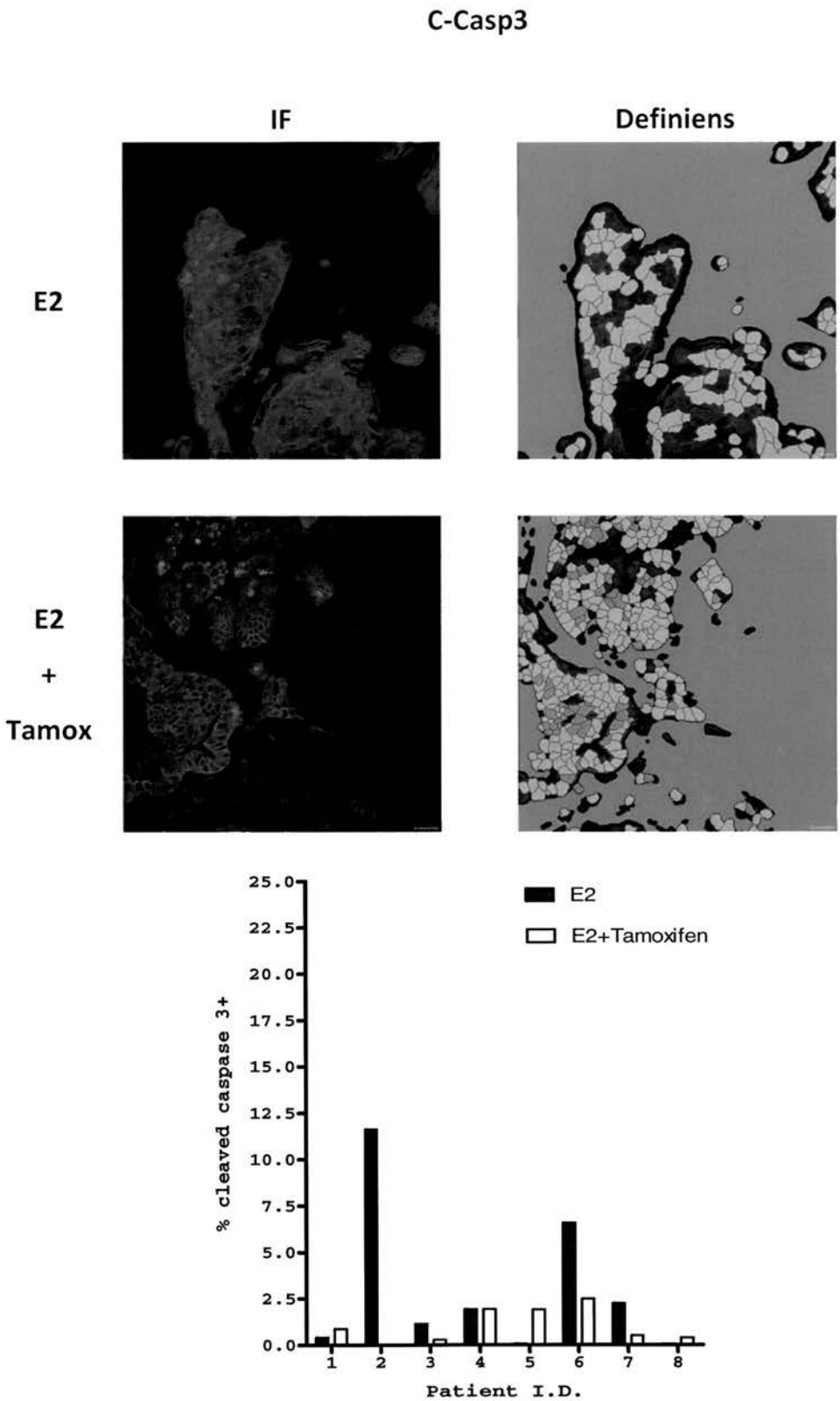


Fig 4.5 Two dimensional quantification of apoptosis in response to tamoxifen *ex vivo*. Representative images following immunofluorescence staining for CCasp3 from same tumour preparations treated with oestrogen or with oestrogen and tamoxifen. Original 40x fields of view are shown in the left panels, with target (CCasp3) in red, cytokeratin in green, nuclei in blue. In the right panels, Definiens Tissue Studio analysis is shown.

The percentage of highly positive cells for CCasp3 is calculated for each treated and untreated preparation across multiple 40x images and analyzed, by patient.

4.4 2D Quantification of Changes in CCasp3 Expression

There was also a wide range of CCasp3 expression between samples. Tamoxifen reduced the percentage of apoptotic cells expressing CCasp3 (oestrogen alone: average 3.0%, range 11.63-0.07%; oestrogen and tamoxifen: average 1.1%, range 1.94-0.29%) [Fig 4.5]. Poisson regression analysis showed that the number of CCasp3 positive cells in untreated samples was 2.88 times that of tamoxifen treated samples (95% CI 1.18 to 7.01, p=0.02). 38% of patients (3/8) demonstrated an increase in apoptosis following tamoxifen exposure and 50% demonstrated a decrease in apoptosis with one patient demonstrating no change in apoptosis levels. There was no correlation between levels of proliferation and apoptosis (Spearman's correlation: rho= 0.34, p=0.19) [Fig 4.6].

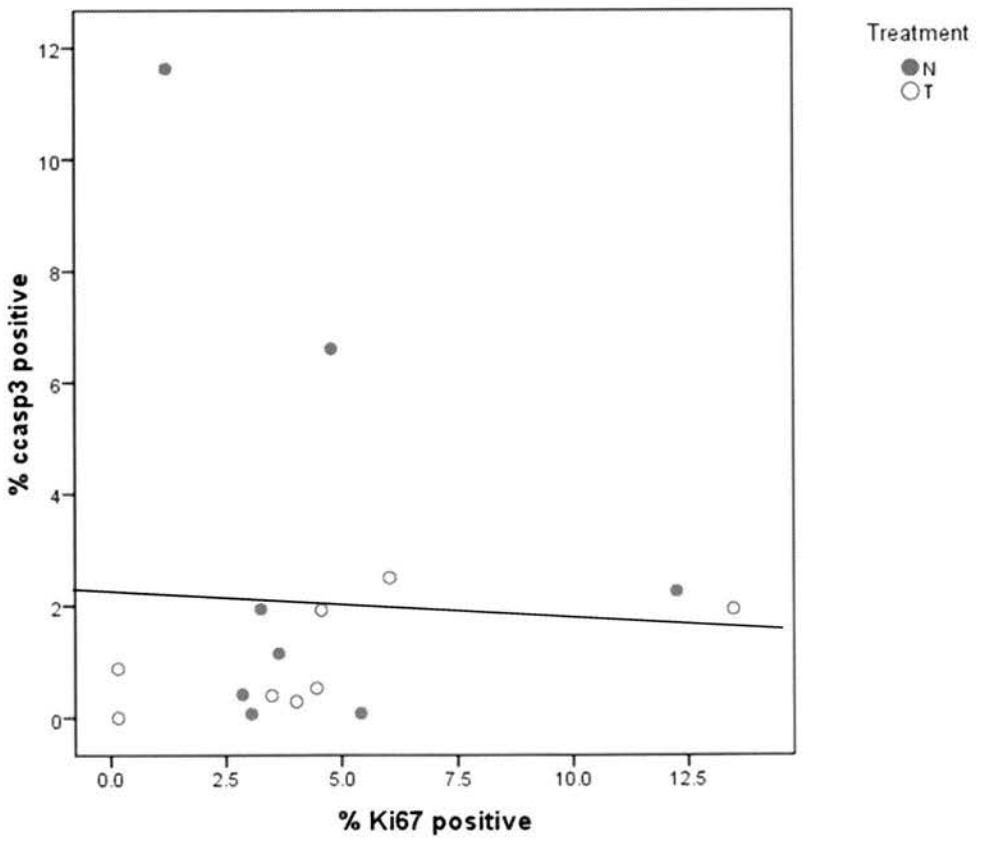


Fig 4.6 Linear regression analysis of expression of markers of proliferation and apoptosis. There was no significant correlation between Ki-67 and CCasp3 expression, either treated, untreated or both.

Table 4.1 Patient demographics and histopathology of biopsy

Patient I.D.	ER Status	HER2 Status	Grade	Age	Tumor size	Histological Type	TNM
1	CAT 8	NEG	1	82	13mm	NST	T1N0M0
2	CAT 7	NEG	2	47	13mm	Lobular	T1N0M0
3	CAT 8	NEG	1	54	11mm	Tubular	T1N0M0
4	CAT 8	POS	2	64	17mm	NST	T1N0M0
5	CAT 8	NEG	3	37	16mm	NST	T1N0M0
6	CAT 8	NEG	3	44	14mm	NST	T1N1M0
7	CAT 8	NEG	3	55	18mm	NST	T1N2M0
8	CAT 8	NEG	3	62	20mm	NST	T1N0M0
9	CAT 7	NEG	2	55	23mm	NST	T1N3M0
10	CAT 8	POS	3	57	18mm	NST	T1N0M0
11	CAT 4	NEG	3	76	20mm	NST	T1N0M0
12	CAT 0	NEG	3	46	15mm	NST	T1N0M0
13	CAT 0	POS	3	44	33mm	NST	T1N0M0
14	CAT 0	POS	3	56	60mm	NST	T1N1M0
15	CAT 2	NEG	3	37	50mm	NST	T1N2M0

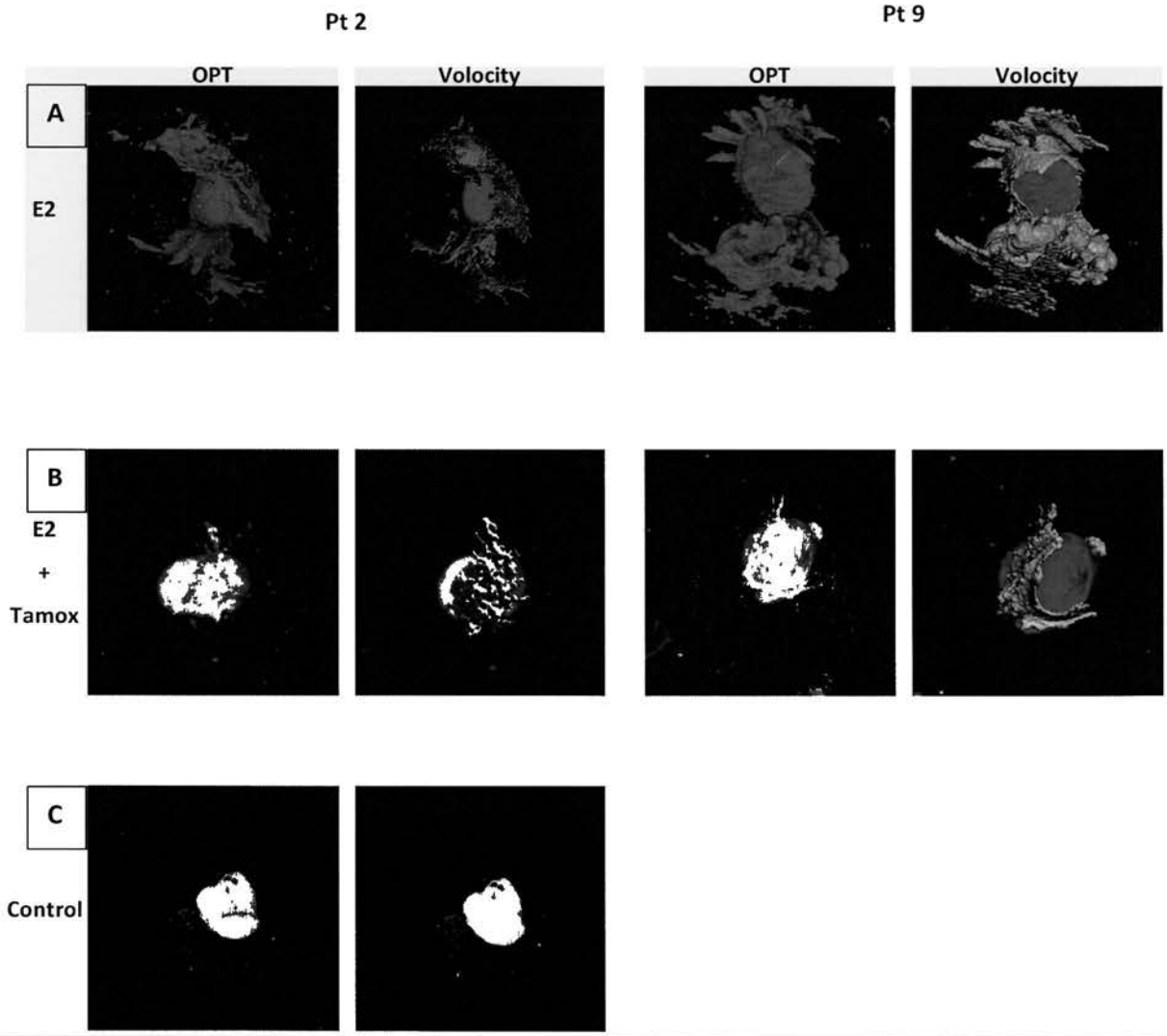


Fig 4.7 3D reconstruction and measurement of epithelial volume. [A] Representative images from Patient 2 and Patient 9 of untreated gels following OPT reconstruction and Volocity quantification of epithelial volume. The quantified volume was compared to the epithelial volume of gels following tamoxifen treatment [B].

[C] Negative control assay (secondary antibody only) from oestrogen preparation originating in the same tumour.

Next, we set out to identify an alternative parameter to quantify tamoxifen sensitivity in individual tumours. Additional samples were utilised from the initial group of 8 ER+ tumours that had been used to investigate Ki-67 and CCasp3 levels. In addition to these, two further ER+ tumours were obtained. Five ER low and negative (ER low/-) tumours were also used as controls. The patient characteristics and histopathology are described in Table 1. For the majority of samples two tamoxifen treated and two untreated preparations per patient were analysed, providing a total of 60 samples.

