Tumour microenvironment interactions in breast cancer and the role of caveolin-1

By

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I am the author of the thesis entitled

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**Abstract**

The progression of breast cancer is driven by the interplay between cancer cells and their microenvironment. The interaction between cancer cells and stromal cells results in stromal cells with a cancer-associated phenotype. Examples of this are the cancer-associated fibroblast (CAF) and the tumour-associated macrophage (TAM), which are important players in the tumour microenvironment. CAFs and TAMs play a role in all stages of tumour progression, from epithelial-to-mesenchymal transition (EMT) and cancer cell proliferation, to metastasis and treatment resistance.

Caveolin-1 (CAV1) is the structural protein of caveolae, 60-80 nm diameter invaginations of the cell membrane. Caveolae play a role in clathrin-independent endocytosis, cholesterol homeostasis, cellular signalling, and mechanosensing.

The role of CAV1 in breast cancer is a complex one. CAV1 has been described as an oncogene, as well as a tumour suppressor in breast cancer cells. The loss of CAV1 expression in the tumour microenvironment of breast cancer patients has been shown to correlate with a reduction in progression-free survival. In addition, CAV1 expression has been proposed as a marker of CAFs, and the downregulation of CAV1 in fibroblasts has been shown to promote tumour progression, *in vivo*. However, conflicting with these findings, other studies have indicated that CAV1 expression in the tumour microenvironment is associated with cancer progression and that low fibroblastic CAV1 levels are suppressive of breast cancer.
The mechanisms behind the regulation of CAV1 expression in cancer-associated fibroblasts are unclear. This study describes fibroblastic CAV1 expression in a cell culture model that includes major cell types in the tumour microenvironment: the cancer cell, the fibroblast and the macrophage.

The non-contact co-culture of primary human breast fibroblasts with human breast cancer cells did not result in significant changes in fibroblastic CAV1 levels. However, the introduction of differentiated monocytes into the co-culture system resulted in a decrease of CAV1 expression by fibroblasts.

Similar to the non-contact co-culture system, the contact co-culture of primary human breast fibroblasts with breast cancer cells did not result in changes in fibroblastic CAV1 expression. Conversely, the contact co-culture of fibroblasts with monocytes decreased fibroblastic CAV1 levels.

The reduction of fibroblastic CAV1 levels after non-contact co-culture suggests that fibroblastic CAV1 expression is regulated by the secretion of a soluble factor. This study measured the levels of several cytokines in the conditioned medium from non-contact and contact co-cultures of fibroblasts, cancer cells and differentiated and undifferentiated monocytes. The secretion profiles of fibroblast and cancer cell co-cultures were markedly different from the secretion profiles of co-cultures of fibroblast with differentiated monocytes. The inclusion of differentiated monocytes in the fibroblast and cancer cell co-cultures resulted in the increased secretion of CAF-associated cytokines. Additionally, co-cultures that contain fibroblasts, cancer cells, and differentiated monocytes resulted in the increased secretion of cytokines that are
known to drive the polarization of macrophages to a tumour-associated macrophage (TAM) phenotype, compared to co-cultures of fibroblasts and differentiated monocytes alone. Several cytokines, some of which have been previously implicated in the regulation of CAV1 expression, were secreted in increased amounts in co-cultures with decreased fibroblastic CAV1 levels. The cytokine profiles generated by this study provide candidates to be investigated further for their ability to regulate CAV1 levels in fibroblasts.

The effect of the decrease of fibroblastic CAV1 levels on their ability to induce EMT in breast cancer cells was investigated using fibroblasts transfected with CAV1 siRNA. The conditioned medium from transfected fibroblasts was used to treat human breast cancer cells. The primary human breast fibroblasts proved resistant to CAV1 knockdown, and a relatively small reduction in CAV1 protein levels was achieved. However, this small reduction in fibroblastic CAV1 protein levels resulted in the secretion of factors in the culture medium that increased vimentin expression, and decreased E-cadherin expression, in human breast cancer cells.

In both the non-contact and contact co-culture systems, high levels of variability in CAV1 expression levels between experimental replicates, and between different batches of fibroblasts were observed. The variability between experimental replicates was reduced by the elimination of foetal bovine serum, a commonly used cell culture additive, from the co-culture system. However, the variability in CAV1 levels between experimental replicates, and between fibroblast batches, found in this study, in addition to the resistance to CAV1 knockdown by siRNA, points to complex regulatory mechanisms underlying CAV1 expression. This, together with the
conflicting reports in the field about the complex tumour-promoting and tumour-suppressing roles of CAV1 in the breast cancer microenvironment, underline the importance of further investigation of the regulatory mechanisms behind CAV1 expression in the breast cancer microenvironment. Understanding the regulation of CAV1 expression in fibroblasts may provide novel therapeutic targets for the treatments of a subset of breast cancer patients.
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>αSMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine sulfoximine</td>
</tr>
<tr>
<td>CAF</td>
<td>cancer-associated fibroblast</td>
</tr>
<tr>
<td>CAV1</td>
<td>caveolin-1</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CK8</td>
<td>cytokeratin 8</td>
</tr>
<tr>
<td>CTCF</td>
<td>corrected total cell fluorescence</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epithelial growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FGF-2</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon γ</td>
</tr>
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</table>
IL interleukin
IQGAP1 Ras GTPase-activating-like protein
JAK Janus kinase
MAPK mitogen activated protein kinase
MCP-1 monocyte chemotactic protein-1 (also known as CCL2)
MCT monocarboxylate transporter
MEF mouse embryonic fibroblast
MEK Raf/mitogen-activated and extracellular signal-regulated kinase kinase
MET mesenchymal-to-epithelial transition
MIP-1α macrophage inflammatory protein-1α (also known as CCL3)
MMP matrix metalloproteinase
MMTV mouse mammary tumour virus
NO nitric oxide
NOS nitric oxide synthase
PDGF platelet-derived growth factor
PI3K phosphoinositol 3-kinase
PKB protein kinase B (also known as Akt)
PP1 protein phosphatase 1
PP2A protein phosphatase 2A
PR progesterone
qPCR quantitative polymerase chain reaction
RNA ribonucleic acid
ROS reactive oxygen species
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>RSE</td>
<td>relative standard error</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immune deficiency</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromal cell-derived factor 1</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAM</td>
<td>tumour-associated macrophage</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>THPD</td>
<td>carrier-treated THP-1 cells</td>
</tr>
<tr>
<td>THPT</td>
<td>TPA-treated THP-1 cells</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>Y14</td>
<td>tyrosine 14</td>
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Chapter 1: Literature review
**Breast cancer**

Breast cancer is by far the most predominant form of cancer in women, and only comes second to lung cancer as the most prevalent cause of cancer deaths globally (Ferlay et al., 2015). Data from the World Health Organisation GLOBOCAN project shows that, worldwide, 1.67 million cases of breast cancer were diagnosed, and 522,000 deaths were due to breast cancer, in 2012 (Ferlay et al., 2015).

**Classification**

Breast cancer should not be considered a single disease, but rather a collection of subtypes, with distinct histopathologies, genetic characteristics, and treatment requirements (Goldhirsch et al., 2011; Vargo-Gogola and Rosen, 2007). Different subtypes can be classified via a number of methods. Over the recent years, a number of multigene arrays, for example, have proposed several very distinct subtypes in the literature (Goldhirsch et al., 2011). However, gene expression array information is not always obtainable in a clinical setting.

In 2011, a panel of experts, the St Gallen Consensus, has reached general consensus on the classification of 4 subtypes of breast cancer, on the basis of oestrogen receptor (ER) and progesterone receptor (PR) expression, human epidermal growth factor receptor 2 (HER2) expression levels, and Ki-67 labelling index, a marker for cell proliferation (Goldhirsch et al., 2011). The subsequent St Gallen conferences, in 2013 and 2015, have further refined the criteria to identify breast cancer subgroups and have provided current insights into the most appropriate treatments for different subtypes of breast cancer (Coates et al., 2015; Goldhirsch et al., 2013). The clinical distinction between ER-, PR- and HER2-positive breast cancers has been expanded
on with the classifications luminal A-like, luminal B-like, HER2-enriched, and basal-like. These classifications are based on extensive gene profiling of breast cancers, and while there is not a perfect overlap with the clinical subtypes, most luminal tumours are ER- and PR-positive, HER2-enriched tumours show HER2 overexpression, and most basal-like tumours are triple negative (Perou et al., 2000; Tyanova et al., 2016).

Carcinogenesis

The normal breast is composed of milk glands, surrounded by stroma composed mainly of adipose tissue and a framework of fibres called Cooper’s ligaments (Ramsay et al., 2005). Milk glands consist of a network of ducts and lobules for the production, storage, and secretion of milk during lactation. Breast cancer is neoplastic growth, originating most commonly in the epithelia of the milk gland. The milk duct and lobule are the most common sites for this initial lesion to occur, ductal carcinoma and lobular carcinoma being the most frequent forms of breast cancer diagnosed. However, on rare occasions breast tumours originate from the myoepithelial compartment (Gudjonsson et al., 2005).

Cancer cells are associated with gene mutations and epigenetic changes that alter the intracellular signalling mechanisms of healthy cells, and interrupt homeostasis (Jones and Baylin, 2002). In normal tissues, cell numbers are homeostatically controlled by a range of cell signals and growth factors. An important feature of cancer cells is their ability to escape these homeostatic mechanisms, and sustain chronic proliferation.

Cancer cells characteristically show sustained proliferation and limited apoptosis, leading to tumour growth (Hanahan and Weinberg, 2011). Mechanisms utilized by
cancer cells to generate proliferative signalling are the production of their own growth factor ligands, the stimulation of cells in tumour-associated stroma to provide growth factors, and the elevation of receptor proteins levels (Hanahan and Weinberg, 2011).

The continued and uncontrolled proliferation of cancer cells that have escaped homeostatic regulation can lead to the formation of tumours. Tumours, however, are more than masses of proliferating cancer cells; they are complex organs, consisting of multiple cell types. Like organs, tumours create their own supply of oxygen and nutrients by initiating the formation of vasculature, through a process called angiogenesis. Populations of cancer cells within the tumour can acquire high motility and migrate into the vasculature, spreading to local and distant sites, in a process referred to as metastasis (Nguyen and Massague, 2007).

**Metastasis**

Although survival rates for breast cancer are slowly improving, the prognosis of breast cancer worsens dramatically once the carcinoma becomes invasive (Ferlay et al., 2015; Massagué et al., 2017). Common sites of breast cancer metastasis are bone, lung, liver, and brain (Nguyen et al., 2009). Although tissues have many mechanisms in place that are highly unfavourable to invasion by foreign cell types, some tumours, due to their heterogeneity and genetic instability, will generate cell types that are able to overcome these challenges (Nguyen and Massague, 2007; Sethi and Kang, 2011). In addition, primary tumours secrete factors, such as cytokines, and extracellular vesicles, that will affect distant sites before metastasis, creating niches that are favourable to secondary tumour growth (Peinado et al., 2017).
**Epithelial-to-mesenchymal transition**

Epithelial cells are well anchored in the surrounding tissue by the formation of intercellular junctions and extracellular matrix (ECM) attachment. The loss of epithelial cell characteristics and progression to a more mesenchymal phenotype, in a process referred to as epithelial-to-mesenchymal transition (EMT), is a normal part of embryogenesis and tissue regeneration (Kalluri and Weinberg, 2009). EMT is a reversible process and cells can move back and forth between EMT and mesenchymal-epithelial transition (MET) states (Kalluri and Weinberg, 2009).

EMT has been proposed to be associated with the development of metastasis (Peinado et al., 2007). Downregulation of E-cadherin, a hallmark of EMT, has been linked to invasiveness in a number of studies (Heimann et al., 2000; Oka et al., 1993). However, several studies have shown evidence of epithelial morphology in invasive or metastatic cancer lesions, as reviewed by Christiansen and Rajasekaran (2006). More recent studies have found a close association between cancer stem cells and EMT (Liu et al., 2014b; Mani et al., 2008).

**Cancer stem cells**

Recently, the importance of a subset of cancer cells, referred to as cancer stem cells, has become apparent (Hanahan and Weinberg, 2011). Cancer stem cells are cancer cells that can form new tumours, which is the reason they are also referred to as tumour initiating cancer cells (Mani et al., 2008). Only a small portion of cells within the tumour possess the ability to establish themselves at distant sites and form new tumours. Upon *in vitro* induction of EMT, breast epithelial cells have been found to display both characteristics of EMT-like cells, and of breast cancer stem cells (Mani...
et al., 2008). Additionally, this overlap between EMT and cancer stem cell characteristics was found in a cell population isolated from clinical samples of human invasive breast carcinoma (Liu et al., 2014b). In addition to this EMT-like cancer stem cell population, an epithelial-like cancer stem cell population was found, and the authors have proposed that breast cancer stem cells are likely to reversibly transition between EMT and MET states (Liu et al., 2014b). As metastasis worsens cancer prognosis significantly, a focus on specifically targeting cancer stem cells may improve cancer treatment.

**Cancer and inflammation**

In recent years, scientists have become increasingly aware of the importance of the role of signals from outside the tumour on the progression of cancer. For many years, cancer research focussed predominantly on the cancer cell, but for the past two decades, more and more attention has been given to the tumour as part of a larger system.

Inflammatory mediators in the tumour microenvironment are important players in setting the context for cancer cells (Bissell and Hines, 2011; Shalapour and Karin, 2015). Cancer and inflammation are interconnected, the expression of different oncogenes drives inflammation, and inflammation can promote cancer (Mantovani and Sica, 2010; Shalapour and Karin, 2015). The increased occurrence of cancer at the site of inflammation is well described (Arias et al., 2012; Bromberg and Wang, 2009). Commonly cited examples to illustrate the link between inflammation and cancer include the increased risk of bowel cancer in chronic inflammatory bowel
disease patients (Koliaraki et al., 2017), and gastric cancer in patients with *Helicobacter pylori* infection (Lee et al., 2016).

Inflammation is a normal part of wound healing. Interestingly, there are many similarities between the composition of tumour stroma and healing wound tissue (Bissell and Radisky, 2001; Dvorak, 1986). Both tumour stroma and healing wound tissue promote angiogenesis, changes in the ECM, and increased cell motility (Kessenbrock et al., 2010). Similar to wound healing, tumour stroma is characterised by the induction of myofibroblasts, the upregulation of matrix metalloproteinases (MMPs), and the presence of invading immune cells and chemotactic factors, such as PDGF and TGF-β (Bissell and Radisky, 2001; Kalluri, 2016).

Inflammation can disrupt the normal interaction between fibroblasts and epithelial cells. In the case of prolonged interruption, this can lead to the proliferation of cells. Abnormal signalling can cause epithelial cells with an innate tumorigenic potential to start to display a carcinogenic phenotype, as well as cause genetic instability, which can give rise to a tumorigenic potential (Bissell and Radisky, 2001). An inflammatory microenvironment leads to a constant presence of reactive nitrogen and oxygen species, cytokines, and chemokines (Hussain and Harris, 2007). This, in turn, leads to the activation of pro-carcinogenic transcription factors, like NF-κB and STAT3 (Moore et al., 2010). It is no wonder that tumours are often referred to as wounds that never heal (Bissell and Hines, 2011; Dvorak, 1986).
**Tumour microenvironment**

Normal breast tissue is highly organized. Glandular tissue is separated from the surrounding connective tissue, or stroma, by the basement membrane. A major cell type within the breast stroma is the fibroblast. Fibroblasts maintain the structure of the stroma by synthesizing ECM components, like polysaccharides, fibronectin, and collagen. Fibroblasts can become activated, for example in wound healing, and secrete higher than normal amounts of ECM components. The activated fibroblast is also referred to as myofibroblast, characterised by the upregulation of α-smooth muscle actin (αSMA) (Orimo et al., 2005). The newly formed matrix, generated by myofibroblasts, then serves as a base for tissue regeneration. Other important factors in tissue remodelling are MMPs, proteinases that can degrade ECM proteins, such as collagen, fibronectin, and laminin.

The strong association between cancer and inflammation suggest that the stroma surrounding the tumour is a critical partner in tumour progression. In the initial stages of cancer, the primary tumour is generally contained within the epithelia, separated from other parts of the tissue by the basement membrane. During cancer progression, the stroma around a tumour undergoes changes that resemble the reorganisation of tissue that takes place during inflammation (Bissell and Radisky, 2001). This changing stroma, also referred to as reactive stroma, contains an increased number of fibroblasts, many displaying an activated, or myofibroblast, phenotype, and is characterised by large deposits of fibronectin and collagen, and the presence of proteases, such as MMPs, and invading immune cells (Coussens and Werb, 2002; Kalluri, 2016).
Signalling between the tumour and the microenvironment evolves progressively with the development toward a more invasive cancer, with the signalling from the tumour supporting an altered microenvironment, and signalling from an altered microenvironment supporting tumour progression and, possibly, metastasis (Bissell and Hines, 2011).

*Cancer-associated fibroblasts*

The subset of fibroblasts that is involved in most cancers is termed cancer-associated fibroblasts (CAFs). The CAFs found in tumour stroma are a heterogeneous population; with a large proportion of CAFs displaying a myofibroblastic phenotype (i.e., express αSMA), intermixed with other CAFs that do not express αSMA (Erez et al., 2010; Sugimoto et al., 2006).

CAFs play a role in all stages of cancer, including invasion and metastasis (Kalluri, 2016). Like their non-cancer-associated counterparts, CAFs greatly influence the structure of the ECM (Kalluri, 2016). Normal breast tissue shows collagen fibres wrapped around the epithelium of mammary glands in a circular fashion, suggesting a containing and anchoring function (Provenzano et al., 2008). Thickened collagen fibres that lay perpendicular to the tumour, however, are commonly found in desmoplastic stroma, and are associated with an increase in ‘stiffness’ of the matrix (Malik et al., 2015). The remodelled ECM, displaying increased ‘stiffness’, has been used to detect cancer (Butcher et al., 2009), and collagen alignment has been proposed as a diagnostic marker for breast cancer (Conklin et al., 2011). Functionally, the rearrangement of collagen fibres, to align perpendicular to the tumour, may
enhance cancer cell invasion of the surrounding stroma by providing cancer cells with an ECM track (Goetz et al., 2011; Provenzano et al., 2006).

**Matrix metalloproteinases**
Other factors in the stroma that play an important role in the remodelling of the ECM are MMPs. Both wounding and the presence of tumour cells can trigger increased levels of MMPs in the ECM. Expressed by both cancer cells and stromal cells, MMPs exert their pro-carcinogenic function by remodelling the ECM to make it more favourable to cancer cell invasion (Egeblad and Werb, 2002; Malik et al., 2015; Sternlicht and Werb, 2001). Of the family of MMPs, MMP2 and MMP9 are most frequently implicated for their role in breast cancer (Merdad et al., 2014; Pellikainen et al., 2004; Wiercinska et al., 2011).

The ECM is not merely a structural framework that supports cells and arranges tissues, but also provides vital information to cells about their environment. Remodelling of the ECM, such as by MMPs, therefore, does not merely result in a structural framework that physically favours cancer cell invasion, but also changes cell behaviour through a change in the informational content relayed to nearby cells (Sternlicht and Werb, 2001).

**Tumour-associated macrophages**
Besides the presence of CAFs, the tumour-associated stroma is characterized by increased numbers of macrophages. Monocytes continuously patrol the blood and lymph system, invading tissues and differentiating into macrophages in response to inflammation signals. Macrophages are phagocytic immune cells that play an
important role in tissue homeostasis, by clearing apoptotic cells and producing growth factors. When stimulated by an antigen, they elicit an immune response through the production of inflammatory cytokines (Geissmann et al., 2010). Macrophages can express both pro- and anti-tumour activity (Mantovani and Sica, 2010). A subset of tumour-associated macrophages (TAMs), referred to as ‘alternatively’ activated (M2) macrophages, promotes EMT and metastasis, and is correlated to poor prognosis (Chen et al., 2011; Su et al., 2014).

**Caveolin-1 and cancer**

Caveolin-1 (CAV1) is the 22-24 kDa structural protein of caveolae, 60-80 nm diameter invaginations of the cell membrane. Caveolea where first described in 1953 (Palade), and named for their resemblance to little caves (cavae) in electron microscopy. A more recent study has, however, shown the shape of caveolae to be affected by aldehyde-fixation, and suggests the invagination to range from almost flat, to most commonly cup-shaped, to rarely omega-shaped, *in vivo* (Schlormann et al., 2010). When the expression of CAV1 is lost, e.g., in CAV1 null mice, no caveolae are formed (Razani et al., 2001a). See Figure 1 for a transmission electron image of caveolae in a primary human breast fibroblast.

Caveolae are abundant in fibroblasts, adipocytes, smooth and striated muscle cells, and endothelial cells (Couet et al., 1997). Unlike clathrin-coated pits, caveolae are not present on the cell membrane of every cell type with the same abundance, and the distribution of caveolae across the cell membrane is uneven, with more caveolae at the rear of migrating cells (Martinez-Outschoorn et al., 2015). The number of caveolae present on the cell membrane is variable, and influenced by environmental
cues. Loss of integrin-mediated attachment to the ECM, for example, results in the internalisation of caveolae (del Pozo et al., 2005).

Caveolae are invaginated lipid rafts. Lipid rafts are microdomains of the plasma membrane that are more ordered and tightly packed than surrounding membrane regions, and are rich in cholesterol, sphingolipids, and membrane proteins (Kirkham and Parton, 2005). Caveolae have been shown to be involved in endocytosis, cholesterol homeostasis, cellular signalling, and mechanosensing (Bastiani and Parton, 2010), and the functions of caveolae are still studied with much interest.

Figure 1: Transmission electron microscopy image of Fre85 primary human breast fibroblast. The arrows show caveolae at the cell membrane.
The caveolin gene family consists of CAV1, CAV2 and CAV3. CAV1 contains alternative initiation codons, resulting in the isoforms CAV1α and CAV1β (Scherer et al., 1995). CAV1 and CAV2 are co-expressed in several cell types, whereas CAV3 is muscle specific. The CAV1 gene is localised to locus D7S522 of human chromosome 7q31.1, a known tumour suppressor locus (Engelman et al., 1998b). The transcription of CAV1 is regulated by multiple transcription factors, including E2F/DP1, Sp1, FoxO3a, EGR-1, p53, PPARγ, and promoter shore CpG methylation (Bist et al., 2000; Dasari et al., 2006; Dasgupta et al., 2015; Rao et al., 2013). CAV1 transcription is upregulated in the presence of free cholesterol (Fielding et al., 1999).