Optical Projection Tomography was utilised to measure the volume of residual epithelium in both the treated and untreated samples for each patient following termination of the experiment at day 20. OPT generated a 3D reconstruction of each preparation, with division of the tumour into acellular connective tissue and fluorescently labelled epithelium [Fig 4.7]. Using Volocity software enabled the measurement of epithelial compartment volume that remained in continuity with the original biopsy stroma.

Across the ER+ group, tamoxifen significantly reduced the volume of the epithelial compartment when compared against their untreated matched pairs ($p = 0.001$, Mann-Whitney test) [Fig 4.8A]. In the ER low/- group, there was no significant change in tumour volume between the untreated and tamoxifen treated preparations ($p=0.78$, Mann-Whitney test) [Fig 4.8B].

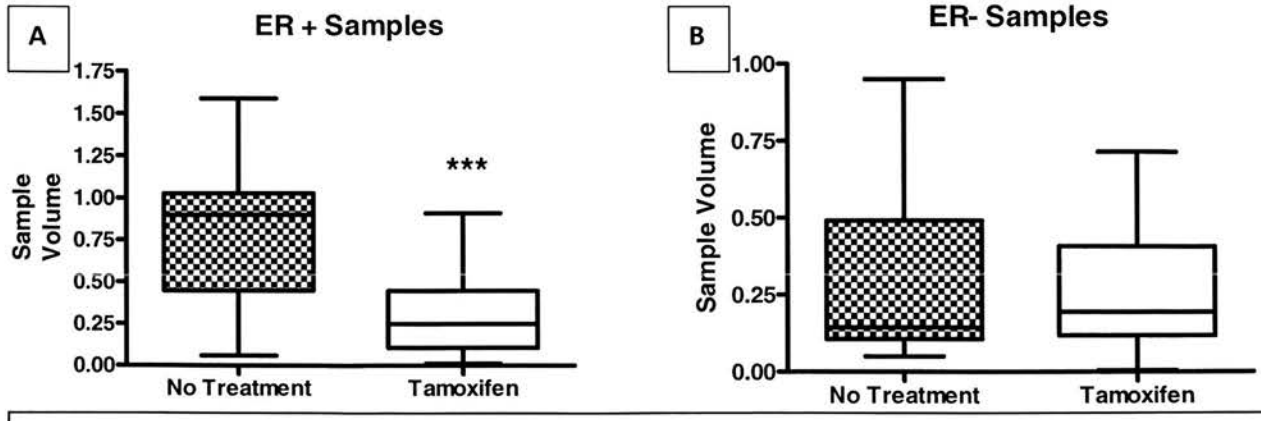


Fig 4.8 Comparison of epithelial volume changes in tamoxifen treated and untreated samples. [A] In biopsies taken from ER+ samples there was an overall significant reduction in epithelial volume in gels exposed to tamoxifen in comparison to untreated gels ($p = 0.001$, Mann-Whitney test), indicating tamoxifen sensitivity.

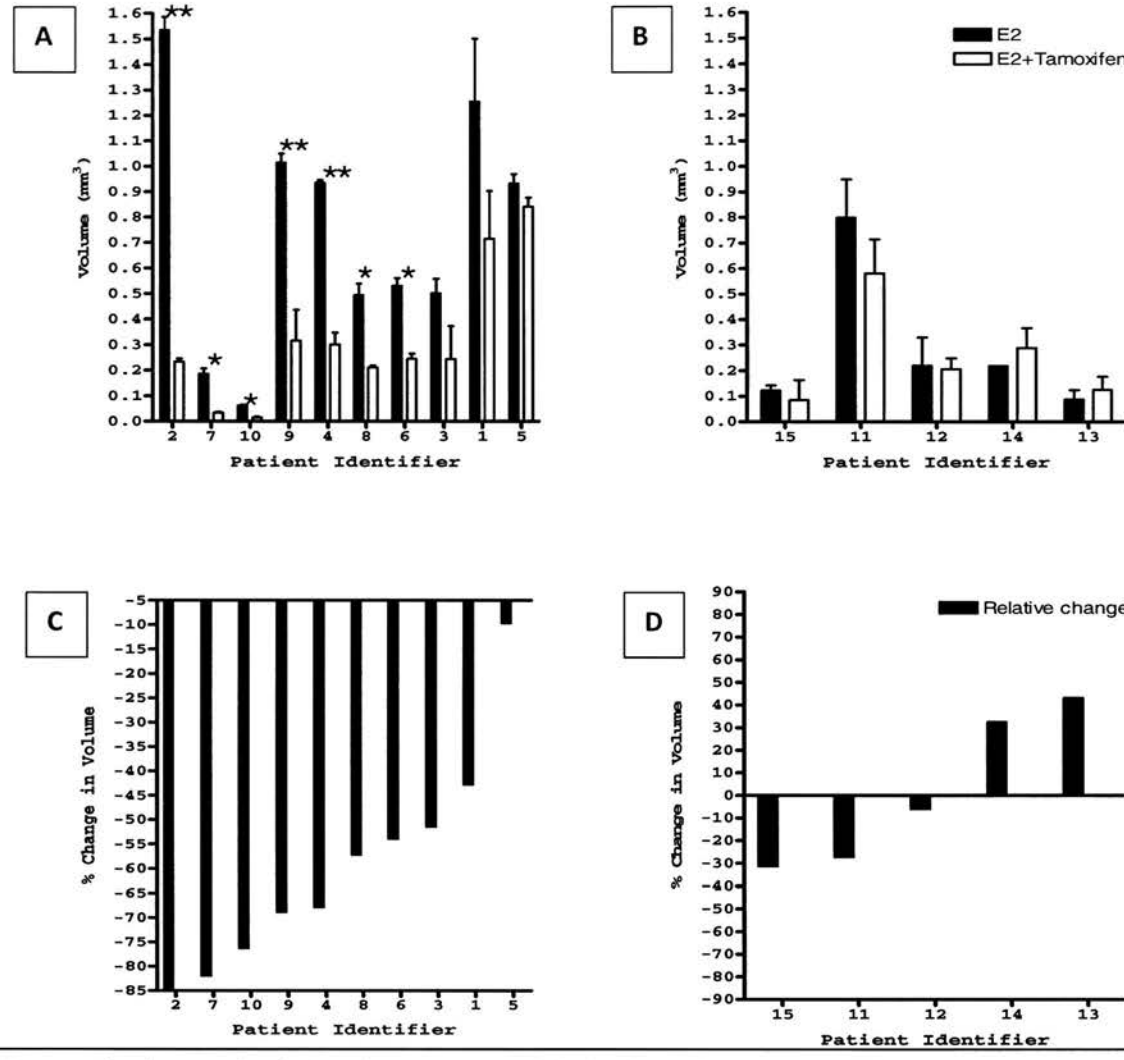


Fig 4.9 Absolute and relative changes in epithelial volume between individual patients. For each patient, treated and untreated epithelial volumes were compared using ANOVA. Tamoxifen exposure led to significant reductions in 70% of the ER+ group [A, C] and non-significant changes in ER low/- [B,D] patients. Asterisks indicate significant changes in tumour volume (* = $p < 0.05$, ** = $p < 0.01$).

Percentage reduction in tumour volume was calculated by comparing the average volume of tamoxifen treated preparations against the average volume of their untreated preparations, originating from the same tumour [Fig 4.9A,B].

In the ER+ group, after tamoxifen treatment relative reduction in assay volume ranged from 85% to 9%, indicating a range of tumour sample sensitivity between patients [Fig 4.9C]. Using analysis of variance (ANOVA) for individual assay volumes, patients could be divided in to those that demonstrated a significant change (7 patients, $p<0.05$) in tumour volume following tamoxifen and those that had non-significant changes (3 patients). Statistical analysis is summarised in Table 4.2.

In the ERlow/- group, preparations created from samples with low levels of ER positivity (patients 11 and 15) demonstrated non-significant reductions in tumour volume, and ER negative samples (patients 12-14) demonstrated non-significant increases in tumour volume [Fig 4.9B,D].

Association between change in Ki-67 and change in volume for the original eight ER+ patients was also analysed. There was no significant correlation between change in volume and change in Ki-67, either by Pearson's (linear, $p=0.60$) or Spearman's (non-linear, $p=0.57$) test.

The length of follow-up for all patients is less than one year from the date of curative resection. No patients have demonstrated recurrence and all ER+ patients remain on anti-oestrogen therapy.

Patient ID	Mean Tumour Vol. E2	Mean Tumour Vol. E2+T	Mean Tumour Vol. (E2)-(E2+T)	p-value
1	1.25 (0.32, 2.19)	0.72 (-0.22, 1.66)	0.54 (-0.79, 1.86)	0.22
2	1.53 (1.37, 1.70)	0.23 (0.07, 0.40)	1.30 (1.07, 1.53)	**0.002**
3	0.50 (0.07, 0.93)	0.24 (-0.19, 0.68)	0.26 (-0.35, 0.87)	0.21
4	0.94 (0.79, 1.08)	0.30 (0.15, 0.45)	0.64 (0.43, 0.84)	**0.006**
5	0.93 (0.78, 1.08)	0.84 (0.69, 1.00)	0.09 (-0.13, 0.31)	0.22
6	0.53 (0.42, 0.64)	0.24 (0.13, 0.36)	0.29 (0.13, 0.44)	**0.02**
7	0.19 (0.12, 0.25)	0.03 (-0.03, 0.10)	0.15 (0.06, 0.26)	**0.02**
8	0.49 (0.36, 0.63)	0.21 (0.07, 0.35)	0.28 (0.09, 0.48)	**0.03**
9[1]	1.02 (0.77, 1.26)	0.32 (0.07, 0.56)	0.70 (0.35, 1.05)	**0.0051**
10	0.06 (0.05, 0.08)	0.01 (0.002, 0.03)	0.05 (0.03, 0.07)	**0.008**
11	0.80 (0.19, 1.41)	0.58 (-0.03, 1.19)	0.22 (-0.65, 1.08)	0.39
12	0.22 (-0.14, 0.58)	0.21 (-0.15, 0.57)	0.01 (-0.50, 0.52)	0.92
13	0.09 (-0.11, 0.28)	0.12 (-0.07, 0.32)	-0.04 (-0.31, 0.24)	0.62
14[2]	0.22 (-1.19, 1.62)	0.29 (-0.70, 1.28)	-0.07 (-1.79, 1.65)	0.69
15	0.12 (-0.13, 0.37)	0.08 (-0.17, 0.33)	0.04 (-0.32, 0.39)	0.69

Table 4.2. Analysis of variance (ANOVA) for individual patients. Mean tumour volumes (and 95% confidence intervals) are shown for untreated (E2) and tamoxifen treated (E2+T) preparations from individual treatments.

1 This patient only had one E2 assay analysed.

2 This patient had 3 preparations per treatment analysed.

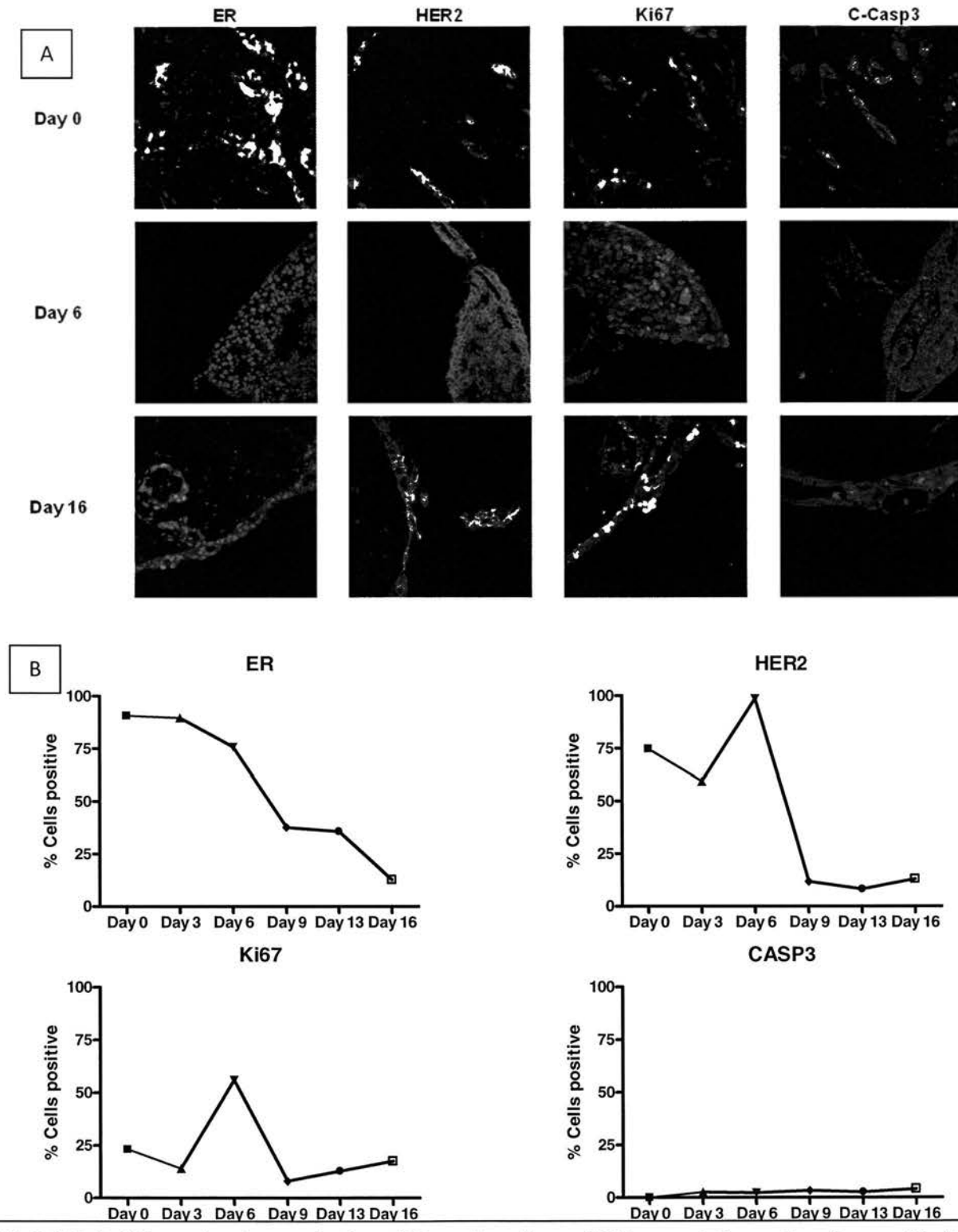


Fig 5.1 Clinical biomarker characterization of 3D *ex vivo* cultures. [A] Representative sections from an ER+/HER2+ biopsy sample stained for ER, HER2, Ki67 and CCasp3 are shown for day 0, day 6 and day 16 (Red, ER/HER2/Ki67/ CCasp3; green, epithelial mask; blue, DAPI). [B] Tissue Studio analysis of expression levels over time course shown in lower panels.

Chapter 5: Factors affecting tumour cell migration and proliferation

Having established an assay that supports primary tumour biopsy material and developed a means of quantifying the response of samples to tamoxifen, we set out to characterise which processes drive epithelial proliferation, cellular migration and invasion from the centre of the original tumour biopsy in to the surrounding collagen.

5.1 Quantification of changes over time within assay

It had previously been noted that while levels of Ki67 and CCasp3 remained relatively constant over the course of the experiment, expression of ER and HER2 appeared to decrease over the experimental time course (see Fig 3.8). In order to quantify these changes, preparations derived from an ER+/HER2+ biopsy sample were formalin fixed at intervals over the time course of an experiment. Serial histology sections were cut from the original biopsy sample and additionally assays were fixed at five time intervals, creating a series of samples over the experimental time course at day 0, day 3, day 6, day 9, day 13 and day 16. Histological assessment of haematoxylin and eosin stained sections demonstrated a gradual increase of epithelial cells at the core/gel interface and a concomitant decrease in epithelial cells in the central core. Immunofluorescence 2D quantitative techniques were used to quantify markers of proliferation and apoptosis and in addition HER2 and ER expression. Briefly, serial sections at each time point were stained and multiple 40x images captured at fixed exposure using an automated fluorescence microscope. Using Tissue Studio and fixed parameters these images were analysed and the cells categorised by positivity for the target antibody. [Fig 5.1A] demonstrates representative images captured by the automated microscope, for sections stained with A)ER, B) HER2, C) Ki67 and D) CCasp3. Definiens was used to quantify changes in expression pattern over time [Fig 5.1B]. As noted previously following qualitative analysis using IHC, levels of Ki67 and CCasp3 fluctuated around their original level. Additionally, quantitative analysis supported previous observations that ER and HER2 levels decreased over the time course.

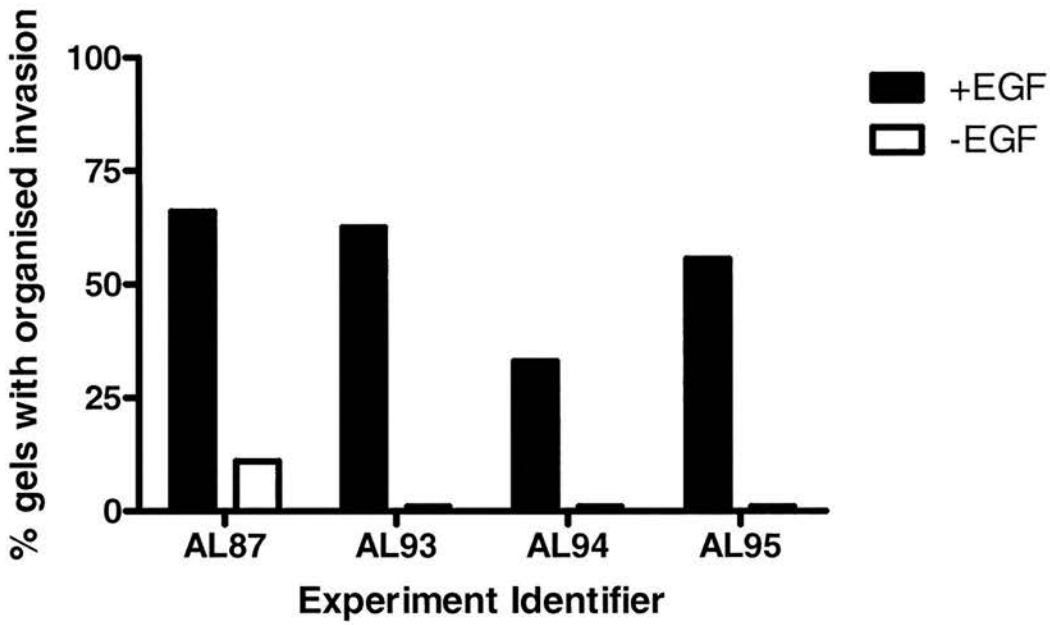


Fig 5.2 Effect of EGF on structural invasion. A total of 76 gels from four ER+/HER2- samples were divided into two groups, receiving either MEGM Complete or EGF depleted MEGM. Assays in EGF free media did not demonstrate structural invasion to the same degree as those exposed to EGF.

5.2 Role of EGF in driving invasion

We hypothesised that loss of ER and HER2 expression may be key steps in epithelial migration and invasion. Furthermore, we hypothesised that this loss of expression was induced by the assay itself, either 1) through integrin interaction with collagen or 2) through growth factors within the media. As demonstrated previously (see Fig 3.3) invasive potential did not appear to correlate with collagen concentration and therefore hypothesis 1) was discounted.

To investigate the role of growth factors in the media, two experiments were performed: MEGM® is a defined medium that is serum-free and consists of a basal medium (MEBM) with five additional growth factor supplements supplied separately (BulletKit®). These are hEGF, bovine pituitary extract (BPE), insulin, hydrocortisone and gentamycin with amphotericin. Using both ER+ and an ER- tumour samples, multiple assays were created and divided in to five groups with six assays in each group. In each group media minus one of the five growth supplements was used.

In the group of gels that had gentamycin/amphotericin withheld, bacterial contamination became evident and these experiments were discarded. Groups in which insulin, hydrocortisone or BPE were withheld continued to demonstrate structural invasion. However, in the group exposed to MEGM minus EGF there was no evidence of structural invasion. Further H&E processing of these gels revealed adequate cellularity, but no margination of cells from within the biopsy to the gel interface. From these initial experiments we deduced that stimulation of the epithelial component of the assay with EGF may play a role in driving structural invasion into the surrounding collagen gel.

To further investigate EGF's role, four ER+ batteries were created. In each experiment one half of the gels received MEGM Complete, the second half received MEGM without EGF supplement. In gels incubated in EGF-free media, initial single cell invasion was evident, but structural invasion was a very rare event. In contrast, structural invasion was evident in the majority of EGF exposed controls [Fig 5.2]. These experiments further supported our hypothesis that EGF drives epithelial invasion.

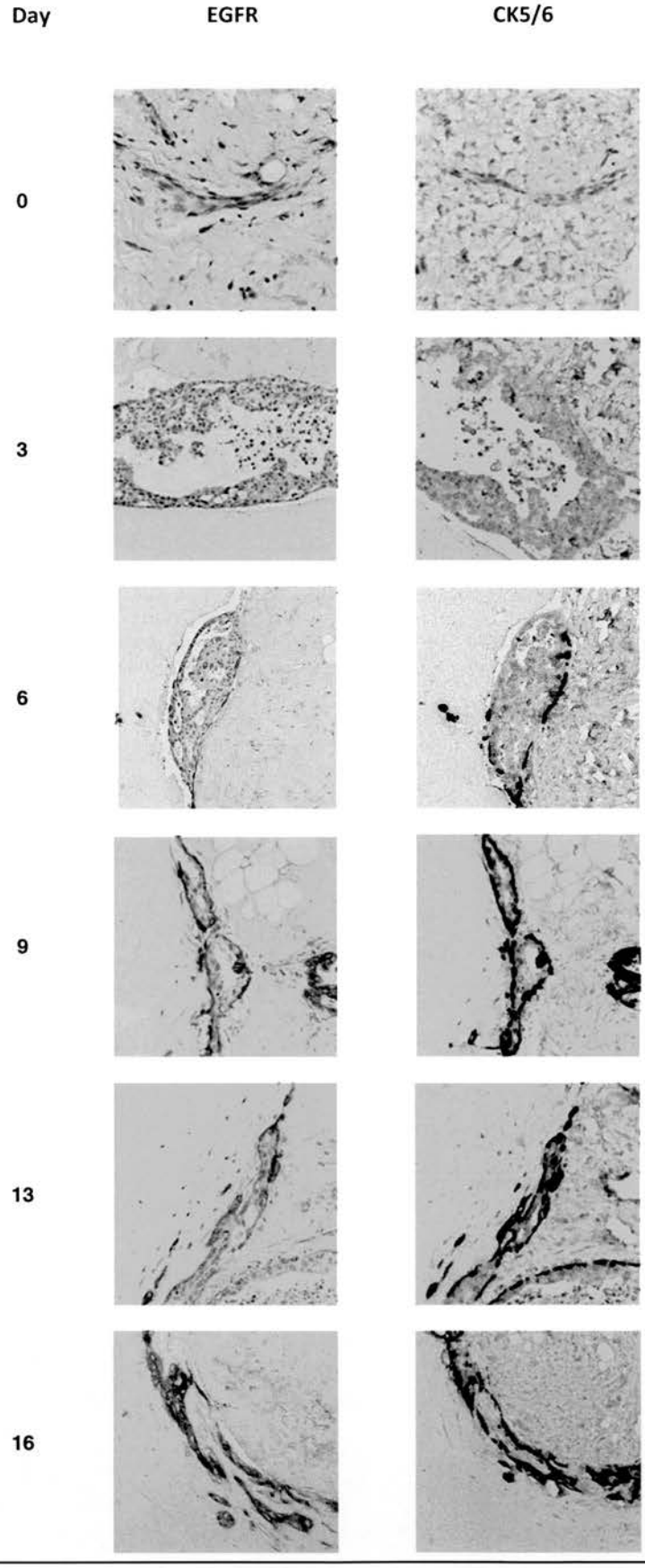


Fig 5.3 Changes in EGFR and CK5/6 expression over time. Additional sections from the previously investigated time series were stained for basal markers EGFR and CK5/6. Over the sixteen day time course intensity and proportion of positive cells increased.

In order to better characterise this transformation of epithelial phenotype, additional serial sections were taken from the time series used previously in Fig 5.1 and expression of EGFR and CK 5/6 elicited using immunohistochemistry [Fig 5.3]. Over the time course, both the proportion of cells and the intensity of expression within these positive cells increased. Taken together with IHC performed previously in Chap 3, this indicated a change from a luminal phenotype (ER+, PR+, HER2+, EGFR-, CK5/6-) to a basal phenotype (ER-, PR-, HER2-, EGFR+, CK5/6+) The basal phenotype was most marked in invading epithelial structures. In order to quantify the change to a basal phenotype, levels of EGFR were quantified as before using Definiens Tissue Studio and over the time course provided objective evidence that the proportion of EGFR positive cells was increasing [Fig 5.4]. Attempts to perform IF quantification using CK5/6 were abandoned due to technical issues with the interaction between CK5/6 and the pan-CK mask.

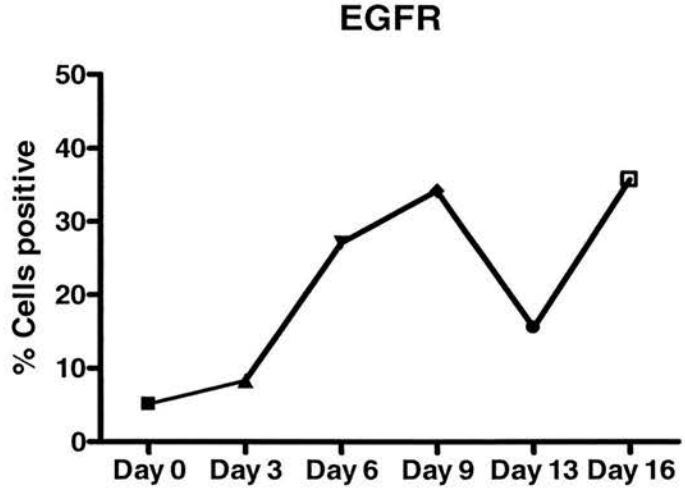


Fig 5.4 Quantitative changes in EGFR over time. Over the time-course strong EGFR expression rose from <5% of cells to almost 40%.