Upon translation, CAV1 is targeted to the endoplasmic reticulum by a signal recognition particle (Monier et al., 1995). From the ER, CAV1 is transported to the Golgi (Ostermeyer et al., 2001). Many cell types show accumulation of CAV1 in the Golgi. These pools of CAV1 differ structurally from the protein inserted in the cell membrane. Exiting from the Golgi, CAV1 is assembled into an exocytic structure, containing high levels of cholesterol and sphingolipids, to be inserted into the membrane and form caveolae (Parton and Simons, 2007). The budding of these exocytic caveolar-carriers has been shown to be dependent on cholesterol (Bastiani and Parton, 2010).

At the plasma membrane, hetero-oligomeric complexes of a group of proteins called cavins associate with the newly formed caveolar structures (Hayer et al., 2010). The cavin protein family consists of four members; cavin-1 is required for the formation of caveolae (Hill et al., 2008), cavin-2 plays a role in cavin-1 localisation to the plasma membrane (Breen et al., 2012), cavin-3 localises to caveolae, but is not
required for caveolar formation (Liu et al., 2014a), and cavin-4 is a muscle-specific component of the caveolar complex (Bastiani et al., 2009). Cavin-1 is ubiquitously expressed, and its absence in knock-out animals leads to a complete loss of caveolae formation in all tissues (Hill et al., 2008).

CAV1 is phosphorylated by the tyrosine kinase Src at its tyrosine 14 (Y14) residue. As CAV1β is composed of residues 32-178, only CAV1α (1-178) can be phosphorylated by Src (Li et al., 1996). Other tyrosine kinases capable of phosphorylating CAV1 on Y14 are Fyn and c-Abl (Goetz et al., 2008). Serine phosphorylation at serine 80 (S80) causes CAV1 to be excreted as a soluble protein (Schlegel et al., 2001). Palmitoylation is required for CAV1 oligomerization, but not for localisation to caveolae (Dietzen et al., 1995; Goetz et al., 2008).

**Caveolae signalling hypothesis**

CAV1 has been implicated as a regulator of several different signalling pathways. It has been suggested that CAV1 in caveolae directly binds to a number of different signalling molecules, as CAV1 has been shown to immunoprecipitate with several of these signalling molecules (Martinez-Outschoorn et al., 2015; Sargiacomo et al., 1995). However, functional experiments have not confirmed this for many of the signalling molecules in question, as reviewed by Parton and Simons (2007).

The CAV1 scaffolding domain, amino acid sequence 80-101 on the N-terminal side, has been implicated as the binding site for several signalling molecules (Smart et al., 1999). Moreover, CAV1-binding motifs have been identified in the active catalytic domain of several signalling molecules (Smart et al., 1999). However, the CAV1
scaffolding domain also has membrane-binding activity, possibly causing it to insert into the caveolar membrane, and may not even be accessible to those molecules it has been proposed to interact with (Collins et al., 2012; Parton and del Pozo, 2013). This is supported by the observation that antibodies against the CAV1 scaffolding domain do not stain the cell membrane, but do stain the Golgi network (Pol et al., 2005), indicating the CAV1 scaffolding domain is hidden once CAV1 takes part in the formation of caveolae. This lead Parton and Simons (2007) to speculate that it may be the non-caveolar pools of CAV1 that have a functional effect on some of the implicated signalling pathways. See Figure 2 for a schematic representation of CAV1 in caveolae.
Figure 2: CAV1 inserted in the cell membrane. CAV1 has a hairpin-like structure spanning the cell membrane. CAV1 contains an oligomerization domain and a scaffolding domain. Caveolar membrane regions contain increased concentrations of sphingolipids and cholesterol.

In addition to the controversy around the functional role of the CAV1 scaffolding domain, there is doubt around the functional availability of the CAV1 binding domain that has been identified in many putative CAV1-binding proteins. Structural analysis of several putative CAV1-interacting proteins showed that the CAV1 binding domain is tightly packed within the hydrophobic core of the protein (Collins et al., 2012).

Taken together, the model of caveolae as signalling nodes, where signalling molecules are bound, by their CAV1 binding domain to the CAV1 scaffolding domain, needs to be reassessed. Alternatively, the widespread effect of the loss of CAV1 may indirectly result in aberrant signalling (Parton and del Pozo, 2013).
Examples of this include the roles of CAV1 in endocytosis of surface receptors, in exocytosis and ubiquitylation pathways, as well as the changes in membrane structure, particularly in lipid rafts, that occur when CAV1 levels are altered (Parton and del Pozo, 2013). A recent study by Ariotti et al. (2014) illustrated the relationship between caveolae, the composition of the plasma membrane, and Ras signalling. Interestingly, the loss of CAV1, the loss of cavin1, and the loss of caveolae, all affect plasma membrane organisation and Ras signalling, suggesting a caveolae-dependent, rather than a CAV1 dependent effect (Ariotti et al., 2014).

**The role of CAV1 in signalling pathways**

A functional interaction has been shown *in vitro* between CAV1 and a number of signalling pathways. Functional effects of CAV1 have been described for the MEK1/ERK1/2, PI3K/Akt, nitric oxide synthase, JAK/STAT and WNT/β-catenin pathways, as reviewed by Shatz and Liscovitch (2008). It is important to note, however, that these signalling pathways are not impaired in cells that naturally lack caveolae, nor in CAV1 knock-out cells (Parton and Simons, 2007).

**MAPK**

The Raf/mitogen-activated and extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signalling pathway is commonly dysregulated in breast cancer (Saini et al., 2013). As part of the mitogen activated protein kinase (MAPK) signalling cascade, activation of the signalling molecules MEK and ERK results in the activation of transcription factors that regulate the transcription of molecules involved in proliferation, apoptosis, and migration/invasion, including growth factors, cytokines, and cyclins.
CAV1 is able to inhibit the MEK1/ERK1/2 pathway through direct interaction with MEK1 and ERK1/2 (Engelman et al., 1998a). On the other hand, CAV1 has been shown to play a role in the activation of this same pathway, by coupling the integrin alpha subunit to the tyrosine kinase Fyn, and setting into motion a sequence of events that links integrins to the MAPK/ERK pathway (Wary et al., 1998). More recently, CAV1 knockdown in Ewing sarcoma cells was shown to decrease MEK1/2 and ERK1/2 phosphorylation. Its co-localisation with CAV1 and ability to modulate ERK2 activity, suggest that the scaffold protein Ras GTPase-activating-like protein (IQGAP1) may provide a link between CAV1 and MEK/ERK (Lagares-Tena et al., 2016).

**PI3K/Akt**

Another signalling pathway that is highly implicated in cancer is the phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB, also known as Akt) signalling pathway. Akt is an important signalling molecule involved in many pathways regulating cellular processes, such as apoptosis, protein synthesis, cell motility, and proliferation.

Similar to its role in the MEK1/Erk1/2 pathway, CAV1 can both inhibit and stimulate the PI3K/Akt pathway. In a study using a human prostate cancer cell line, protein phosphatases PP1 and PP2A were inhibited by vector-induced CAV1 expression, leading to increased levels of activated Akt (Li et al., 2003). In a study using an inflammatory breast cancer cell line, downregulation of CAV1 was found to inactivate Akt (Joglekar et al., 2015). A study using prostate cancer cells found that treatment with the bioactive flavonoid baicalein resulted in reduced proliferation and migration, in addition to decreased CAV1 expression and reduced Akt.
phosphorylation (Guo et al., 2015). Conversely, a study in a cardiomyocyte-derived cell line suggests that CAV1 dependent targeting of PP2A to caveolae can lead to inhibition of Akt (Zuluaga et al., 2007).

eNOS
The nitric oxide synthases (NOSs) are a family of enzymes that catalyse the production of nitric oxide (NO). NOS overproduction, and the resulting increase in reactive oxygen species (ROS), may lead to higher mutation rates, increased cell proliferation, and tumour progression (Brown and Bicknell, 2001). Co-expression of endothelial nitric oxide synthase (eNOS) with CAV1 in COS-7 cells inhibits the basal release of NO, demonstrating a functional effect of CAV1 on the NOS pathway (García-Cardeña et al., 1997). A mouse study that selectively overexpressed CAV1 in endothelial cells lining the tumour blood vessels in vivo, showed decreased NO production, decreased tumour microvessel density, and decreased tumour blood flow (Brouet et al., 2005).

TGF-β
Transforming growth factor β (TGF-β)-activated Smad signalling plays a role in a wide range of biological functions, including proliferation, differentiation, ECM production, and cell death. In fibroblasts, TGF-β induces increased αSMA expression and myofibroblast differentiation (Evans et al., 2003; Midgley et al., 2013). Cancer cell-derived MMP9 has been shown to activate TGF-β in fibroblasts, and drive myofibroblast differentiation (Dayer and Stamenkovic, 2015). CAV1 has been shown to inhibit TGF-β/SMAD signalling in NIH-3T3 fibroblasts (Razani et al., 2001b).
Activated TGF-β receptors are endocytosed through two distinct pathways: the clathrin-coated vesicle pathway, and the caveolar pathway (Di Guglielmo et al., 2003). Endocytosis of activated TGF-β receptors through clathrin-coated pits leads to Smad activation, and transcription of TGF-β-responsive genes, whereas endocytosis through caveolae leads to receptor ubiquitylation and degradation (Di Guglielmo et al., 2003; Meyer et al., 2013).

**JAK/STAT**

The Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) signalling pathway is a highly conserved signalling pathway that relays signals, usually cytokines, from outside the cell to the nucleus, to activate gene transcription. This pathway is the main alternative to the second messenger system.

JAK and Src activation of STAT3 has been shown to contribute to breast cancer cell growth and survival *in vitro* (Garcia et al., 2001). Levels of activated STAT3 are increased in CAV1 -/- pulmonary fibroblasts, compared to wild type fibroblasts, after PDGF-BB and TGF-β treatments (Ryter et al., 2014). Signals from outside the cell that are implicated in activating STAT3 through JAKs in breast cancer cells, include EGF (Garcia et al., 2001), and IL-6 (Bromberg and Wang, 2009). In conjunction with these findings, EGF has been reported to downregulate the expression of CAV1 in human cancer cell lines (Lu et al., 2003). EGF is a strong chemotactic factor and is expressed by many tumour types (Goswami et al., 2005). EGF is also produced by cells in the tumour microenvironment, such as T cells and macrophages. Interestingly, both CAFs (Studebaker et al., 2008), and CAV1-null-mammary fibroblasts (Sotgia et al., 2009), have been shown to secrete increased levels of IL-6.
Functional analysis of the interaction of STAT3 with the CAV1 promoter, combined with evidence of the effect of CAV1 expression on the invasiveness of brain metastatic cell lines, suggests that STAT3 influences brain metastasis by binding the CAV1 promoter directly, and negatively regulating CAV1 transcription (Chiu et al., 2011). Conversely, overexpression of CAV1 inhibits STAT3 (Chiu et al., 2011).

This combined evidence suggests that CAV1 expression in breast cancer may be regulated through the JAK/STAT3 pathway by growth factors and other cytokines. Both the study of CAV1 knockout fibroblasts, and the co-culture of cancer cells and fibroblasts, have found an increase in the secretion of numerous growth factors and other cytokines (Martinez-Outschoorn et al., 2011; Sotgia et al., 2009). Therefore, signalling through the JAK/STAT pathway could both underlie the CAV1 downregulation, and be a consequence of CAV1 downregulation. However, the downregulation of CAV1 through STAT3 has so far only been shown in epithelial cells, and not in CAFs.