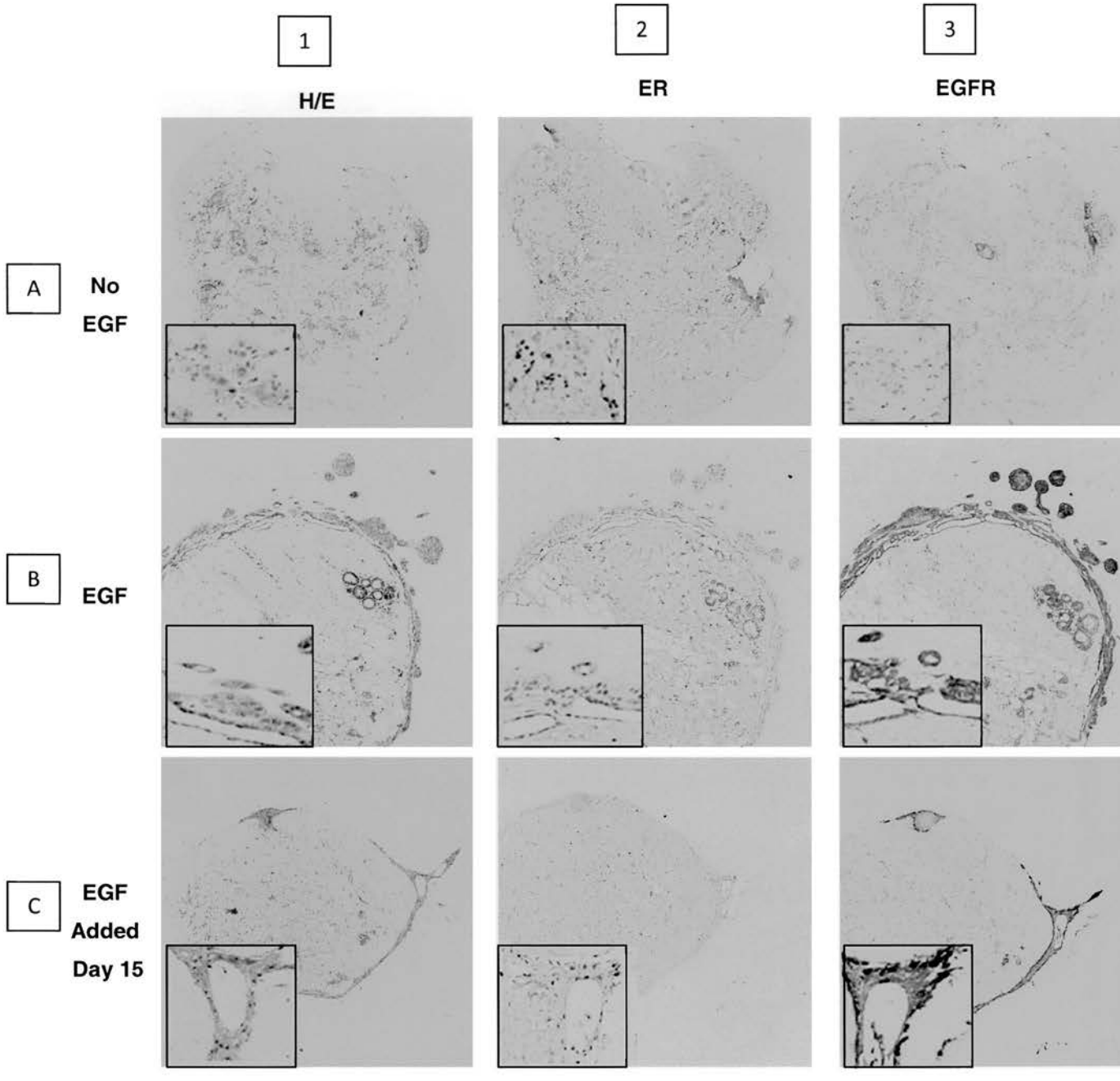


Fig 5.5 Effect of EGF on EGFR and ER expression. Gels were incubated over 20 days in [A] EGF deplete MEGM, [B] normal MEGM or [C] EGF deplete MEGM with exposure to EGFR delayed until day 15. Serial sections from representative gels were cut and analysed. Viable epithelium was demonstrable in all three groups, but margination and invasion were only evident in EGF exposed gels, along with a reduction in ER and an increase in EGFR expression. (Inlays = Magnified views)

5.3 Role of EGF in transforming assay from luminal to basal phenotype

We hypothesised that there were two likely explanations for this transformation from a luminal to basal phenotype: either the original luminal cells were gradually transforming to a basal phenotype over the time course, or that EGF stimulated a small population of basal cells to proliferate and expand with concurrent apoptosis and consequent involution of the original ER+ cell population.

To investigate this further, multiple ER+ assays were split into three cohorts. The first received MEGM Complete, the second cohort received MEGM without EGF, and the third group had delayed exposure to EGF; receiving EGF depleted MEGM for fifteen days followed by five days of MEGM Complete. Viable epithelium was evident in all three cohorts at Day 20 on H&E assessment [Fig 5.5-1]. In the EGF-exposed cohort epithelium accumulating as a peripheral crust was evident. In contrast, in the EGF free cohort the majority of the epithelial content remained scattered throughout the core. Interestingly, the cohort that underwent delayed EGF exposure also demonstrated a degree of epithelial crust formation. Serial sections underwent IHC for ER [Fig 5.5-2] and EGFR [Fig 5.5-3]. In the EGF-free cohort, a high proportion of the epithelium remained ER+. As had been noted previously in the EGF-exposed cohort there were very few ER+ cells. In the EGF-delayed group, ER+ cells were again largely absent. These experiments provided circumstantial evidence as to the fate of the ER+ proportion of cells: The presence of ER+ cells in the EGF-free gels indicates that the assay itself does not exert a cytocidal effect on ER+ cells. If EGF stimulation were inducing expansion of a basal subpopulation of cells, there would still be similar numbers of the ER+ cells in EGF+ and EGF- assays, but this was not the case. To explain the reduction in ER+ cell numbers, EGF may stimulate a directly cytocidal effect on ER+ cells. However, the absence of ER+ cells in the EGF-delayed cohort indicate that the cytocidal effect of EGF would have to be very rapid and the presence of a crust indicate that the expansion of a basal phenotype would have to be very fast. Alternatively EGF exposure stimulates luminal cells to convert to a basal phenotype, in keeping with the low levels of CCasp3 seen throughout the experimental time-course.

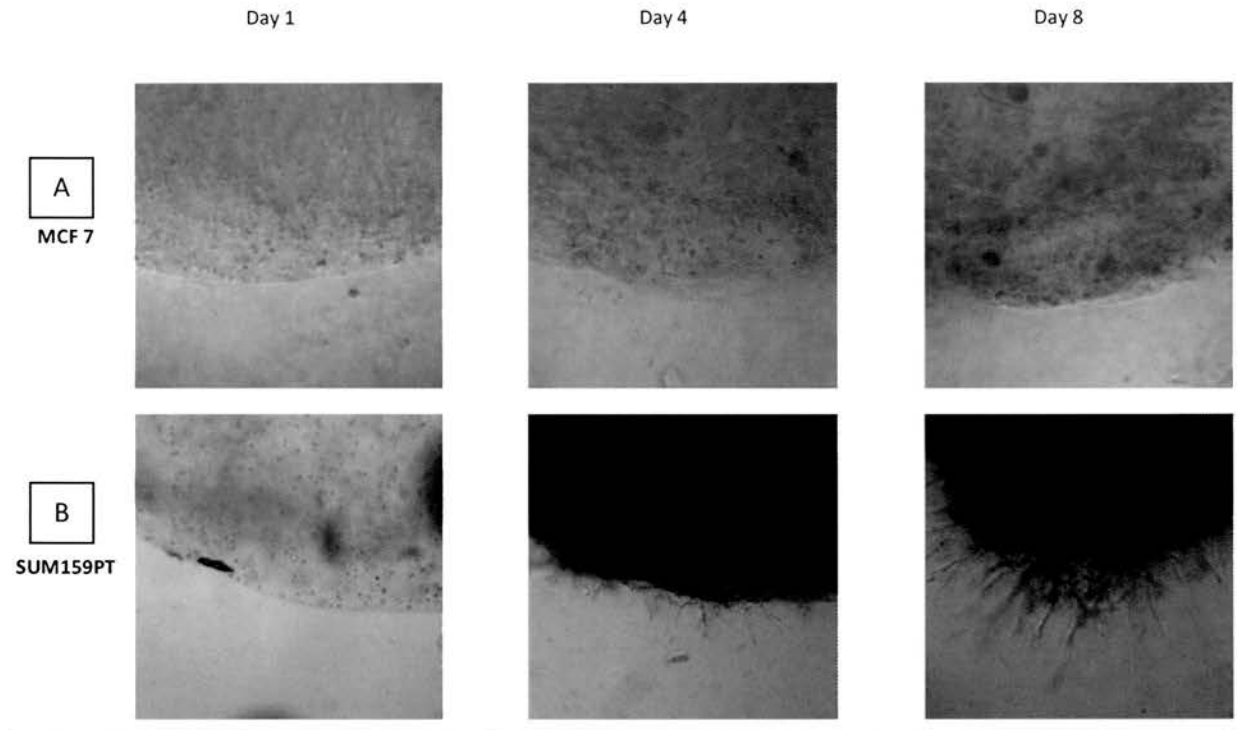


Fig 5.6 Cell plug invasion assay. Plugs of [A] MCF-7 and [B] SUM159PT were observed over time within the invasion assay. MCF-7 cell plugs were non-invasive as predicted. In contrast SUM159PT cell plugs showed initial single cell invasion (Day 4) followed by additional structural invasion (Day 8).

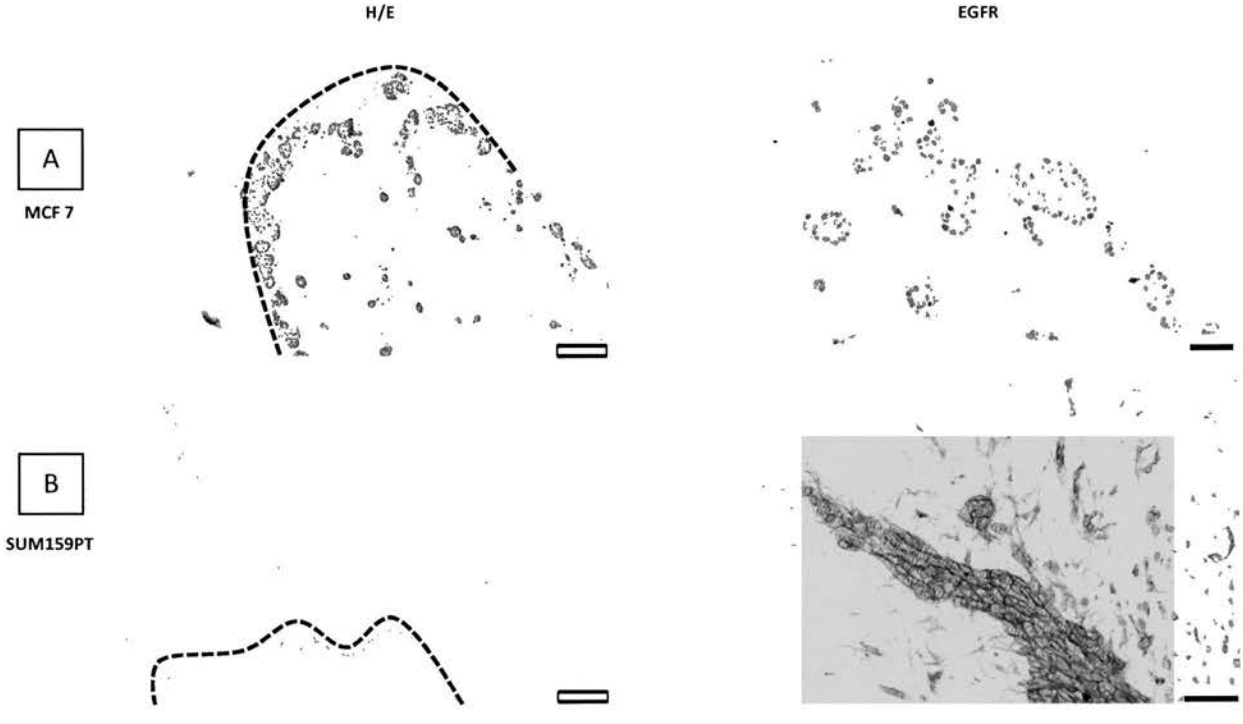


Fig 5.7 Histopathological assessment of cell plug invasion assays. [A]There was no evidence of MCF-7 cells invading from the cell plug border (dotted line) into the surrounding collagen. [B] In contrast, both single cell and structural invasion were evident in SUM159PT cross-sections. EGFR expression levels were checked at a proteomic level confirming negativity in MCF-7 cells and positivity in SUM159PT cells. White bar 100μm, Black bar 50μm.

In order to explore the trend between EGFR up-regulation and invasive potential of epithelium, cell line experiments were performed using a modified collagen based assay using two cell lines, SUM159PT and MCF-7. Both are immortalised lines derived from primary breast cancer. SUM159PT cells are of a basal phenotype, with positive expression of EGFR and negative expression of ER and MCF-7 cells are classed as luminal phenotype (ER+, EGFR-) [128]. Dense plugs of collagen and individual cell lines were created in 96 well plates to recapitulate the volume and cell density of the primary biopsy material. After 24hr these 200μl plugs were embedded in to the centre of 1ml collagen cushions.

Over the early experimental time course the assay behaviour was observed intermittently, using brightfield microscopy at 10x magnification [Fig 5.6]. MCF-7 cell plugs demonstrated no evidence of invasion into the surrounding collagen or associated collagen organisation. In contrast, SUM159PT cells demonstrated a pattern of behaviour similar to that seen in primary biopsy samples: An initial wave of invasion by cells of mesenchymal phenotype from the cell plug edge into the surrounding collagen was observed, followed by collective invasion of multicellular structures and associated collagen organisation and contraction. EGF supplementation to MCF-7 assays did not induce transformation to an invasive phenotype. H&E staining of sections obtained from paraffin embedded samples confirmed single cells and epithelial structures invading into the surrounding collagen in assays from SUM159PT samples but no evidence of cellular invasion from MCF-7 assays. IHC using EGFR antibodies confirmed EGFR positivity in SUM159PT assays, and EGFR negativity in the MCF-7 assays – despite exposure to EGF [Fig 5.7].

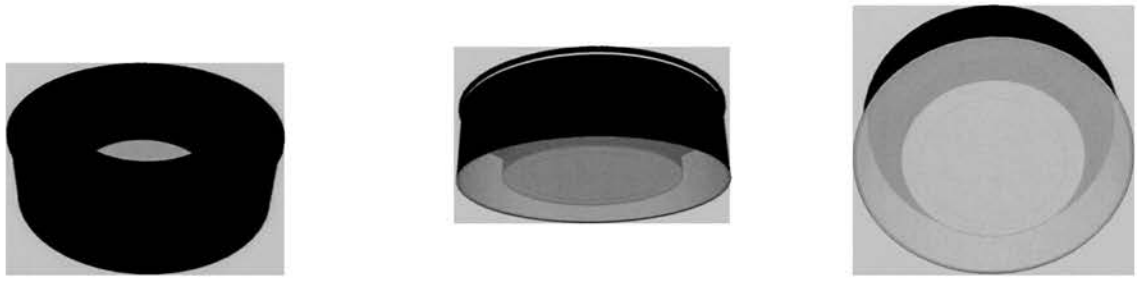


Fig 5.8 Design of viewing chamber insert for 6-well plate. In order to observe assay behaviour over time using a macroscope we designed an optical insert that would not impede gas exchange and maintain humidity, whilst fixing a gels position in order to produce time delay capture movies. The gel would be sandwiched in a 5mm gap between the insert and the bottom of the 6wp, surrounded by a reservoir of fresh media.

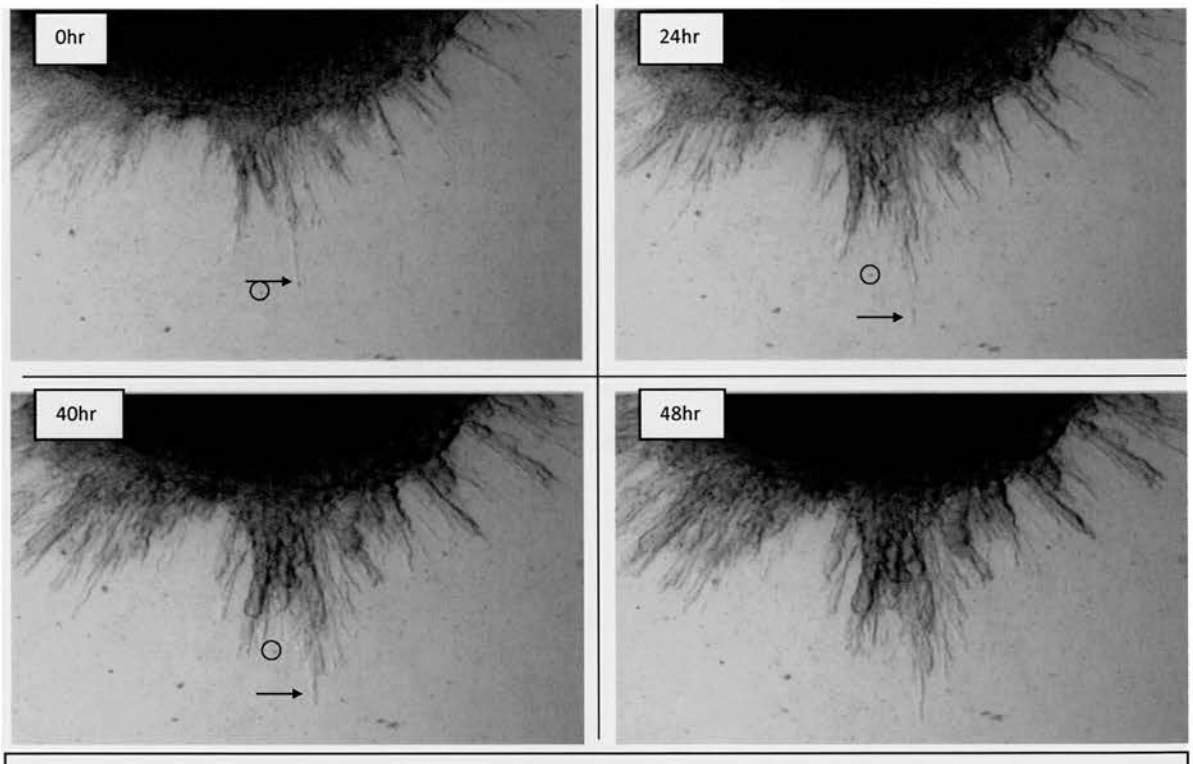


Fig 5.9 Snapshots of cell line behaviour over time in viewing chamber. 200,000 Sum159PT cells were mixed with 200ml Collagen Type I in a 96 well plate. The cell plugs were left to gel for 24hr and subsequently embedded into the centre of a 1ml of Collagen Type I cushion in a 24 well plate. At day 7 the assay was subjected to 48hr of extended depth of focus time delay macroscopy.

When compared to inert debris in the collagen (***circle***), single cells (***arrow***) are observed detaching from structural outgrowths, invading in to the surrounding matrix and then returning and reintegrating with invading structures.

5.4 Modes of invasion in cell lines and clinical samples

To uncover the mode by which SUM159PT invaded, we set about modifying the assay further to permit direct surveillance of the gel over an extended time period. Viewing chambers were designed by developing glass bottom inserts suitable for six well plates [Fig 5.8]. These held individual assays in a fixed position within a well by sandwiching an assay between the insert and bottom of each well. Small apertures in the top of the inserts allowed media to be changed over the time course and free gas and temperature exchange with the surrounding incubator, thereby maintaining homeostasis.

Cell line assays were observed over a 48 hr time course from day 7 by which time invasion from the cell plug had been established. Direct observation of invasive behaviour using the macroscope revealed the interaction between cells of mesenchymal and epithelial phenotype. The mesenchymal cells appeared to originate from invading epithelial structures, invade nearby collagen before returning to and reintegrating with the epithelial component. The epithelial component subsequently continued to invade in a direction previously explored by single cells [Fig 5.9].

The significance of this mode of invasion was unclear, as no direct evidence of this mechanism had been noted in cultures derived from primary breast biopsy material. Therefore time delay experiments were set up using primary tissue and images captured over 60hr from day 8. A similar interplay between individual mesenchymal cells and epithelial structures was evident [Fig 5.10].

The SUM159PT cell plugs demonstrated a similar pattern of invasion as primary breast biopsies, with an initial wave of predominantly mesenchymal single cells followed by epithelial looking structures invading into the surrounding collagen. Previously it had been assumed that in the primary biopsy assays the mesenchymal single cells were fibroblasts. However, to have a similar pattern of invasion in cell line based assays indicated that either the cell line itself was contaminated with fibroblasts, or the single cells were in fact of epithelial origin, but demonstrating mesenchymal characteristics.

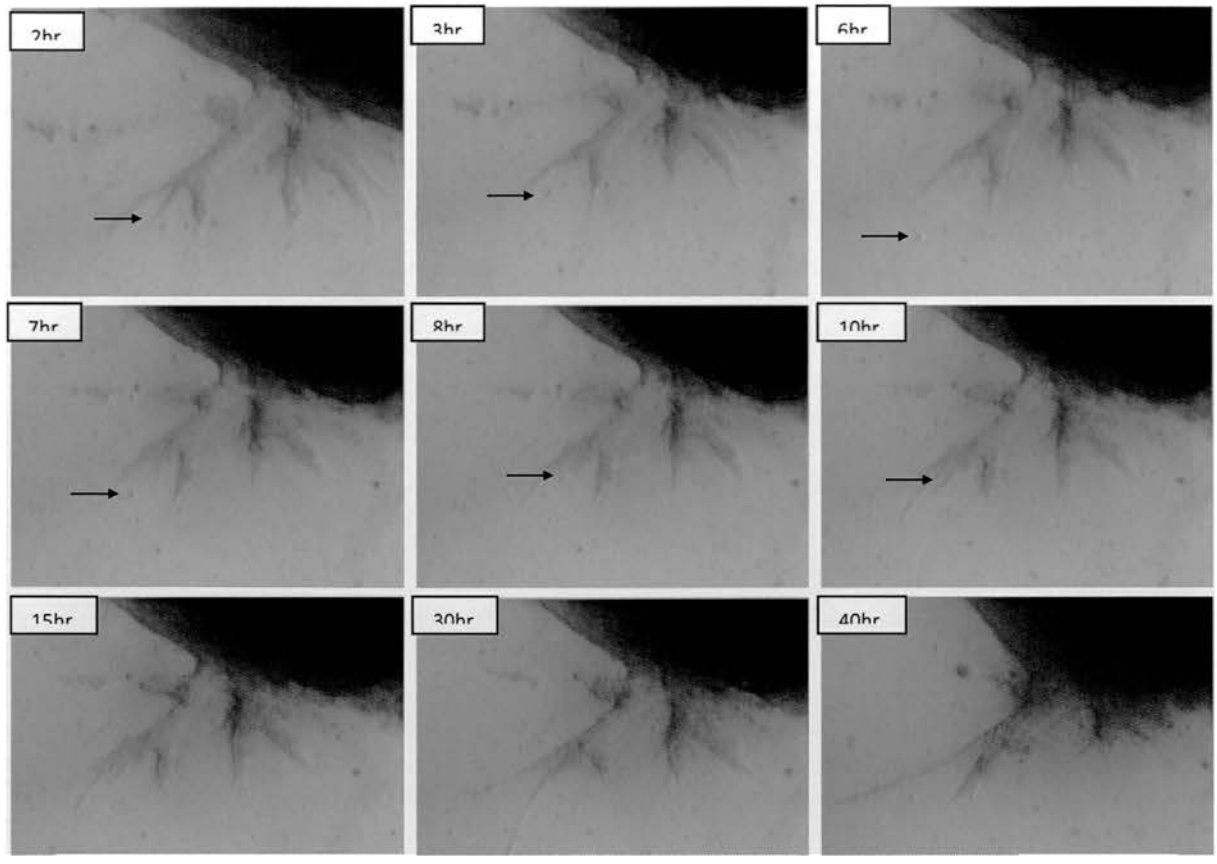


Fig 5.10 Snapshots of primary tumour behaviour over time in viewing chamber. In order to compare cell plug and primary biopsy behaviour, an ER+ primary tumour sample was used for imaging from day 8 using the same experimental set up as the cell plugs over a 60hr period. Again, cells of mesenchymal phenotype were observed originating from invading structures, exploring the surrounding collagen before returning and fusing with the invasive front (***arrows***).

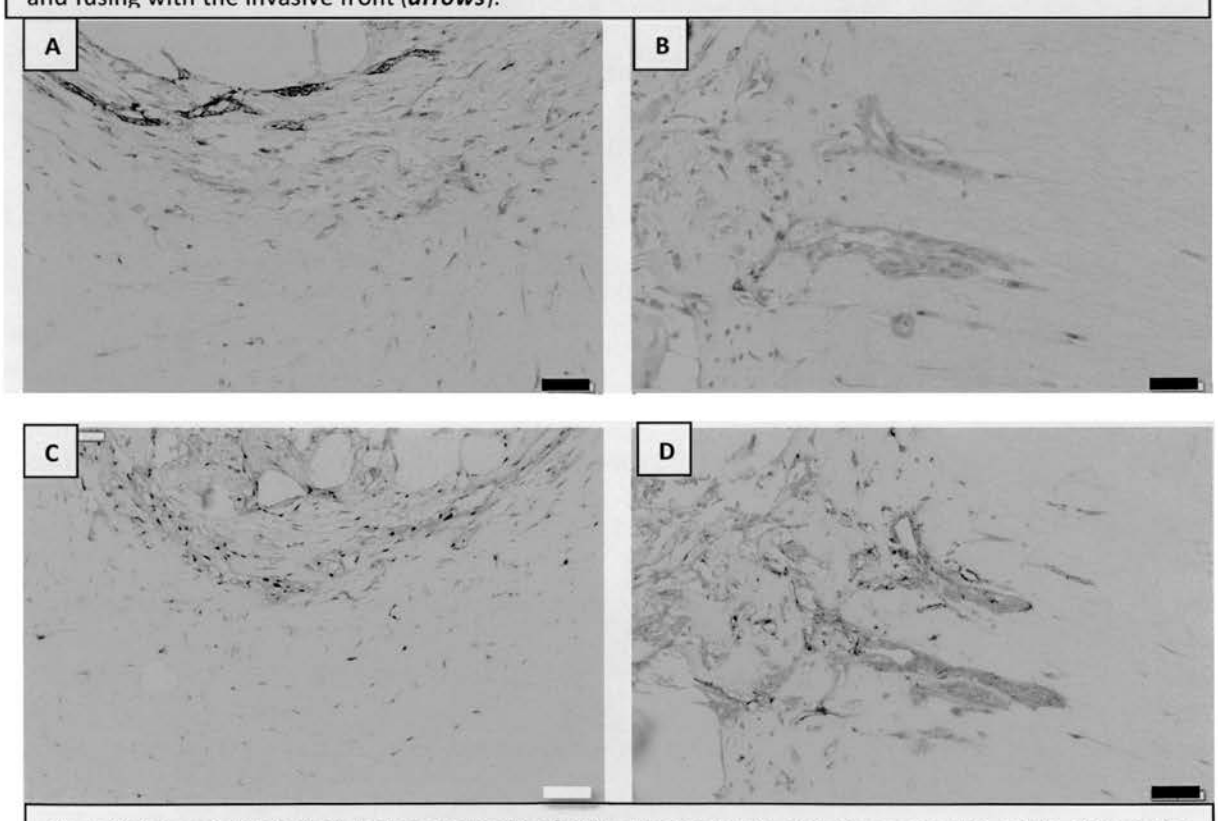


Fig 5.11 Immunohistochemical assessment of primary assays. In order to assess the origin of invading structures expression levels of the luminal cytokeratin 8/18 was assessed. Expression was evident in both invading structures and single cells [A+B]. These cells also expressed [C] MMP15 and [D] MMP-MT1. White bar 100μm, Black bar 50μm.