**CAV1 and mitochondrial function**

The observation that subsets of cancer cells produce lactate, even in the presence of oxygen, has been well described, and is widely known as the Warburg effect (Vander Heiden et al., 2009). However, this does not mean that cancer cells are less dependent on oxidative phosphorylation. Mutations in the TCA cycle occasionally occur in some cancers, but are interestingly correlated to increased patient survival (Yan et al., 2009). A study by Bosch et al. (2011) has suggested that a lack of CAV1 leads to increased mitochondrial cholesterol, which in turn leads to mitochondrial dysfunction.
and aerobic glycolysis. Mitochondrial dysfunction causes the accumulation of ROS in the cell, ultimately leading to cell death.

In recent years, it has been hypothesized that cancer cells initiate aerobic glycolysis in stromal cells leading to oxidative stress in the tumour stroma, which in turn causes autophagy of stromal cells, and the release of metabolites, such as lactate, ketones, and fatty acids, to be used by cancer cells for oxidative phosphorylation (Martinez-Outschoorn et al., 2016).

Loss of CAV1 in stromal fibroblasts may result in aerobic glycolysis via mitochondrial dysfunction, causing autophagy and the potential transfer of metabolites to cancer cells (Sotgia et al., 2011). These energy-rich metabolites are transported across the cell membrane via the monocarboxylate transporters (MCT) -1 and -4. MCT4 has been proposed as a biomarker for identifying high-risk breast cancer patients, and the expression of stromal MTC4 is inversely related to the expression of stromal CAV1 (Martins et al., 2013; Witkiewicz et al., 2012).

CAV1 and integrin-mediated endocytosis
Endocytosis through clathrin-coated pits is well characterised, however clathrin-independent endocytosis, such as through caveolar budding, is less well described. Endocytosis, through the budding and internalisation of caveolae, is clathrin-independent and dynamin dependent. Both caveolae and non-caveolar CAV1 can be targeted to early endosomes upon endocytosis, and then recycled back to the plasma membrane (Parton and del Pozo, 2013). Alternatively, monoubiquitylation of CAV1 will result in targeting to multivesicular bodies (endosomes containing luminal
vesicles, also referred to as late endosomes) for degradation (Parton and del Pozo, 2013). The endocytosis of caveolae may play a role in modulating signalling pathways by uncoupling signalling intermediates at the lipid raft, in a process regulated by integrins (Salanueva et al., 2007).

Integrins provide the physical connection of the cell with the ECM, and modulate signalling pathways of membrane receptor kinases. Integrins interact with multiple growth regulatory pathways, inhibiting growth when anchorage is lost. In cancer, on the other hand, these growth regulatory pathways become constitutively activated, and adhesion through integrins is no longer required for growth (Guadamillas et al., 2011). Therefore, anchorage-independent growth is strongly related to tumorigenicity and metastasis.

A study by del Pozo et al. (2005), carried out in WT and CAV1 -/- cell lines of human and murine origin, suggests that caveolar endocytosis plays a role in anchorage-independent growth through the internalisation of lipid rafts upon cell detachment from the ECM. The internalisation of lipid rafts inhibits growth regulatory pathways, such as Rac, Erk and Akt signalling pathways (del Pozo et al., 2005). Integrin-dependent endocytosis of lipid rafts is dependent on the phosphorylation of CAV1 at Y14. In the absence of CAV1, internalisation of lipid rafts does not take place, and growth regulatory pathways remain activated (del Pozo et al., 2005). Src kinases and PKC are known to phosphorylate CAV1 at Y14 and, therefore, are likely to play an important part in the regulation of anchorage-independent growth by CAV1.
CAV1 and exosomes

Exosomes are secreted vesicles that can contain cytoplasmic components and genetic material (Simons and Raposo, 2009). Exosomes are released from multivesicular bodies once fusion with the cell membrane has taken place (Théry et al., 2009). Both cancer cells and stromal cells secrete exosomes that promote cancer progression (Luga et al., 2012; Peinado et al., 2012). Exosomes play a role in cancer through the transfer of signalling molecules and RNAs, and the release of exosomes is dramatically increased in cancer (Logozzi et al., 2009). The secretion of exosomes by stromal cells has been shown to drive metastasis, and chemotherapy and radiation resistance, of breast cancer cells (Boelens et al., 2014; Luga et al., 2012).

A study by Svensson et al. (2013) found that exosomes enter the cell by clathrin-independent endocytosis, and that exosome uptake is negatively regulated by CAV1. Exosomes isolated from plasma from SCID mice engrafted with melanoma tumours strongly expressed CAV1, while CAV1 was undetectable in exosomes from control animals, and increased amounts of CAV1-positive exosomes were found in the plasma from melanoma patients (Logozzi et al., 2009). It is not yet clear how stromal CAV1 levels might affect exosome signalling in the tumour microenvironment.

CAV1 expression by breast cancer cells

The role of CAV1 in breast cancer is a complex one. CAV1 has been described as an oncogene, as well as a tumour suppressor, in breast cancer cells. Studies have shown CAV1 to be over-expressed by a subtype of breast cancer that is associated with poor prognosis (Pinilla et al., 2006). CAV1 expression was strongly associated with a basal-like phenotype in the immunohistochemical evaluation of 556 BrCa1/BrCa2 –
associated carcinomas (Pinilla et al., 2006), and of 245 invasive breast cancers (Savage et al., 2007).

Other studies, however, have found CAV1 expression by cancer cells to be associated with a less aggressive phenotype. Analysis of CAV1 mRNA and protein expression of 4 normal mammary epithelial, and 6 breast cancer, cell lines, showed that CAV1 expression was lower or not detectable in the breast cancer cell lines (Lee et al., 1998). In addition, the overexpression of CAV1 in human breast cancer cells that showed no detectable CAV1 expression before, led to substantial growth inhibition (Lee et al., 1998).

Loss of CAV1 in a breast cancer mouse model, using CAV1 -/- mice interbred with metastatic mammary tumour-forming MMTV-PyMT mice, resulted in accelerated tumorigenesis and metastasis (Williams et al., 2004). PyMT-CAV1 -/- mice lack CAV1 expression in both the epithelial and stromal compartment. To determine the effect of CAV1 expression in stromal fibroblast versus epithelial cells on tumour burden, nude mice were injected with PyMT-transformed CAV1 wild type, and CAV1 -/- MEFs, and with mammary adenocarcinoma cells transfected with a CAV1 construct. It was found that the presence of CAV1 in fibroblasts, as well as the presence of CAV1 in cancer cells, reduced the tumour burden (Williams et al., 2004).

A review by Shatz and Liscovitch (2008) on CAV1 levels in several cell lines and patient cancers concluded that CAV1 appears to be acting as a tumour suppressor in early stages of cancer, but is upregulated in metastatic and multi-drug resistant cell lines and patient tumour samples. More recently, CAV1 has been found to be
upregulated in breast cancer cell lines after chemotherapy, and to be enriched in breast cancer stem cells (Wang et al., 2014).

**Evidence for a protective role of CAV1 in the microenvironment**

The clinical relevance of CAV1 expression in the breast tumour microenvironment has been shown by several unrelated studies examining separate patient cohorts. CAV1 expression in patient breast tissue biopsies, as determined by immunohistochemical analysis, was compared to disease outcome. Loss of CAV1 expression in the tumour stroma has been found to be strongly associated with advanced tumour stage, lymph node metastasis, and reduced progression-free survival (El-Gendi et al., 2012; Sloan et al., 2009; Witkiewicz et al., 2009b). Qian et al. (2011) confirmed a strong correlation between reduced disease-free survival and a loss of CAV1 expression in the tumour stroma, but combined with a high level of CAV1 expression in the tumour.

A similar study, looking specifically at CAV1 expression in CAFs in tissue biopsies from breast cancer patients, also showed a strong link between the loss of CAV1 expression and the reduction in overall survival and disease-free survival (Simpkins et al., 2012), pointing to a key role for fibroblasts. Analysis of CAV1 levels in fibroblasts isolated from human breast cancer lesions has shown that CAV1 was downregulated in 8 out of 11 samples (Mercier et al., 2008). A study on ER+ DCIS patients showed that 7 out of 8 patients that underwent recurrence to invasive breast cancer had reduced or absent levels of stromal Cav1, whereas 35 out of 36 ER+ DCIS patients with high levels of stromal Cav1 did not show recurrence to invasive breast cancer during the time of the study (Witkiewicz et al., 2009a). This combined
evidence points to the potential use of CAV1 as an independent predictor of disease outcome in breast cancer patients. Furthermore, the association between CAV1 expression in the stroma and cancer progression has led to the question of what mechanisms might be behind this.

To investigate the effects of cancer cells on CAV1 expression in fibroblasts, human fibroblasts were cultured with a human breast cancer cell line (MCF7). This resulted in downregulation of CAV1 expression in the fibroblasts, mimicking what occurs in the stroma of high-risk breast cancer patients (Martinez-Outschoorn et al., 2010b). Interestingly, treatment with an inhibitor of the lysosomal pathway rescued CAV1 expression, suggesting that downregulation of CAV1 in co-cultures was mediated by autophagic/lysosomal degradation. Co-culture of fibroblasts with cancer cells was reported to result in the upregulation of myofibroblast markers, such as α-SMA, calponin, and vimentin, in the fibroblasts, suggesting a phenotype switch. It is important to note however, that no quantitative analysis of these reported changes in protein expression after co-culture, including the downregulation of CAV1, was provided.

Similar to the effect of co-culture with cancer cells, fibroblasts treated with CAV1 siRNA upregulated myofibroblast markers (Martinez-Outschoorn et al., 2010b). This suggests that the shift to a myofibroblast phenotype after co-culture with cancer cells is due to the loss of CAV1 expression, and that the loss of CAV1 expression is not merely a marker for the myofibroblast phenotype, but is a driver of it.
Like CAFs, CAV1-deficient fibroblasts have been shown to be pro-tumorigenic and angiogenic in vivo. Co-injection of CAV-1-deficient fibroblasts with a human breast cancer cell line (MDA-MB-231) into nude mice resulted in larger tumours, as measured by weight and volume, and an increase in angiogenesis, as measured by vessel area and vessel number (Bonuccelli et al., 2010). Additionally, a study of CAV1 -/- mice has reported abnormal growth and differentiation of stromal cells (Yang et al., 2008). The authors noted that in many organs abnormalities in epithelial cell growth and differentiation were also present, while epithelial CAV1 expression was normally low or non-detectable. It was therefore hypothesised that the loss of CAV1 leads to disruption of normal stromal-epithelial interactions (Yang et al., 2008).

Furthermore, CAV1 deficient fibroblasts have been shown to increase invasiveness of cancer cells and induce EMT in vitro (Simpkins et al., 2012; Sotgia et al., 2009). Treatment of cancer cells with conditioned media from monocultures of CAV1 -/- murine mammary fibroblasts, or CAV1 siRNA-transfected immortalised human breast fibroblasts, resulted in less cohesion, and induced the expression of αSMA and downregulation of E-cadherin, markers of EMT (Simpkins et al., 2012; Sotgia et al., 2009).

Immortalized human skin fibroblasts co-cultured with MCF7 cells have been further reported to have increased ROS levels, combined with decreased CAV1 levels, compared to monocultures (Martinez-Outschoorn et al., 2010a). In this study, CAV1 expression in fibroblasts was rescued by treatment with a ROS scavenger, and similarly with the anti-oxidant metformin, whereas treatment with the pro-oxidant
buthionine sulfoximine (BSO) downregulated CAV1 expression, and induced DNA damage (Martinez-Outschoorn et al., 2010a). Furthermore, treatment with CAV1 siRNA induced DNA damage, mitochondrial dysfunction, and a glycolytic switch in fibroblasts.

Proteomic and transcriptional analysis of CAV-1-deficient fibroblasts has been reported to show an upregulation of the expression of glycolytic enzymes (Bonuccelli et al., 2010). CAV1 is an inhibitor of NOS (García-Cardeña et al., 1997). NO overproduction can lead to DNA damage, mitochondrial dysfunction, and an increase in ROS levels. Together, this points toward the induction of oxidative stress in fibroblasts by the presence of cancer cells, with CAV1 acting as an important marker and potential regulator. Again, only immunofluorescence was used to detect changes in protein expression levels after co-culture with cancer cells, as in the experimental findings described above. Even though there is strong evidence of oxidative stress in fibroblasts that have lost CAV1 expression (Bonuccelli et al., 2010; Martinez-Outschoorn et al., 2010a; Pavlides et al., 2010), and the role of CAV1 as a NOS inhibitor (García-Cardeña et al., 1997), more research is required to determine what triggers the cycle of CAV1 downregulation and oxidative stress in fibroblasts.

**Evidence for a pro-carcinogenic role of CAV1 in the microenvironment**

In an apparent contradiction to the finding that a downregulation of CAV1 in the tumour stroma is strongly correlated to reduced survival, research by Goetz et al. (2011) suggests that the presence of CAV1-expressing fibroblasts in the stroma leads to a more invasive cancer cell. An analysis of 132 breast tumours and 35 normal breast tissues showed an increased expression of CAV1 in CAFs, and further analysis
indicated that CAV1 expression in CAFs is associated with an increased mortality risk for patients (Goetz et al., 2011). This is a finding that seems to directly contradict the inverse relationship between CAV1 and patient survival found by other studies (El-Gendi et al., 2012; Qian et al., 2011; Simpkins et al., 2012; Sloan et al., 2009; Witkiewicz et al., 2009b). It is important to note that most of these studies, with the exception of Simpkins et al. (2012), did not link CAV1 expression to a positive CAF marker, and, with the exception of El-Gendi et al. (2012) did not correlate stromal CAV1 with metastasis.

To elucidate the effects of endogenous CAV1 on fibroblasts, wild type and CAV1 -/- mouse embryonic fibroblasts (MEFs) were grown in a cell-free 3D matrix generated by 6-8 day confluent cultures of fibroblasts. In addition, MEFs were grown in matrices generated by wild type and CAV1 -/- MEFs, to elucidate the effect of an ECM generated by CAV1 -/- fibroblasts on wild type and CAV1 -/- fibroblasts. Goetz et al. described the effect of endogenous CAV1, and a CAV1 -/- MEFs-generated microenvironment on cell shape, cell contractility, and matrix organization. Their results show that both CAV1 -/- MEFs, and MEFs seeded in matrices produced by CAV1 -/- MEFs, display decreased cell elongation and lower cell contractility.

A decrease in cell contractility is consistent with the role of CAV1 in Rho mediated contractility (Grande-Garcia et al., 2007). CAV1 promotes actomyosin contraction through the sequestration of the endogenous Rho inhibitor p190RHOGAP outside lipid rafts (Goetz et al., 2011). In the absence of CAV1, p190RHOGAP associates with the lipid raft and inhibits Rho-mediated actomyosin contraction. Actomyosin contraction results in ECM-remodelling by integrins (Goetz et al., 2011; Parton and
Compared to CAV1-/- MEFs, WT MEFs further generated a more parallel orientation of fibronectin fibres in the matrix. The reorganisation of the tumour stroma by CAFs is a normal process during the progression of cancer (Kalluri and Zeisberg, 2006). Matrices generated by CAFs have been reported to be rich in fibronectin and collagen (Amatangelo et al., 2005), and collagen fibre deposition perpendicular to the tumour has been linked to tumour cell invasiveness (Provenzano et al., 2008). Highly aligned and thickened collagen fibres result in increased matrix ‘stiffness’, which is a feature of a desmoplastic stroma (Malik et al., 2015).

Consistent with their hypothesis that CAV1 is required for a highly ordered and pro-carcinogenic matrix, Goetz et al. (2011) found that wild type MEFs promoted tumour cell invasion and metastasis. Tumours generated in CAV1-/- mammary glands displayed less stromal fibre alignment, compared to those generated in wild type mammary glands, and the transplantation of ‘wild type’ tumours into nude mice resulted in more metastases (Goetz et al., 2011).

It is important to note that MEFs are an embryonic cell type, and potentially elicit a different response, compared to adult mammary fibroblasts. However, in accordance with a pro-tumorigenic role of CAV1, others have found that the injection of melanoma cells into CAV1-/- mice led to reduced tumour growth and diminished angiogenesis, compared to WT mice (Chang et al., 2009). Additionally, in line with the findings by Sloan et al. in the breast cancer stroma, high CAV1-expression in the stroma of malignant pleural mesothelioma is associated with worse patient outcome.
CAV1 clearly is of significance in breast cancer, and several studies point to a potential use of CAV1 expression levels in the tumour stroma as a predictor for clinical outcome, and as such may be useful in determining treatment strategies (El-Gendi et al., 2012; Qian et al., 2011; Simpkins et al., 2012; Sloan et al., 2009; Witkiewicz et al., 2009b). Furthermore, evidence from studies on CAV1 KO/KD systems \textit{in vitro} (Goetz et al., 2011; Martinez-Outschoorn et al., 2010a; Martinez-Outschoorn et al., 2010c; Simpkins et al., 2012; Sotgia et al., 2009), and \textit{in vivo} (Bonuccelli et al., 2010; Goetz et al., 2011), suggests that CAV1 is not merely a marker of a progressive tumour stroma, but plays an active role in the mechanism(s) that favour tumour cell invasiveness and metastasis.

The exact nature of these mechanisms is still largely unclear. There is mounting evidence of the association of CAV1 with oxidative stress, hypoxia, and autophagy in fibroblasts (Sotgia et al., 2011). It has been hypothesized that cancer cells utilize the increased autophagy/mitophagy in the tumour stroma through a form of metabolic coupling, whereby the stroma cells undergo aerobic glycolysis, and secrete high energy metabolites, directly feeding adjacent cancer cells (Sotgia et al., 2011).

The role of CAV1 in the remodelling of the ECM (Goetz et al., 2011), and the effect of changes in the ECM on tumour cell invasiveness (Provenzano et al., 2008), is another mechanism that can potentially explain how a change in CAV1 levels in the stroma affects tumour progression and metastasis. Additionally, the observation that a range of cytokines are differentially secreted between fibroblasts isolated from CAV1
-/- mice, and WT fibroblasts (Sotgia et al., 2009) opens up a multitude of additional regulatory mechanisms that may play a role in the interplay between cancer cells and their microenvironment.

The functions of CAV1 in the cell appear to be diverse. Caveolae have been shown to be involved in endocytosis, cholesterol homeostasis, cellular signalling and mechanosensing (Bastiani and Parton, 2010) and a role in cancer may be associated with each of these functions. Moreover, CAV1 outside of caveolae may have different functions to CAV1 associated with caveolae. Therefore, the role of CAV1 in cancer is a complex one, potentially involving multiple mechanisms related to different roles of CAV1 in the cell. The aim of the research described in this thesis was to investigate the effect of interactions between fibroblasts, breast cancer cells, and (differentiated) monocytes on fibroblastic CAV1 levels using a co-culture system, as well as to investigate the effect of loss of fibroblastic CAV1 on breast cancer cell EMT in vitro.
Chapter 2: Materials and methods
Isolation of primary murine fibroblasts

Primary murine fibroblasts were harvested from the mammary fat pads of female BALB/c mice, aged 5-15 weeks. Procedures were performed in accordance with Deakin University animal ethics approval number AEX03-2013. For each batch of fibroblasts, a single mouse was euthanized with isoflurane (MilliporeSigma, Darmstadt, Germany), and the 2 inguinal fat pads were separated from the peritoneal wall and skin. The mammary fat pads were minced in 1 mL phosphate-buffered saline (PBS) (Amresco, Solon, OH, USA) with 2% (v/v) foetal bovine serum (FBS) (Bovogen Biologicals, East Keilor, VIC, Australia), and penicillin (100 units/mL) (Thermo Fisher Scientific, Waltham, MA, USA) and streptomycin (100 units/mL) (Thermo Fisher Scientific, Waltham, MA, USA). The tissue was digested with 5 mL Dulbecco's Modified Eagle Medium/F-12 (DMEM/F12, Thermo Fisher Scientific, Waltham, MA, USA) with collagenase I (3 mg/mL) (MilliporeSigma, Darmstadt, Germany) and dispase (4 mg/mL) (Thermo Fisher Scientific, Waltham, MA, USA), at 37 °C for 1 hour, with agitation. The digested tissue was centrifuged for 5 minutes at 300xg. The pellet was resuspended in DMEM/F12 with penicillin (100 units/mL) and streptomycin (100 units/mL). The cell suspension was centrifuged twice for the duration required for the centrifuge to reach 400xg, and the pellet was resuspended in 10 mL DMEM/F12 with penicillin (100 units/mL) and streptomycin (100 units/mL) each time; fibroblast fractions were isolated by collecting the supernatant after each centrifugation. Fibroblast fractions were combined, and centrifuged at 300xg for 5 minutes. The pellet was resuspended in 15 mL DMEM/F12 with 10% (v/v) FBS, penicillin (100 units/mL), streptomycin (100 units/mL), insulin (0.2 units/mL) (Eli Lilly Australia), and basic fibroblast growth factor (FGF2) (1 ng/mL) (Thermo Fisher Scientific, Waltham, MA, USA), and transferred to a culture flask. Murine fibroblasts
were cultured at 37°C with 5% CO₂ and 5% O₂ for at least one week prior to use in experiments.

**Cell culture**

67RN, 168FARN, 66CL4 and 4T1.2 murine cancer cells were a kind gift from Dr Robin Anderson, and were cultured in α-MEM (Thermo Fisher Scientific, Waltham, MA, USA) with 5% foetal bovine serum (FBS) (Bovogen Biologicals, East Keilor, VIC, Australia). hTERT-BJ immortalized human skin fibroblasts were a kind gift from Dr Robin Anderson, and were cultured in DMEM/199 (4:1) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS. PMC42-LA human breast cancer cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS. MCF7 and MDA-MB-231 human breast cancer cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS. Fre85 primary human breast fibroblasts were a kind gift from Dr Lily Huschtscha, and were cultured in DMEM with 10% FBS. THP-1 human leukemic monocytes were a kind gift from Dr Robin Anderson, and cultured in RPMI with 10% FBS. All cells were cultured at 37°C with 5% CO₂, in a humidified incubator. For subculturing, cell lines were detached by incubation with 0.05% trypsin/EDTA (Thermo Fisher Scientific, Waltham, MA, USA).

**Macrophage differentiation**

THP-1 cells, at a cell concentration of 0.5-1x10⁶ cells/mL, were treated with 100 nM phorbol 12-myristate 13-acetate (MilliporeSigma, Darmstadt, Germany) (200 µM stock in dimethyl sulfoxide (DMSO, MilliporeSigma, Darmstadt, Germany)) in RPMI
(Thermo Fisher Scientific, Waltham, MA, USA) for 18-24 hours. Carrier-treated THP-1 cells were used as a control in experiments.