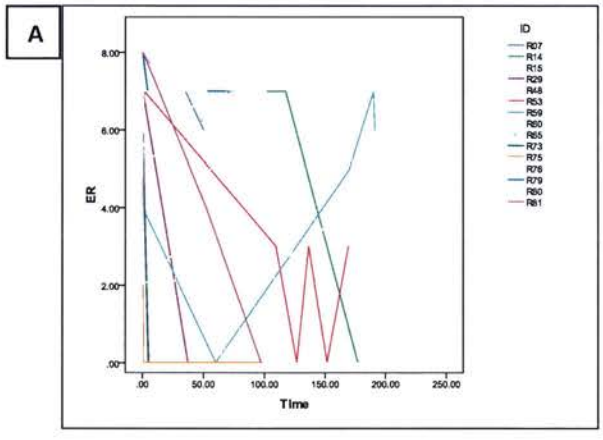
To further investigate the origins of both individual and invading groups of cells in the assay, immunohistochemistry was employed to elucidate whether these cells expressed cytokeratins of a luminal lineage. Both individually and collectively invading cells demonstrated positive expression of luminal markers CK 8/18 [Fig 5.11A+B], indicating that both the cells of mesenchymal and epithelial phenotype were of luminal epithelial origin as opposed to fibroblasts derived from the mesoderm. Levels of matrix metalloproteinases (MMP), a family of peptidase enzymes responsible for the degradation of extracellular matrix components, including collagen, gelatin, fibronectin, laminin and proteoglycan were also investigated. MMP-15 and MT1-MMP were positively expressed in both single and collectively invading epithelia [Fig 5.11C+D], providing a mechanism by which expressing cells may invade through the dense fibrillar mesh of type 1 collagen. Additionally levels of fibronectin within the collagen matrix appeared to increase with time indicating increased secretion by invading cells which may exert a protective effect from collagen induced apoptosis.

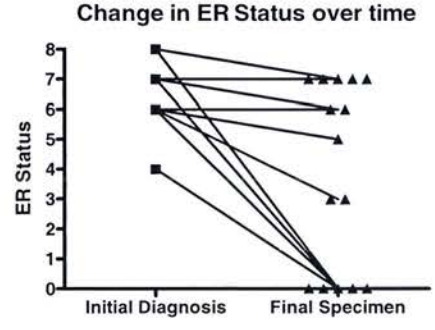
Taken together these experiments seemed to suggest that EGF appeared to have a significant role in transforming ER+ breast cancer cells in to a basal phenotype, with upregulation of EGFR expression and reduction in ER expression. Despite retaining luminal cellular markers these cells demonstrated increased invasive activity through both single cell and collective invasion and had the ability to shift between epithelial and mesenchymal phenotypes.

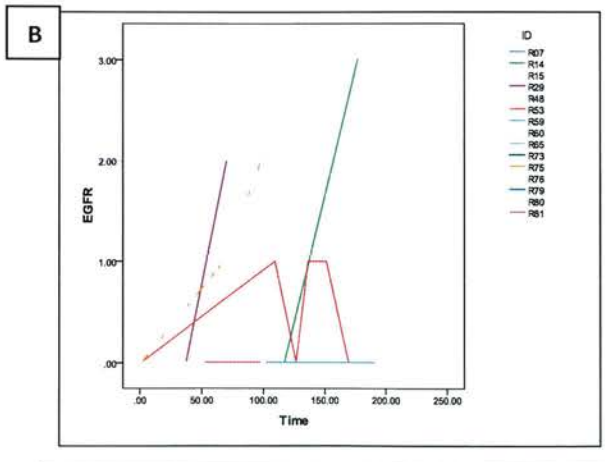
Table 1: Patient demographics and histopathology

(Blue = ER+ retained, Yellow = Developed EGFR expression, Orange = Transformed to ER-/EGFR-)

Patient ID	Outcome	Age	Neoadjuvant Therapy	Diagnosis to death /f.u. (months)	No. of resections	ER Status on original core	ER Status on final resection	EGFR Status on original core	EGFR Status on final resection
R07	Died	71	Tamoxifen	71.00	2	7	6	0	0
R14	Died	85	Tamoxifen	186.00	2	7	0	0	3
R15	Died	75	Tamoxifen	284.00	3	6	5	0	1
R29	Died	94	Letrozole	78.00	2	7	0	0	2
R48	Alive	62	Nil	104.00	2	8	7	0	0
R53	Died	69	Nil	205.00	6	6	3	0	0
R59	Alive	60	Nil	245.00	5	6	6	0	0
R60	Died	80	Nil	147.00	4	8	7	0	0
R65	Alive	90	Tamoxifen	131.00	3	8	7	0	0
R73	Died	80	Letrozole	29.00	2	6	0	0	0
R75	Alive	50	Nil	132.00	3	4	0	0	2
R76	Died	62	Nil	54.00	2	6	3	0	2
R79	Alive	66	Letrozole	113.00	4	8	7	0	0
R80	Alive	64	Nil	87.00	2	7	7	0	0
R81	Alive	55	Nil	129.00	4	8	0	0	0







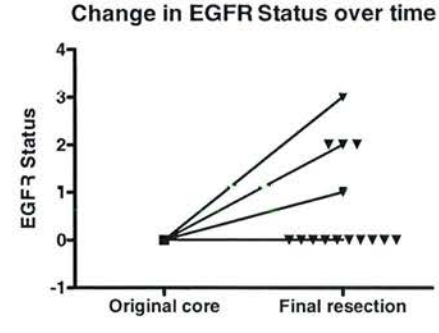


Fig 5.12 Dynamic and absolute changes in ER and EGFR status over time. [A] ER levels demonstrated a decreasing trend in expression with a significant reduction in expression levels between initial and final resection specimens (p=0.001 paired T-test). [B] EGFR levels were significantly higher at final resection (p=0.027 paired T-test).

5.5 Impact of increasing EGFR levels on Overall Survival in the Clinical Setting

We hypothesised that expression of EGFR may provide a key step in the development of hormone therapy resistance in ER+ tumours and provide a mechanism of invasion to distant sites, leading to metastasis and patient death.

To explore this further, we set about identifying patients from the Edinburgh Breast Unit who had undergone curative resection and received adjuvant hormone therapy for ER+ breast cancer but subsequently developed at least one local recurrence which had undergone resection. Using their paraffin embedded resection specimens, we aimed to quantify changes in ER and EGFR expression over time. We hypothesised that development of hormone resistance would be linked to a decrease in ER expression and an increase in EGFR expression. Furthermore we hypothesised that higher levels of EGFR expression would lead to reduced overall survival secondary to disseminated disease.

A total of fifteen patients were identified from the LETMA series [129] with subsequent recurrence in the ipsilateral breast despite curative resection and adjuvant therapy, see Table 1. The median number of resections per patient was 3 (mean 3, range of 2-6 resections). The median Allred score of ER status (0-8 scale) at initial resection was 7, (mean 6.8, range 8-4). The median Allred score on most recent resection was 5 (mean 3.9, range of 7-0) [Fig 5.12A]. This represents a statistically significant reduction in ER status on paired T-test (P-Value = 0.001, 95% CI: 1.389, 4.478). EGFR status (0-3 scale) was 0 for all initial resection specimens, but on final resection the mean EGFR status was 0.667 (median 0, range 0-3) [Fig 5.12B], a statistically significant difference on paired T-test (P-Value = 0.027, 95% CI: 0.087,1.246).

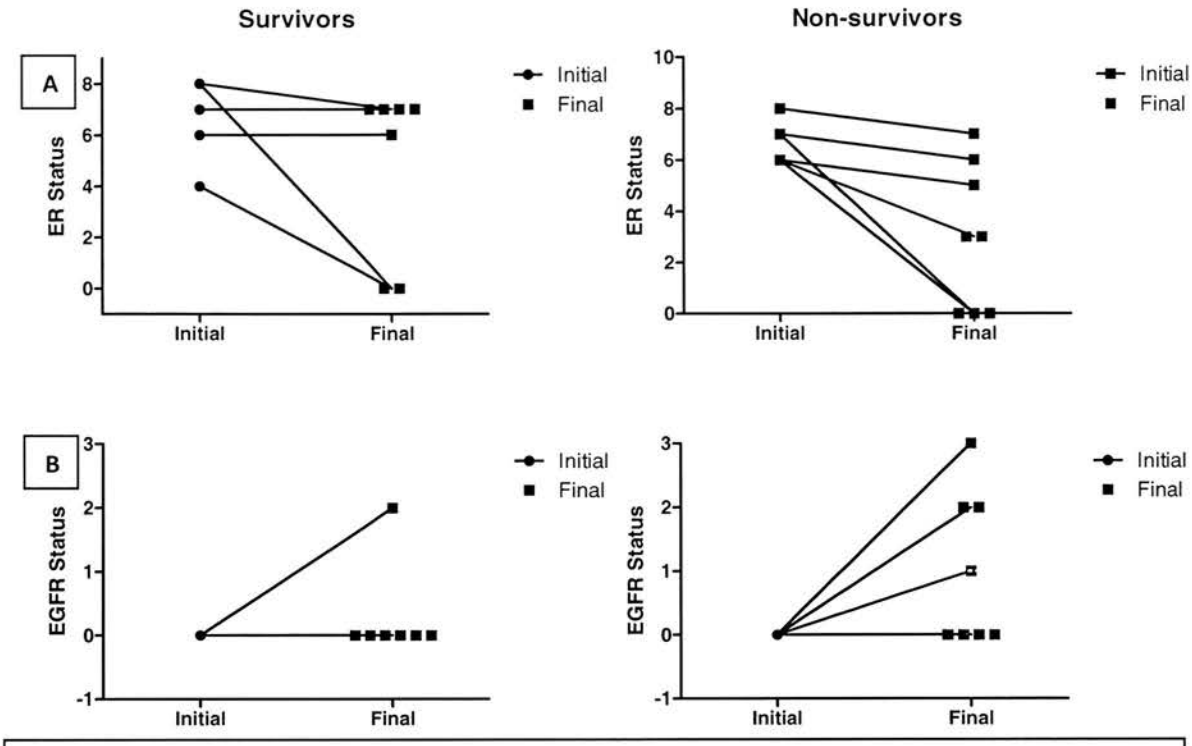


Fig 5.13 Changes in ER and EGFR status in Survivors and Non-survivors. [A] No significant difference in ER status between survivor group and non-survivor group (p= 0.273 T-test). [B] No significant difference in EGFR status at final resection between survivor group and non-survivor group (p= 0.189 T-test).

We hypothesised that local recurrence in itself would not impact on overall survival; only if the disease were to develop the ability to metastasize (through expression of EGFR) would survival be reduced. Therefore we hypothesised that there would be higher levels of EGFR expression in those that had not survived versus those that had. A total of 8 patients had died (non-survivor group), half of whom had expression of EGFR at final resection. Their mean EGFR status was 1 (median 0.5, range 0-3). By comparison, of the 7 patients who had survived (survivor group), one had demonstrated EGFR expression at time of final resection. Their mean EGFR status was 0.29 (median 0, range 0-2) [Fig 5.13]. Numbers in each group were too small to demonstrate a statistically significant difference on 2-group T-test (P-Value = 0.189, 95% CI: -0.408, 1.837).

In summary, insufficient resection specimens were identified to provide sufficient data to perform meaningful survival analysis. However we were able to conclude that for recurrence following hormone therapy to occur neither up-regulation of EGFR expression nor ER independence were essential. However, both statistically significant increases in EGFR expression and reductions in ER status were seen in the recurrent cancers when compared with their initial resection.

5.6 Conclusion

The results laid out in this chapter appear to implicate EGF as a factor in luminal cell conversion from an ER+ to a basal-like phenotype with concommitent upregulation of invasive behaviour. Further experiments using cell lines confirmed that in cells with a basal-like phenotype a similar mode of invasion may be induced. Furthermore, in cell lines lacking an intact EGFR signalling pathway, there was no evidence of invading epithelium. Further analysis of both cell line and primary biopsy assay behaviour demonstrated the plasticity of epithelial cells in converting to and from mesenchymal states during their invasive phase. This may provide direct evidence implicating the role of EMT and MET in cancer progression, with EGFR expression as a major driver.

These experiments implicated EGFR as a causative agent in inducing ER+ cancer to develop invasive characteristics and metastasize. In order to ascertain the clinical relevance of these laboratory based observations, a correlation between EGFR expression, ER down-regulation and invasive behaviour in

clinical resection specimens was sought. Statistically significant reductions in ER expression along with significant increases in EGFR were found over time, however sample size was too small to find statistically significant reduced overall survival in the EGFR positive group.

In conclusion, further interrogation of this 3D model has provided new insight into a possible role of EGFR as a driver of EMT and metastasis. Not only may this help explain why patients with EGFR positive disease fare worse, but it also offers a new line of enquiry regarding the use of EGFR inhibitors in ER+ disease. Indeed the use of prophylactic EGFR inhibitors in ER+ disease may theoretically reduce the risk of tamoxifen treated patients developing hormone resistance and distant visceral metastasis.

Chapter 6: Discussion

6.1 Rationale for Development of 3D assay

Culturing primary tumour explants to determine an individual's sensitivity to treatment is not a new concept[130]. However, the instability of primary tissue in culture over an extended time period[131] and the lack of adequate means to quantify response to treatment[132] have frustrated attempts to develop these assays into a useful clinical tool.

We set out to adapt three-dimensional culture techniques in order to develop a predictive model of *de novo* tumour resistance to drug therapy. Developing a culture model to study cancer behaviour through combination of a three-dimensional assay and primary tumour material would have many advantages. From a practical point of view, placing freshly harvested biopsy material in a matrix that partially recapitulates the donor environment minimises cellular disruption and may result in prolonged sample survival. Additionally, by using intact biopsy material, the complex interaction between the heterogeneous mix of epithelial and mesenchymal cells is preserved. This stromal context, recognised as an essential component of tumourigenesis and propagation[133], is absent in monoclonal cell line cultures.

During development of the culture model we investigated a number of factors that may influence assay outcome. The role of collagen concentration in promoting cancer invasion remains controversial. In the clinical setting, increased breast tissue density is associated with more aggressive tumours, associated DCIS, lymphatic and vascular invasion and a greater risk of developing a second breast carcinoma[134]. However increasing concentrations of collagen in the *in vitro* setting may have variable effects, with some authors describing an increase in invasive behaviour[121], whilst other researchers describe an optimal level at which to use collagen dependent on cell line used, with reduced invasiveness beyond this concentration [135]. Following

our experiments, we concluded that initial collagen concentration did not have a significant impact on invasive behaviour.