**Co-cultures**

For non-contact co-culture, $6 \times 10^5$ fibroblasts were seeded on a 24 mm diameter, 0.4 µm pore, polyester Transwell permeable support (Corning Life Sciences, Corning, NY, USA), while $3 \times 10^5$ breast cancer cells and/or monocytes were seeded on the bottom of the 9 mm$^2$ well of a 6-well plate, with 4 mL of culture medium with 10% (v/v) foetal bovine serum (FBS) (Bovogen Biologicals, East Keilor, VIC, Australia).

See Figure 1. The cells were incubated for 3 days at 37°C, with 5% CO$_2$.

**Figure 1:** A schematic overview of non-contact co-culture of fibroblasts with cancer cells and (differentiated) monocytes. Transwell permeable supports were inserted into the 9 mm$^2$ well of a 6-well plate. (A) For the monoculture control fibroblasts were seeded on the Transwell permeable support. (B) For the non-contact co-culture fibroblasts were seeded on the Transwell permeable support and cancer cells and (differentiated) monocytes were seeded in the bottom of the well.
For mixed-non-contact co-cultures, $6 \times 10^5$ fibroblasts were seeded on a 24 mm diameter, 0.4 µm pore, polyester Transwell permeable support, while $6 \times 10^5$ fibroblasts were seeded in the bottom of the 9 mm$^2$ well of a 6-well plate, with $3 \times 10^5$ breast cancer cells and/or monocytes. Four mL of culture medium with 10% (v/v) FBS was added to the well. The cells were incubated for 3 days at 37°C, with 5% CO$_2$.

For non-contact co-culture with minimum separation between fibroblasts and cancer cells/monocytes, $6 \times 10^5$ breast cancer cells were seeded on an inverted, 24 mm diameter, 0.4 µm pore, polyester Transwell permeable support, sitting in a petri dish containing culture medium. After overnight incubation at 37°C, with 5% CO$_2$, the Transwell permeable support was placed into the well of a 6-well plate, and $6 \times 10^5$ fibroblast were seeded on the Transwell permeable support, on the side opposite to the breast cancer cells. Cells were incubated for 3 days at 37°C, with 5% CO$_2$.

For contact co-cultures, fibroblasts and cancer cells/monocytes were seeded together at 2:1 or 6:1 fibroblast to cancer cell and/or monocyte ratios. The total number of cells seeded was $6 \times 10^5$ cells per 9 mm$^2$ well of a 6-well plate. The cells were incubated at 37°C, with 5% CO$_2$, for 5 days.

For experiments in serum replacement medium, the cells were washed twice with phosphate-buffered saline (PBS) (Amresco, Solon, OH, USA), 18-24 hours after the
cells were seeded, and 4 mL of medium containing Serum Replacement 1 (MilliporeSigma, Darmstadt, Germany) was added.

Serum starvation

Fre85 cells were grown under normal conditions, in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) with 10% foetal bovine serum (FBS) (Bovogen Biologicals, East Keilor, VIC, Australia). Cells were harvested with 0.05% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA), and seeded at 6x10^5 cells per 9 cm² well in DMEM/F12 with 10% FBS. After 18-24 hours, cultures were washed twice with phosphate-buffered saline (PBS) (Amresco, Solon, OH, USA), and cultured in DMEM/F12 with 0.2% FBS for 24 hours.

Heat-inactivation of serum

Fetal bovine serum (FBS) (Bovogen Biologicals, East Keilor, VIC, Australia) was incubated at 56°C, for 30 minutes, with gentle mixing every 5 minutes. Aliquots of heat-inactivated serum were stored at -20°C until use.

Western analysis

Cells in experimental and control wells were washed twice with phosphate-buffered saline (PBS) (Amresco, Solon, OH, USA), and lysed in a buffer containing 10 mM Tris, pH 7.5 (Biochemicals, Gymea, NSW, Australia) and 1% (w/v) sodium dodecyl sulfate (SDS) (Amresco, Solon, OH, USA), using 150 μL per 9 cm² well. After the addition of complete mini protease inhibitor cocktail (Roche, Mannheim, Germany),
as per manufacturer instructions, the lysates were passed through a 20G needle five times, and sonicated for 30 seconds on a Microson XL ultrasonic cell disruptor (Misonix, Farmingdale, NY, USA), at 12.5% of maximum output power. Protein concentrations were determined by the Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA), as per manufacturer instructions.

Proteins were heat-denatured at 95°C, for 5 minutes, in sample loading buffer containing 17 mM Tris, pH 6.8; 0.4% (w/v) SDS, 3% (v/v) glycerol (Ajax Finechem, Taren Point, NSW, Australia), 0.17 mg/mL bromophenol blue (MilliporeSigma, Darmstadt, Germany), and 0.1M β-mercaptoethanol (MilliporeSigma, Darmstadt, Germany). Proteins were separated on a 12% SDS-polyacrylamide gel, and transferred to a 0.45-µm pore-size nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% (w/v) skim milk powder in Tris-buffered saline (50 mM Tris, pH 7.6; 150 mM NaCl (MilliporeSigma, Darmstadt, Germany)) (TBS), for 1 hour at room temperature. The membranes were incubated with the appropriate primary antibody, diluted in TBS, overnight, at 4°C. Following four 10-minute washes with TBS, the membranes were incubated with the appropriate HRP-conjugated secondary antibody, diluted in TBS, for 1 hour at room temperature. Following four 10-minute washes with TBS, the membranes were incubated with HRP chemiluminescent substrate (MilliporeSigma, Darmstadt, Germany), and imaged on a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

For re-probing, membranes were stripped with ReBlot Plus Strong (MilliporeSigma, Darmstadt, Germany), for 5 minutes, at room temperature. Following blocking with
5% (w/v) skim milk powder in TBS, for 15 minutes, at room temperature, the membranes were incubated with the appropriate primary antibody, diluted in TBS, for 1 hour, at room temperature. Following four 10-minute washes in TBS, the membranes were incubated with the appropriate HRP-conjugated secondary antibody, diluted in TBS, for 30 minutes, at room temperature.

**Antibodies**

See Table 1 for antibodies used in Western analysis, immunofluorescence and flow cytometry.
Table 1: Antibodies used in Western analysis, immunofluorescence and flow cytometry.
The manufacturer details, and dilutions used for experiments, are shown.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Catalogue number</th>
<th>Dilution in Western analysis</th>
<th>Dilution in immunofluorescence</th>
<th>Dilution in flow cytometry</th>
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<td>Ms-α-CD24-PE</td>
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<td>Sc-11397</td>
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Quantitative polymerase chain reaction

Total RNA was extracted from cell pellets using the RNeasy mini kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Synthesis of cDNA was performed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR (qPCR) was performed with Power SYBR Green master mix (Thermo Fisher Scientific, Waltham, MA, USA), 1 µM forward and reverse primers, and 20 ng cDNA. The sequences of the primers that were used for qPCR are described in Table 2. The program used was 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A melting curve was included in each run (65-95°C, 0.5°C/cycle, 5 seconds). The qPCR was performed on a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA), and data was analysed using CFX manager software (Bio-Rad, Hercules, CA, USA).

Table 2: Forward and reverse primers used in qPCR. The primer sequences for the genes targeted in qPCR are shown.

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<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
<tbody>
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<td>Mouse CAV1</td>
<td>5’ GACCCCAAGCATCTCAACGA 3’</td>
<td>5’ GTTCTGCAATCACATCTTCAAAG 3’</td>
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<tr>
<td>Human CAV1</td>
<td>5’ ACCTCAACGATGACGTGTC 3’</td>
<td>5’ ACAGTAAGGTGAGAAGGCTGG 3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’ CACCATTGGCAATGACGGGTT 3’</td>
<td>5’ AGGTCTTTTGGATGTCCAGG 3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’ GCGAGATCCCTCAAAATCAA 3’</td>
<td>5’ ATGGTTCACCCCATGACGA 3’</td>
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</tbody>
</table>
Confocal microscopy

Fibroblasts (hTERT-BJ or Fre85), breast cancer cells (MCF7 or MDA-MB-231) and/or monocytes/macrophages (undifferentiated or differentiated THP-1) were grown on 12 mm diameter coverslips in 2 cm² wells (1x10⁵ cells per well), or Transwell permeable supports (Corning Life Sciences, Corning, NY, USA), in 9 cm² wells (6x10⁵ cell per well), for 3 to 5 days, and washed twice with phosphate-buffered saline (PBS) (Amresco, Solon, OH, USA). Cells were fixed in 4% (w/v) paraformaldehyde (MilliporeSigma, Darmstadt, Germany) in PBS, for 10 minutes, at room temperature. After fixing, coverslips and permeable supports were washed twice with PBS, and stored in 0.05% (w/v) sodium azide (MilliporeSigma, Darmstadt, Germany) in PBS, at 4°C, until staining.

Cells were permeabilized with 0.1% (v/v) Triton X-100 (MilliporeSigma, Darmstadt, Germany) in PBS, for 5 minutes, and blocked in 1% (w/v) bovine serum albumin (BSA) (MilliporeSigma, Darmstadt, Germany) in PBS, for 10 minutes, both at room temperature. Then the cells were incubated with the primary antibody diluted in 1% (w/v) BSA in PBS, overnight, at 4°C (see Table 1 for antibodies and dilutions). Cells were subsequently incubated with the appropriate Alexa Fluor-conjugated secondary antibody, for 2 hours, and with 1 µM TOPRO-3 nuclear stain (Thermo Fisher Scientific, Waltham, MA, USA), for 20 minutes, both at room temperature.

The cells were imaged on a Leica TCS SP2 AOBS laser scanning confocal microscope. Using ImageJ, an outline was drawn around the fibroblasts. The perimeter around the cell, the area, and the mean fluorescence were calculated, for the
selected cells and adjacent areas of background. The corrected total cell fluorescence (CTCF) was calculated. CTCF = integrated density – (area of selected cell × mean fluorescence of background readings), as described by McCloy et al. (2014).

**Transfections**

Fre85 cells were harvested with 0.05% (w/v) trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA), and kept in a 37°C water bath until they were ready to be transfected.

**RNAiMAX:**

Transfection constructs were prepared in 500 µl Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA), by adding 30 pmol (lamin and non-targeting) or 75 pmol (CAV1) siRNA, and 5 µl Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA), and incubated for 10 minutes, at room temperature (see Table 3 for siRNAs used). A total number of 2.5x10^5 cells were seeded in 9 cm^2 wells, with 500 µl transfection construct, and 2.5 mL RPMI (Thermo Fisher Scientific, Waltham, MA, USA).

**siPORT:**

siPORT transfection reagent (5 µl) (Thermo Fisher Scientific, Waltham, MA, USA) was diluted in Opti-MEM (100 µl). siRNA was diluted, by adding 30 pmol (lamin and non-targeting) or 75 pmol (CAV1) siRNA, to 100 µl Opti-MEM. Diluted transfection
reagent was combined with diluted siRNA, and incubated for 10 minutes, at room
temperature (see Table 3 for siRNAs used). A total number of $2.0 \times 10^5$ cells were
seeded in 9 cm$^2$ wells, with 200 µl transfection construct, and 2.5 mL RPMI.

After 4-6 hours the cells were washed twice with phosphate-buffered saline (PBS)
(Amresco, Solon, OH, USA), and the medium was changed to 4 mL DMEM (Thermo
Fisher Scientific, Waltham, MA, USA) with Serum Replacement 1 (MilliporeSigma,
Darmstadt, Germany).

**Table 3: siRNAs used in transfection.** The manufacturer information for the siRNAs
used in experiments is shown.

<table>
<thead>
<tr>
<th>Target</th>
<th>Catalogue number</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>CAV1</td>
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<td>Lamin</td>
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<td>Non-targeting</td>
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<td>GE Dharmacon, Lafayette, CO, USA</td>
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</table>
PMc42-LA treatments with conditioned medium

PMc42-LA cells (6x10^5) were seeded in 9 cm^2 wells, with RPMI (Thermo Fisher Scientific, Waltham, MA, USA) with 10% (v/v) foetal bovine serum (FBS) (Bovogen Biologicals, East Keilor, VIC, Australia). After 18-24 hours, the medium was changed to 0.2-µm filter-sterilised conditioned medium, from 24-hour transfections of Fre85 with CAV1 siRNA. Cells were incubated for 7 days, at 37°C, with 5% CO_2.

Flow cytometry

After 3- and 7-day treatments with conditioned medium, PMc42-LA cells were harvested with 0.05% (w/v) trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA). The resulting detached cells were washed with phosphate-buffered saline (PBS) (Amresco, Solon, OH, USA), and 1x10^6 cells were resuspended in 100 µl 0.5% (w/v) bovine serum albumin (BSA) (MilliporeSigma, Darmstadt, Germany) in PBS, containing fluorochrome-conjugated antibodies (see Table 1). Cell suspensions were incubated on ice, in the dark, for 30 minutes. The cells were then washed twice with 0.5% (w/v) BSA in PBS, and resuspended in 500 µl 0.5% (w/v) BSA and 1 µg/ml propidium iodide (MilliporeSigma, Darmstadt, Germany) in PBS. Cells were analysed on a BD FACSCanto II (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

Fluorescence-activated cell sorting

Fibroblasts (1x10^6 cells/mL) were labelled with 2 µM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFDA-SE) (MilliporeSigma, Darmstadt, Germany) in phosphate-buffered saline (PBS) (Amresco, Solon, OH, USA), for 10 minutes, at
The cells were then washed twice, with DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) with 10% foetal bovine serum (FBS) (Bovogen Biologicals, East Keilor, VIC, Australia), allowing 10 minutes between washes to let CFDA-SE to diffuse out of the cells. The labelled fibroblasts were seeded together with breast cancer cells and/or monocytes at a 2:1 fibroblast to cancer cell/monocyte ratio. A total number of $4 \times 10^6$ of cells was seeded in a 75 cm$^2$ flask, in DMEM/F12 with 10% FBS. Cells were incubated at 37°C, with 5% CO$_2$, for 3 days. After 18-24 hours, the medium was changed to DMEM/F12 with Serum Replacement 1 (MilliporeSigma, Darmstadt, Germany).

Cells were collected for cell sorting with 0.05% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA), and washed twice with PBS. Cells were resuspended in PBS with 0.5% BSA, and sorted with a BD FACSaria II (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

**Cytokine assay**

Conditioned medium (4 mL from $6 \times 10^5$ Fre85 cells grown in DMEM with Serum Replacement 1 (MilliporeSigma, Darmstadt, Germany) in 9 cm$^2$ wells) was collected from co-cultures, and monoculture controls, and centrifuged twice at 10,000xg. Undiluted conditioned medium, or conditioned medium diluted 1:5 with DMEM culture medium (Thermo Fisher Scientific, Waltham, MA, USA), was assayed on a Milliplex MAP human cytokine/chemokine magnetic bead panel (MilliporeSigma, Darmstadt, Germany), as per manufacturer instructions. Samples were assayed in duplicate. Quality controls, standards (range 3.2-10,000 pg/mL), and matrix solution
(control culture medium), were added to each plate in duplicate. The minimum detectable concentration plus 2 standard deviations was 4.6 pg/mL (EGF), 11.8 pg/mL (FGF-2), 15.0 pg/mL (GM-CSF), 1.1 pg/mL (IFN-γ), 1.6 pg/mL (IL-10), 1.9 pg/mL (IL-13), 17.1 pg/mL (IL-1RA), 1.0 pg/mL (IL-1β), 1.3 pg/mL (IL-6), 0.7 pg/mL (IL-8), 3.4 pg/mL (MCP-1), 6.2 pg/mL (MIP-1α), 1.1 pg/mL (TNFα), 47.9 pg/mL (VEGF), 0.7 pg/mL (PDGF-AA), 2.7 pg/mL (PDGF-AB/BB) and 1.9 pg/mL (RANTES). The assay was read on a Bio-Plex 200 (Bio-Rad, Hercules, CA, USA).

**Statistical analysis**

Statistical analyses were done by Student’s t-test. Statistical significance was defined as p<0.05
Chapter 3: Caveolin-1 expression in fibroblasts in non-contact co-culture with breast cancer cells and monocytes
Introduction

The microenvironment, or stroma, of a tumour plays an important role in the progression of cancer. Stromal cells in the vicinity of the tumour provide it with growth factors, remodel the extracellular matrix (ECM), and promote genetic instability in cancer cells (Bissell and Radisky, 2001). An important cell type in the microenvironment of breast tumours that has been well studied for its part in cancer progression and metastasis is the cancer-associated fibroblast (CAF). CAFs stimulate epithelial-to-mesenchymal transition (EMT) in nearby cancer cells through the secretion of TGF-β, promote angiogenesis through the release of VEGF, and remodel the ECM by the secretion of collagen, fibrin, growth factors, and matrix metalloproteinases (MMPs) (Kalluri, 2016; Madar et al., 2013).

The origin of CAFs is not clear. Studies have shown that breast fibroblasts can increase the expression of CAF markers after being cultured together with breast cancer cells (Martinez-Outschoorn et al., 2010b). Others have shown that autocrine signalling mediated by TGF-β and stromal cell-derived factor 1 (SDF-1) is involved with the transformation of resident fibroblasts into activated CAFs (Kojima et al., 2010). However, other sources of CAFs have also been described. Some CAFs are derived from endothelial cells through endothelial-to-mesenchymal transition (Van Meeteren and Ten Dijke, 2012; Zeisberg et al., 2007) while others are derived from cancer cells through EMT (Drake and Macleod, 2014).

CAFs are difficult to define, and comprise a heterogeneous population of fibroblasts in the tumour micro-environment (Madar et al., 2013). There is no known marker that is specific for the whole CAF population (Sugimoto et al., 2006), but commonly
used CAF markers are α-smooth muscle actin (αSMA), vimentin, and fibroblast-activation protein (Kalluri and Zeisberg, 2006; Shiga et al., 2015).

The stroma surrounding a carcinoma displays similarities to the stroma involved in wound healing, including increased numbers of fibroblasts, angiogenesis, and collagen and fibrin deposits, and is also referred to as reactive stroma (Kalluri and Zeisberg, 2006). Fibroblasts involved in wound healing are referred to as myofibroblasts, and secrete higher amounts of ECM components and proliferate faster (Kalluri and Zeisberg, 2006). A distinguishing feature of myofibroblasts is the expression of αSMA, and αSMA expression is a commonly used identifier of CAFs (Kalluri and Zeisberg, 2006). However, even though CAFs can display similarities to myofibroblasts, and myofibroblasts are often found in the tumour stroma (Kalluri and Zeisberg, 2006), not all CAFs express myofibroblast markers (Sugimoto et al., 2006).

CAFs from different subtypes of breast cancer express different levels of CAF markers. For example, expression of CAF markers is higher in fibroblasts from HER2-type breast cancer stroma, compared to other breast cancer subtypes, such as ER-positive and triple negative (Park et al., 2015). Additionally, the expression of CAF markers differs according to stromal histological phenotype; desmoplastic stroma, which is characterised by fibrosis, shows higher platelet-derived growth factor receptor (PDGFR) α and PDGFRβ expression, whereas inflammatory stroma shows higher fibroblast activation protein α (FAPα) expression (Park et al., 2015).
Macrophages in the tumour microenvironment

The association of inflammation with cancer has been known for a long time, but has become clearer in recent years. A number of studies show a predisposition to tumour development for certain chronic inflammatory conditions (Danese and Mantovani, 2010; Polk and Peek, 2010). An inflammatory microenvironment is increasingly accepted as a generic component of tumours (Hanahan and Weinberg, 2011). Tumour-associated macrophages (TAMs) are a major component of the infiltrating immune cells of tumours (Mantovani and Sica, 2010).

Despite the proinflammatory properties of macrophages, TAMs are protumorigenic and suppress T-cell responses (Noy and Pollard, 2014). In addition, TAMs play a role in matrix remodelling, promote invasiveness and intravasation at the tumour site through the production of MMPs, reactive oxygen species (ROS), and other factors (Condeelis and Pollard, 2006). This apparent contradiction of both tumoricidal and tumorigenic properties of macrophages was resolved with the identification of two distinct populations of macrophages: ‘classically’ activated (M1) macrophages and ‘alternatively’ activated (M2) macrophages (Mantovani et al., 2004). M1 macrophages are proinflammatory and tumoricidal, whereas M2 macrophages are protumorigenic (Comito et al., 2014). TAMs have been shown to have an M2 phenotype, caused by a lack of M1 stimuli and the presence of M2 stimuli in the tumour microenvironment (Sica et al., 2006; Tang, 2013).

CAF\(s\) express a proinflammatory gene signature (Erez et al., 2010). A study by Comito et al. (2014) has shown that CAF\(s\) attract monocytes and induce their
differentiation into macrophages, *in vitro*. This highlights the important interplay between CAFs and TAMs in tumorigenesis.

**Caveolin-1 and CAFs**

Several studies have reported a reduced caveolin-1 (CAV1) expression in CAFs. For example, Mercier et al. (2008) measured lower CAV1 levels in fibroblasts isolated from breast tumour biopsies, compared to fibroblasts isolated from adjacent unaffected tissue, for 8 out of 11 invasive ductal carcinoma patients. A study that evaluated the CAV1 levels in $\alpha$SMA-positive cells in patient biopsies showed that a loss of CAV1 levels predicts reduced disease-free survival (Simpkins et al., 2012). In addition to low CAV1 expression being a characteristic of CAFs, fibroblasts that express lower levels of CAV1, have been shown to display a CAF-like phenotype. Gene expression profiling of primary mammary fibroblasts isolated from CAV1 -/- mice, compared to wild type mice, showed a significant overlap between the transcriptome of CAV1 -/- fibroblasts and CAFs (Sotgia et al., 2009). In addition, the transfection of primary breast fibroblasts with CAV1 siRNA has been shown to induce fibroblastic $\alpha$SMA and vimentin expression (Martinez-Outschoorn et al., 2010b), and promote breast cancer cell invasion (Simpkins et al., 2012).

It is currently not known what the mechanism is behind the loss of CAV1 expression in the tumour microenvironment. It is possible that breast cancer cells induce normal fibroblasts to downregulate CAV1 expression. Alternatively, breast cancer cells may be responsible for inducing low-CAV1 expressing CAFs through EMT or endothelial-to-mesenchymal transition. However, it is also possible that cancer cells indirectly, rather than directly, induce the downregulation of CAV1 in CAFs. As CAFs and
cancer cells have been shown to attract macrophages to the tumour microenvironment, it is possible that macrophages play a role in the downregulation of CAV1 in CAFs.

Aims

The main aim of this study was to determine whether a paracrine interaction between tumour cells and fibroblasts influences the expression of CAV1 in fibroblasts, *in vitro*.

The specific aims were:

- To determine whether an effect of breast cancer cells on fibroblastic CAV1 levels was tissue-specific.

- To investigate whether an effect of breast cancer cells on fibroblastic CAV1 levels was dependent on the invasiveness of the cancer cells.