Over the experimental time course gel contraction was observed, thus increasing fibrillar collagen density. This increasing density over time may result in a self-perpetuating effect on invasive behaviour. These dynamic changes in collagen density secondary to contraction are also seen in the clinical setting, manifested as shortening of the ligaments of Cooper leading to dimpling and skin tethering[136]. This may be mediated through LOX expression, an amine oxidase that initiates intermolecular crosslinking of collagen, often overexpressed in tumours [137]. Further studies have demonstrated that LOX levels are raised in the peritumoural stroma with increased levels of crosslinking resulting in Integrin dependant PI3K activation and increased invasiveness of cells[138]. Similar dynamic changes in the stromal environment of this assay may improve the authenticity of tumour behaviour.

The role of fibroblasts in cancer progression has long been recognised[139]. In both *in vitro* cell co-cultures[140] and xenograft studies[141] cancer associated fibroblasts appear to play an essential role in driving invasion and metastasis, through growth factor signalling and remodelling of the stroma[142]. Activation of pathways associated with the hostile microenvironment allows fibroblasts to facilitate epithelial cells to escape in to surrounding stroma and disseminate systemically [143]. Previously described invasion assays require addition of fibroblasts to induce invasion of epithelial cells [122, 144]. In contrast, during development of our culture model, it was found that additional fibroblasts did not increase epithelial invasiveness. Instead there was an increased risk of contaminating the assay with additional epithelium. These experiments highlighted a number of points worth considering. Firstly, by placing intact primary breast tissue into culture, this provides both the epithelium of interest but also a synchronised stromal support network. Secondly, current fibroblast isolation techniques are suboptimal as cells with alternative lineage may be propagated concurrently. This fact should be borne in mind when interpreting the results of experiments

assessing epithelial penetration of collagen, in which "fibroblast" cultures have already been mixed throughout. Thirdly it highlights the plasticity of cultured epithelial cells and their adaptability to environment. Whilst grown on plastic and following multiple passages, our fibroblast cultures demonstrated only cells of mesenchymal phenotype. However, once placed in 3D collagen we found that a subset of our "fibroblasts" reverted to an epithelial morphology. This observation is in keeping with the concept that neoplastic epithelium may transdifferentiate between mesenchymal and epithelial states in order to optimise survival according to the surrounding environment.

The plasticity of epithelium was further demonstrated at histopathological analysis of the assay at termination of experiment. A variety of migratory modes could be identified at the core/gel interface, from mesenchymal-like single cell invasion to multicellular chain and collective migration as categorised by Friedl et al.[145]. Despite the range of invasive behaviour seen in our assay, immunostaining demonstrated the invading cells continued to express markers of epithelial origin and to a degree retained their original hormone status, although this appeared to be increasingly downregulated at the invasive edge.

As a result of the heterogeneous patterns of invasion, a single cross-section through the assay would not provide sufficient sample data to provide a macroscopic overview of culture model morphology. Instead, using a non-destructive optical microscopy technique designed to image chick embryos, a staining protocol was adapted to allow for 3D reconstructions of the assay to be acquired. The development of this three dimensional imaging technique alongside two dimensional immunostaining would allow for an holistic overview of culture model behaviour at a cellular and tissue level to be ascertained.

In summary, the three-dimensional culture system developed maintains primary tumours' heterogeneous cell mix for an extended period of time and promotes tumoural proliferation and invasion. Furthermore, a range of imaging techniques were adapted to provide a robust method of investigating cell behaviour and provide an overall impression of assay morphology.

6.2 Determining tamoxifen sensitivity of primary breast cancer tissue in culture

Despite tamoxifen being the most established targeted therapy for ER+ breast cancer, there continues to be uncertainty as to who will derive benefit from treatment.

In the UK, 46,000 people are diagnosed with breast cancer every year [146], with 70% of invasive breast cancers demonstrating oestrogen receptor positivity [147]. In these patients targeted anti-hormone therapy is the cornerstone of treatment, with tamoxifen prescribed for both pre- and post-menopausal women and aromatase inhibitors (AI) used for many post-menopausal women [148]. In an epidemiological study in New Zealand, tamoxifen was prescribed to 83% patients with ER+ invasive breast cancer [149]. Recurrence rates are reduced by 31% in patients treated for five years[104], due to the tumouricidal properties of oestrogen antagonism.

However it is well recognised that not all ER+ breast cancers are sensitive to hormone deprivation. Neoadjuvant trials have provided an opportunity to gauge innate resistance of primary breast cancers to anti-hormone therapy. At the Edinburgh Breast Unit, patients treated with neoadjuvant tamoxifen for three months demonstrated a range of response. A reduction in tumour volume >25% was seen in only 67% participants[150].

Moreover, the side-effect profile of tamoxifen is not insignificant. With over 30,000 patients a year eligible to commence on anti-hormone therapy, treatment is considered to be of low toxicity and well tolerated in most women. However, adverse events range from the minor (hot flushes, vaginal discharge and irregular menses) to major life-threatening events (relative risk of endometrial cancer 2.70, stroke 1.49, pulmonary emboli 1.88)[151].

Identification of ER+ patients with tamoxifen insensitivity may allow alternative endocrine therapies to be considered, such as goserelin [152] or fulvestrant [153]. This may reduce the risk of recurrence and limiting adverse events from ineffective treatment. Utilising a predictive biomarker in addition to ER status could improve the specificity of targeted tamoxifen treatment.

Indeed, improved methods of quantitatively predicting hormone resistance in breast cancer are in development: Genomic profiling scores are available within the clinical setting to stratify a patient's risk of recurrence [154]. Unfortunately the associated costs remain prohibitive for the majority of healthcare providers in both developed and developing countries [155]. Other genomic indices which specifically gauge sensitivity to endocrine therapy remain at a pre-clinical stage [156]. It should also be recognised that development of such risk profile scores requires retrospective follow-up analysis of large cohorts of patients over many years. Therefore predictive genomic scores will not be available for a drug until it has been established for an extended period of time in a large population cohort.

Instead, pre-clinical evaluation of therapeutics for antitumoural efficacy has been provided, in part, by cell-based screening strategies such as NCI60[157]. These assays, which may contain fewer than ten cell lines to represent each cancer type, provide a method of quantifying the efficacy of non-specific cytotoxic therapies against a broad spectrum of cancers. However, drug discovery has evolved, with the development of targeted therapeutics often efficacious in only a proportion of cancer subtypes [158]. As a result, small panels of cell lines may be of limited utility in providing pre-clinical data as to the efficacy for these novel drugs. Furthermore these cell lines do not account for *de novo* resistance that may occur as a result of stromal-epithelial cross-talk[159], which may only be detectable in screening systems where the associated tumour microenvironment remains intact.

In order to develop this culture model as a method of screening drugs to detect individual sensitivity, we exposed samples to a drug of presumed efficacy and investigated its effect. Tumour response to treatment is a complex three-dimensional phenomenon, and the use of two-dimensional quantification techniques is frequently inadequate for its full evaluation [160]. Therefore we set about developing and appraising both 2D and 3D quantification techniques to identify an optimal method of assessing tumour response to treatment.

Initial results for patterns of proliferation and apoptosis following tamoxifen exposure demonstrated a wide variation in response. Previously published neoadjuvant studies have also reported a range of individual proliferative response to tamoxifen treatment. In one neoadjuvant study 14% of patients with ER+ breast cancer had an increase in proliferation as measured by Ki67 expression following tamoxifen exposure[161]. However, in contrast to our *ex vivo* culture experiments they found that tamoxifen treatment led to a statistically significant repression in Ki67 levels overall in the treatment groups. In the same study, 2 weeks of exposure to tamoxifen also led to a 14.8% reduction in levels of apoptosis [161]. From this they concluded that oestrogen is not essential as a survival factor for breast cancer cells. Instead, tamoxifen's efficacy is through antagonism of oestrogen leading to a suppression of proliferation.

In keeping with their conclusion, three dimensional analysis of tumour volume in our assay demonstrated that tamoxifen was not in itself cytocidal but did inhibit the tumour's ability to expand. Overall in our study ER+ tumours exposed to tamoxifen had a significant reduction in invading tumour volume when compared to their matched controls. In contrast, ER- tumours, which are not reliant on oestrogen dependant proliferation pathways, did not have a significant reduction in volume following tamoxifen exposure.

Our overall goal was to identify a method of quantifying individual patient sensitivity to anti-hormone treatment. Initially two dimensional analyses of proliferation and apoptosis were assessed. However, the range of response to tamoxifen we identified within a small group of presumed responders indicated that 2D quantification would not provide a suitable biomarker to predict individual response. Indeed these findings mirror the conclusion made by Mitch Dowsett at the Fifth International Conference on Recent Advances and Future Directions in Endocrine Manipulation of Breast Cancer[162]. He acknowledged that whilst Ki67 may provide a method of screening new agents against a large study group to identify potential efficacy, it would not provide a method of identifying individual sensitivity.

To this end, the range of proliferative changes in response to tamoxifen treatment to a group presumed to be sensitive to the drug were sufficiently varied to indicate that its measurement would not provide a robust method of quantifying tamoxifen sensitivity. This may be secondary to sampling errors associated with obtaining 2D sections from an asymmetrical 3D object. Alternatively, a single snapshot of proliferation and apoptosis in this model may not adequately capture the dynamic response of a tumour to tamoxifen.

Therefore, we next set out to both reduce sampling error and also use a clinically validated method of assessing individual sensitivity [163, 164] by quantifying changes in the tumour volume following exposure to treatment. Changes in tumour volume were calculated using a novel approach not previously published, through obtaining 3D reconstructions of tumour samples using OPT and quantifying volumes using a 3D software package. We concluded that quantifying relative reductions in 3D tumour volume was a consistent method of measuring response to tamoxifen treatment. All samples in the ER+ group demonstrated a variable reduction in volume, with 70% (7/10) demonstrating a significant reduction. As expected, preparations derived from tumours with low or negative ER status showed no significant changes in tumour volume [165, 166]. Furthermore, the lack of significant correlation between Ki67 change and volume change following tamoxifen exposure noted in our study was also noted in the ATAC trial [161].

Using the level of reduction in tumour volume as a biomarker, tumour sensitivity could be stratified using a standard statistical method (ANOVA). Similar preparations and their analysis could be applied in the future in the clinical setting for tamoxifen as well as other drugs.

In order to validate this model in the clinical setting a neoadjuvant study is required with biopsy material taken at the time of diagnosis. Assays would be created and cultured in the same treatment as given to the patient, with response in tumour volume compared between the patient and the assay to identify correlative changes.

6.3 Factors affecting tumour cell migration and proliferation

Three-dimensional models have been used extensively to shed light on a number of facets of oncogenesis. Their development has led to a greater understanding of epithelial organisation and polarity, dynamic reciprocity between epithelium and ECM, mechanisms of invasion and potential therapeutic targets [167-170].

Developing a knowledge base about the dynamic nature of cancer has important clinical implications: Currently treatment of locally advanced breast cancer is based on the pathological staging and IHC of the original tumour, with the assumption that this is representative of metastatic disease. However, a research study at the Edinburgh Breast Unit analysing locally advance ER+ tumours alongside paired lymph nodes identified that in 32% of patients nodal metastases had converted to an ER- phenotype [171]. Similar discordance was seen between primary samples and paired nodes in a separate Scandinavian study that also noted overall survival in ER+ patients with ER- nodal metastases was similar to patients with ER- primary cancer[172]. Currently, nodal or metastatic disease is not routinely assessed using IHC.

We therefore set out to identify dynamic changes within the *ex vivo* model which may provide insight into the mechanisms that drive both transformation in tumour phenotype and invasive behaviour alongside the effect on clinical outcome.

Initially, quantitative analysis of expression profiles over time was performed. These showed fluctuating levels of proliferation but no net increase and little apoptosis. A trend in reduction of ER and HER2 expression was also observed in keeping with a conversion from oestrogen receptor positivity to a triple negative phenotype. Transformation corresponded with an increase in invasive behaviour, both of which appeared dependent on exposure to EGF within the culture media and was associated with an increase in EGFR expression at the cell membrane.

The role of EGFR has previously been described both in promoting invasive behaviour *in vitro* with cell culture [173] and also as a physiological mechanism to alter cell adhesion and mobility *in vivo* [174]. Furthermore the inverse correlation between EGFR expression and ER status is also well recognised [175]. As discussed in the introduction, the theory of basal-like breast cancers arising within the myoepithelium whilst ER+ cancers develop from the luminal epithelium is unlikely. Instead, it may be hypothesised that all cancers arise from epithelium within the TDLU, but phenotype differs dependent on alternative signalling pathways for survival[80].

Previously published work using EGFR transfected MCF-7 cells found these cells to be capable of increasing EGFR expression upon oestrogen withdrawal, thus conferring a growth advantage when placed in restrictive growth conditions. These experiments suggested a role for overexpression of EGFR in the growth of breast cancer cells of ER+ origin in the absence of oestrogen [176].

Experiments in this thesis may provide further evidence as to the role for EGFR signalling, providing an alternative survival pathway for ER+ breast cancer which may result in loss of ER expression.

We found that EGFR expression and increased invasive behaviour in cell lines placed in 3D collagen may correlate. Interestingly despite being a monoclonal cell line, SUM159-PT cells were found to show a range of invasive morphology. In-depth macroscopic surveillance of this cell line provided evidence of a continuum of invasive behaviour from single mesenchymal cells through to large epithelial structural invasion, with close interaction between cells using differing modes of invasion being observed. Subsequent observation of primary breast tissue identified similar patterns of behaviour in the *ex vivo* samples. Immunostaining confirmed these cells were of an epithelial origin and also expressed peptidases indicating the method by which they penetrated the collagen ECM. These findings provide strong evidence that the theory of Epithelial to Mesenchymal Transition, frequently witnessed in cell culture but disputed in clinical pathology specimens, may be induced and observed in primary breast cancer.

These observations led us to extrapolate our hypothesis; that cellular expression of EGFR is a key step in the process of a primary breast tumour developing the ability to metastasize. Recurrent breast cancer with resistance to hormone therapy would therefore reduce overall survival only if it developed the ability to metastasize; manifested through EGFR expression. We went on to assess multiple pathology specimens of patients who had developed recurrence following hormone therapy for ER+ breast cancer. Overall survival in patients who went on to express EGFR was only 17% compared with 66% of patients who never expressed EGFR. However, the patient cohort size was too small and follow-up time too short to demonstrate statistical significance.

To date, a wide range of molecular-targeted agents against EGFR, consisting of small molecule EGFR inhibitors (TKIs) and anti-EGFR monoclonal antibodies (MAbs), have been developed. A number of these EGFR inhibitors have been evaluated in breast cancer treatment trials but results have been inconclusive [177]. Mainly carried out in patients with metastatic breast cancer, these phase 1 and 2 trials have used overall survival as the primary endpoint to gauge treatment success, with disappointing results [178-180].

However, efficacy has been identified in patients with non-metastatic breast cancer[181]. Furthermore, there is increasing evidence that EGFR inhibitors may be effective in reversing epithelial to mesenchymal transition. Inflammatory breast cancer (IBC), recognised as the most aggressive breast cancer subtype, has a disproportionately high rate of EGFR expression. Histologically, IBC tumour cells are usually high grade, loosely clustered together with single cells visible within the stroma. IBC may infiltrate breast parenchyma diffusely and lymphovascular tumour emboli are common[182]. *In vitro* studies using IBC cell lines found Erlotinib inhibited cell motility and invasiveness. The mesenchymal phenotype of IBC cells was reversed to epithelial and inhibited spontaneous lung metastasis, even at a low dose that had no significant effect on primary tumour growth[183]. A phase II trial assessing the efficacy of Panitumumab, alongside Nab-paclitaxel and Carboplatin for non-metastatic HER2 negative Inflammatory Breast Cancer is now underway

(NCT01036087). From the experiments performed in this thesis we postulate that the primary efficacy of EGFR inhibitors will not be identified within patients where disease has already spread, but may provide prophylaxis against metastasis in those with early breast cancer.

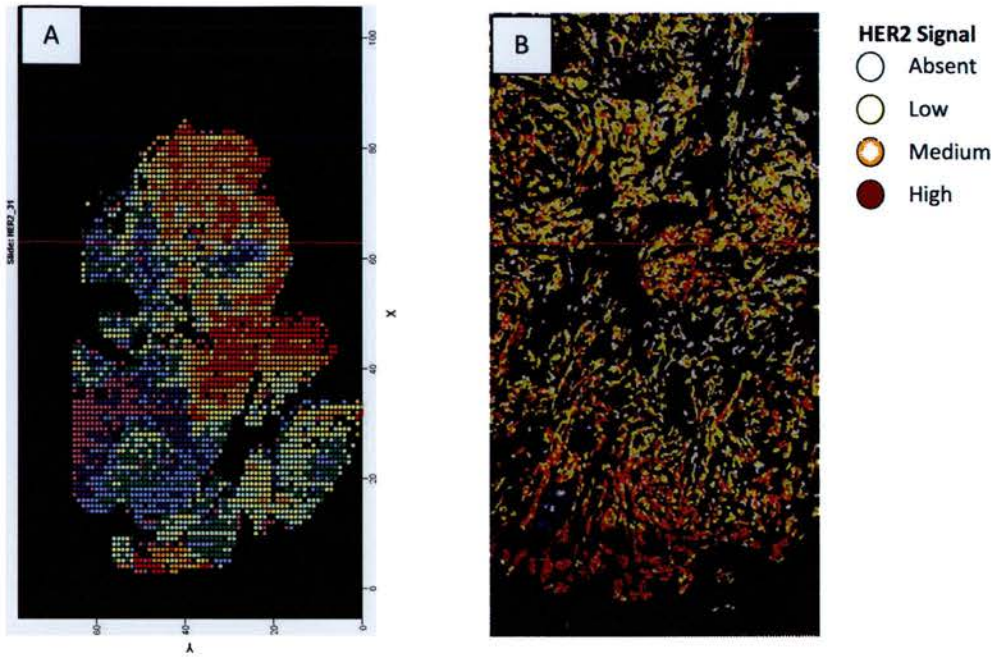


Fig 6.1 [A] Mapping of HER-2 expression in ovarian cancer resection specimens demonstrates the degree of heterogeneity found using cross–sectional analysis. Purple (low) -> Red (high).

[B] Similar investigation of HER-2 mapping in primary breast cancer specimens indicate that higher expression of HER-2 appeared at the leading edge in some specimens

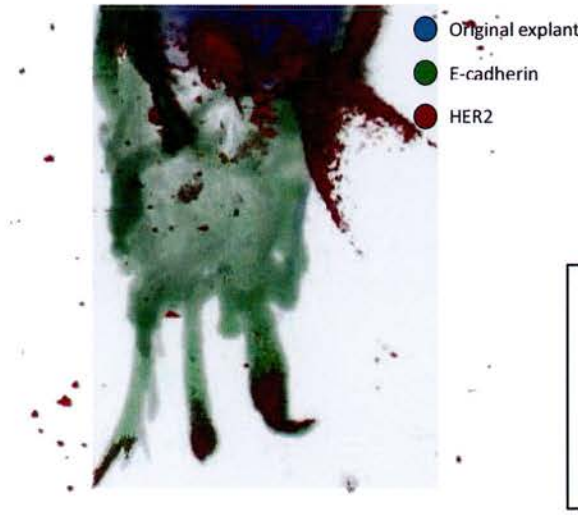


Fig 6.2 Double staining of invasion assays using E-cadherin and HER-2, appeared to show increased expression of HER2 at the tips of some invading structures.

6.4 Future Directions

6.4.1 Exploring the role of HER2 in driving invasion

The members of the epidermal growth factor receptor family do not function exclusively in isolation but may combine and form heterodimers [177]. Heterodimer formation results in recruitment of specific sets of phosphotyrosine-binding effector proteins at the intracellular domain[184], with multiple homo- and heterodimer combinations within the HER family allowing diversification of intracellular signalling. EGFR-HER2 heterodimerisation has been shown to increase metastatic potential in cell lines [175] and treatment using dual HER1/2 inhibitor lapatinib improves survival rates in HER2+ patients refractory to trastuzumab [185].

Provisional experiments investigating the role of HER2 during the development of our model revealed exciting preliminary results. Using 2D quantification, the heterogeneity of HER2 expression seen within ovarian tumours [186] [Fig 6.1A], appeared to have a directional element with increased HER2 expression found at the leading edge of some breast cancer specimens [Fig 6.1B]. As identifying the leading edge in 2D tumour cross-sections may be misleading, we assessed HER2 expression in our invasion assay using 3D reconstruction techniques. Interestingly, HER2 expression appeared strongest at the tips of some invading structures [Fig 6.2]. Investigating the impact of rTK inhibitors on invasive behaviour and HER expression in this model may provide further information as to the efficacy and mode of action of these treatments.

To this end, further work has been carried out in our model to identify the impact of inhibiting downstream effectors of the HER2 signalling pathway. HER2 overexpression in breast cancer may induce complex formation comprising of HER2, Vav2, Rac1, Pak1, actin and actinin, which is implicated in enhanced cell motility and invasiveness as a result of prolonged Rac1 activation[187]. EHT1864, a water soluble pan-Rac inhibitor, was added to our culture model and its effect on invasion was studied. EHT1864 was found to prevent cellular spread into surrounding collagen,

reduced epithelial proliferation and increased apoptosis. In addition, expression of downstream marker STAT3 was reduced. These experiments may provide evidence for further investigation of Rac inhibition as additional treatment in HER2+ patients [188].

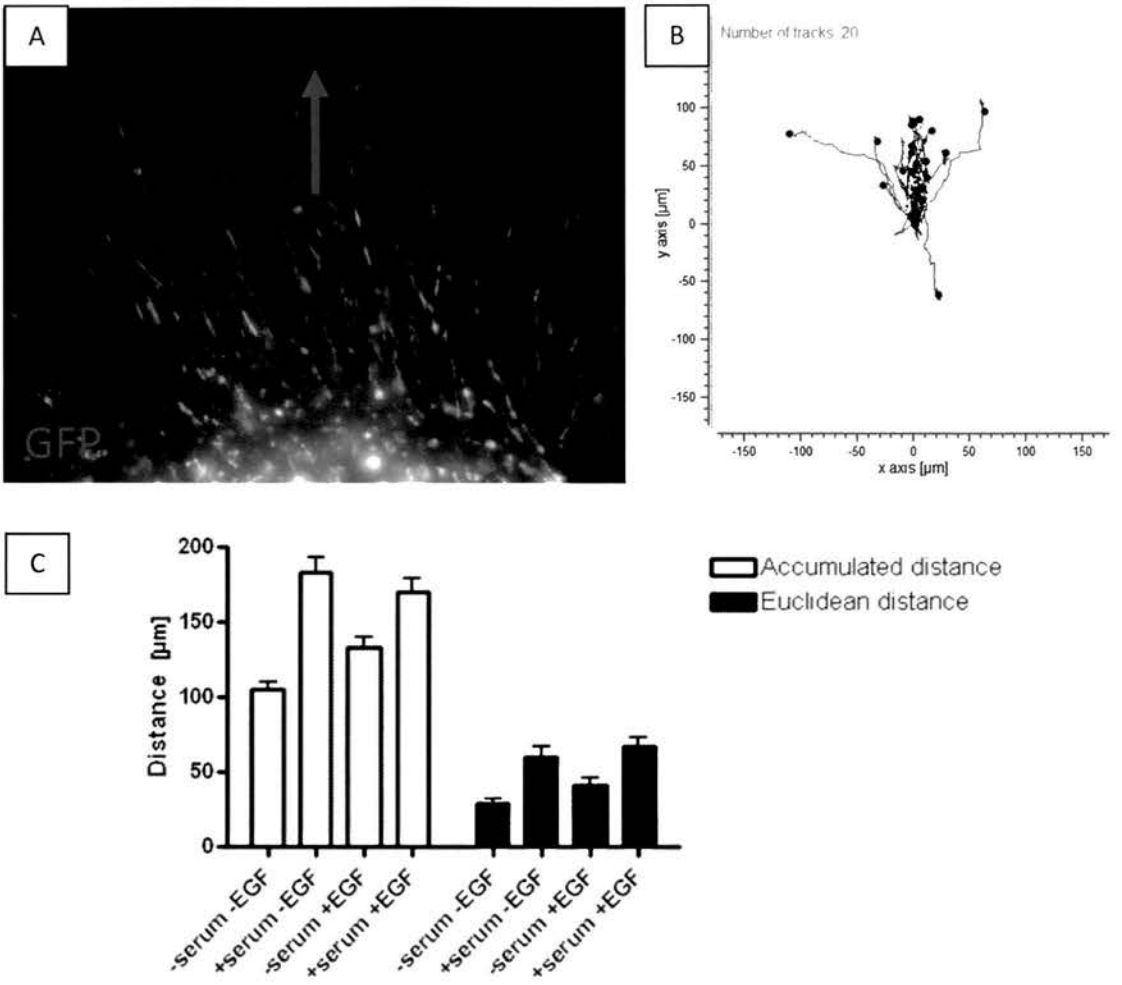


Fig 6.3 [A] Cell lines transfected to express GFP were placed within 3D culture and behaviour over time was analysed using fluoresecence macroscopy. [B] Using 4D tracking software individual cell invasiveness over time was tracked. [C] This allowed for multiple culture variables to be examined to identify factors promoting invasiveness

6.4.2 Improving quantification of invasive behaviour and response to treatment

In order for a breast cancer model to deliver utility, reproducible and objective methods of quantification are required. Whilst we found surveillance of cell behaviour in real time provided important insight into cell behaviour, at the time we had not developed a method of objective analysis. Subsequent work involving GFP transfected cell lines, immunofluorescence macroscopy and 4D cell tracking software is underway and may provide an alternative method of generating objective data on how drug treatment may inhibit invasiveness of cell lines [Fig 6.3]. Affiliated laboratories at the University have attempted to stably transfect whole prostate tissue samples to fluoresce. However, homogeneous transfection of tissue was not achieved due to variable penetration of virus. Further work in this direction is required.

In this thesis response to tamoxifen was determined by measuring tumour volume following exposure. This provided a simplistic method of quantifying sensitivity. However, this technique did not fully exploit the morphological changes available in the collected data. The Centre for Research in Informatics and Systems Pathology (CRISP), based at the University of Abertay have further analysed data presented in this thesis in a collaborative study to calculate the impact of tamoxifen on morphological characteristics other than volume. Using Minkowski functionals, a set of statistical metrics characterising geometry and topology in n-dimensional space, topological characteristics were identified to stratify morphological response of tumours to therapeutic intervention. These advanced morphometric analyses could be developed further in combination with existing range of tumour-response biomarkers [189].

Acknowledgements

First and foremost I would like to acknowledge and thank my supervisors J. Michael Dixon, David J. Harrison and Elad Katz for their guidance.

I thank Joanne Farrell and Sarah E. Wedden for their help using Optical Projection Tomography, Jeremy S. Thomas for his histopathological advice and histo-scoring multiple samples and Linda J. Williams for help with statistics.

I would like to thank Helen Caldwell and other members of the Breakthrough Research Unit for excellent technical support, Lorna Renshaw (WGH Breast Unit) for consenting patients and collecting tumour materials and Paul Perry and Matthew Pearson (MRC-HGU) for help with the Volocity software.

I would also like to thank the Margaret Butters Reekie Charitable Trust and the RCSEd for providing funding to finance the research reported in this thesis.

Declaration

I, Alexander Leeper, confirm the research and data documented in this thesis to be my own. The contributions of other researchers/technical staff are acknowledged. This work has not been submitted for any other degree or professional qualification.

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