- To establish whether oxygen levels during co-culture had an effect on fibroblastic CAV1 levels.

- To investigate a potential role of differentiated monocytes in influencing fibroblastic CAV1 levels.
• To determine whether paracrine signalling by contact co-cultures of fibroblasts, cancer cells and differentiated monocytes had an effect on CAV1 levels in physically separated fibroblasts.

Results

Mouse non-contact co-culture system

A mouse non-contact co-culture system was developed by culturing primary murine fibroblasts together with, but physically separated from, murine mammary cancer cells. The mouse non-contact co-culture system showed large variability in CAV1 levels between experiments. CAV1 mRNA levels after non-contact co-culture of primary murine fibroblasts with different murine mammary cancer cell lines varied between experiments from almost completely non-detectable to slightly higher than control levels. Analysis of the data from 3 separate experiments, using 3 different mice shows that the variability in CAV1 mRNA and protein levels in fibroblasts after co-culture was larger between individual mice than between different cancer cell lines.

Murine fat pad fibroblasts were co-cultured for 48 hours, in a non-contact fashion, with murine cancer lines with different invasive capacities. After non-contact co-culture of murine fibroblasts with 67NR cancer cells, fibroblastic CAV1 mRNA levels were 0.94 ± 0.33 fold the monoculture control (Fig. 1A). After non-contact co-culture of murine fibroblasts with 168FARN cancer cells, fibroblastic CAV1 mRNA levels were 0.79 ± 0.25 fold the monoculture control (Fig. 1A). After non-contact co-culture of murine fibroblasts with 66cl4 cancer cells, fibroblastic CAV1 mRNA levels were 0.82 ± 0.39 fold the monoculture control (Fig. 1A). After non-contact co-culture
of murine fibroblasts with 4T1.2 cancer cells, fibroblastic CAV1 mRNA levels were
0.83 ± 0.22 fold the monoculture control (Fig. 1A).

Western blot analysis to measure CAV1 protein showed a similarly large variability
between experiments (Fig. 1B). After non-contact co-culture of murine fibroblasts
with 67NR cancer cells, fibroblastic CAV1 protein levels were 1.10 ± 0.41 fold the
monoculture control (Fig. 1B). After non-contact co-culture of murine fibroblasts
with 168FARN cancer cells, fibroblastic CAV1 protein levels were 1.26 ± 0.37 fold
the monoculture control (Fig. 1B). After non-contact co-culture of murine fibroblasts
with 66cl4 cancer cells, fibroblastic CAV1 protein levels were 1.05 ± 0.35 fold the
monoculture control (Fig. 1B). Fibroblastic CAV1 protein levels were decreased after
non-contact co-culture of murine fat pad fibroblasts with 4T1.2 (0.32 ± 0.04 fold, 
p<0.05), the most invasive cancer cell line (Fig. 1B). Co-culture of embryonic mouse
fibroblast cell line NIH/3T3 with murine cancer cell lines 67NR, 168FARN, 66cl4,
and 4T1.2 did not change CAV1 mRNA or protein levels in fibroblasts (data not
shown). Figure 1C shows a representative Western image, and Figure 1D shows a
schematic representation of the non-contact co-culture system.
Figure 1: Non-contact co-culture of murine primary mammary fibroblasts (NMF) with a non-invasive (67NR), a mildly invasive (168FARN), an invasive (66cl4) and a highly invasive (4T1.2) murine breast cancer cell line at 20% oxygen. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 against control (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. No significant changes in fibroblastic CAV1 mRNA levels were detected after 48 hours of co-culture. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Fibroblastic CAV1 protein levels were significantly reduced after 48 hours of co-culture with 4T1.2 cancer cells. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. (D) Schematic representation of the non-contact co-culture system. On the left, the monoculture control is shown, on the right, the non-contact co-culture is shown.
Tissue oxygen levels are lower than ambient oxygen levels. The increased oxygen levels that cells are exposed to in ambient oxygen cell culture incubators have been shown to affect the physiological responses of cells. To determine whether oxygen levels had an effect on fibroblastic CAV1 levels in the co-culture system, experiments were carried out at 5% and 20% oxygen levels. When physiological oxygen levels (5% O2) were used for incubation, instead of the ambient oxygen levels regularly used in cell culture, the decrease of fibroblastic CAV1 protein levels seen in Figure 1 did not occur (Fig. 2). CAV1 mRNA levels after non-contact co-culture of primary murine fibroblasts with different murine cancer cell lines for 48 hours showed large variability between experiments (Fig. 2A). Differences in fibroblastic CAV1 mRNA after non-contact co-culture of murine fat pad fibroblasts with 67NR (1.11 ± 0.19), 168FARN (1.15 ± 0.31), 66cl4 (1.14 ± 0.37), and with 4T1.2 (0.92 ± 0.06) murine cancer cells were not significant compared to the monoculture control (Fig. 2A). Fibroblastic CAV1 protein levels after non-contact co-culture of murine fat pad fibroblasts with 67NR (0.97 ± 0.10), 168FARN (1.06 ± 0.12), 66cl4 (1.09 ± 0.03), and with 4T1.2 (1.00 ± 0.16) murine cancer cells were not significantly different from the monoculture control (Fig. 2B). Figure 2C shows a representative Western image.
Figure 2: Non-contact co-culture of murine primary mammary fibroblasts (NMF) with a non-invasive (67NR), a mildly invasive (168FARN), an invasive (66cI4) and a highly invasive (4T1.2) murine breast cancer cell line at 5% oxygen. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. No significant changes in fibroblastic CAV1 mRNA levels were detected after 48 hours of co-culture. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in fibroblastic CAV1 protein levels were detected after 48 hours of co-culture. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control.

As large variability in CAV1 levels was observed between experiments, baseline CAV1 protein levels were assessed in mammary fibroblasts from 4 different BALB/c mice. The mouse with the highest CAV1 levels generated a 7.4 times stronger CAV1 band in Western analysis, compared to the mouse with the lowest CAV1 levels (Fig. 3). The other two mice generated bands that were 2.6 and 3.8 times stronger than the mouse with the lowest CAV1 levels (Fig. 3).
Figure 3: Monocultures of primary breast fibroblasts from different mice. Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Up to 7 fold differences in CAV1 protein levels were detected in 4 different mice. Results are representative of a single measurement.

**Human non-contact co-culture system**

The effects of different human fibroblast cell lines on human breast cancer cell lines were assessed in a non-contact co-culture system. Non-contact co-culture, for 3 days, of immortalised skin fibroblasts hTERT-BJ with different breast cancer cell lines, MCF7, and MDA-MB-231, did not result in significant changes in fibroblastic CAV1 mRNA levels (Fig. 4). Fibroblastic CAV1 mRNA levels were 0.97 ± 0.11 fold the monoculture control after non-contact co-culture with MCF7 breast cancer cells, and 1.12 ± 0.09 fold with MDA-MB-231 breast cancer cells (Fig. 4A).

Fibroblastic CAV1 protein levels after non-contact co-culture of hTERT-BJ with breast cancer cell line MCF7 were 1.38 ± 0.22 fold the monoculture control (Fig. 4B, C). Conversely, hTERT-BJ CAV1 protein levels were significantly increased (1.69 ± 0.27, p<0.05) after non-contact co-culture with MDA-MB-231 (Fig. 4B). Figure 4C shows a representative Western analysis image.
In addition to the invasive breast cancer cell lines MCF7 and MDA-MB-231, hTERT-BJ was co-cultured with the non-invasive breast cancer cell line PMC42-LA and no significant changes in fibroblastic CAV1 mRNA and protein levels were detected (data not shown).

Figure 4: Non-contact co-culture of human immortalized skin fibroblasts (hTERT-BJ) with a mildly invasive (MCF7) and a highly invasive (MDA-MB-231) human breast cancer cell line. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 against control (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. No significant changes in fibroblastic CAV1 mRNA levels were detected after 3 days of co-culture. Results are representative of 7 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Fibroblastic CAV1 protein levels were significantly increased after 3 days of co-culture with MDA-MB-231. Results are representative of 6 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. BJ=hTERT-BJ, MCF=MCF7, MDA=MDA-MB-231

Primary fibroblast cell line Fre85 showed no significant changes in CAV1 mRNA after 3 days of non-contact co-culture with breast cancer cell lines MCF7 and MDA-
MB-231, when compared to a monoculture of Fre85 fibroblasts (Fig. 5A). Fibroblastic CAV1 mRNA levels after non-contact co-culture of Fre85 fibroblasts with mildly invasive breast cancer line MCF7 were 0.91 ± 0.16 fold the monoculture control, and with highly invasive breast cancer line MDA-MB-231 were 0.84 ± 0.09 fold the monoculture control (Fig. 5A).

Fibroblastic CAV1 protein levels after non-contact co-culture with MCF7 were 1.27 ± 0.34 fold the monoculture control (Fig. 5B). Fibroblastic CAV1 protein levels after non-contact co-culture with MDA-MB-231 were 1.29 ± 0.46 fold the monoculture control (Fig. 5B). These changes were not statistically significant. Figure 5C shows a representative Western analysis image.

In addition to the invasive breast cancer cell lines MCF7 and MDA-MB-231, Fre85 was co-cultured with the non-invasive breast cancer cell line PMC42-LA and no significant changes in fibroblastic CAV1 mRNA and protein levels were detected (data not shown).
Non-contact co-culture with (differentiated) monocytes

THP-1 human monocytes were differentiated with 12-<i>O</i>-Tetradecanoylphorbol-13-acetate (TPA), and introduced into the non-contact co-culture system of fibroblasts with MCF7 and MDA-MB-231. The THPT cells were washed twice before inclusion in the co-culture system, to remove any traces of TPA. Additionally, fibroblasts were treated with 1 nM and 100 nM TPA and no significant effect on CAV1 levels was found (data not shown).
Non-contact co-culture with carrier-treated THP-1 cells (THPD) increased CAV1 mRNA 1.44 ± 0.10 fold (p<0.05) in hTERT-BJ immortalized human skin fibroblasts, compared to the monoculture control (Fig. 6A). Co-culture with TPA-treated THP-1 cells (THPT) did not result in changes in CAV1 mRNA relative to monoculture control (0.93 ± 0.12 fold, Fig. 6A). Fibroblastic CAV1 mRNA levels after co-culture with MCF7 and THPD, and MDA-MB-231 and THPD, were 1.18 ± 0.25 and 1.08 ± 0.15 fold the monoculture control, respectively, while fibroblastic CAV1 mRNA levels after co-culture with MCF7 and THPT, and MDA-MB-231 and THPT, were 0.86 ± 0.19 and 0.82 ± 0.12 fold the monoculture control, respectively (Fig. 6A).

Fibroblastic CAV1 protein levels after 3 days of non-contact co-culture of hTERT-BJ with MCF7 or MDA-MB-231 breast cancer cells, in the presence of TPA- or carrier-treated THP-1 cells, were not significantly different compared to the fibroblast monoculture control. Non-contact co-culture of hTERT-BJ with THPD led to fibroblastic CAV1 protein levels that were 2.14 ± 0.45 fold the monoculture control, and with THPT fibroblastic Cav1 levels were 1.87 ± 0.32 fold the monoculture control (Fig. 6B). In the presence of both MCF7 and THPD, fibroblastic CAV1 protein levels were 2.69 ± 1.03 fold the monoculture control, and fibroblastic CAV1 protein levels after co-culture with MCF7 and THPT were 2.04 ± 0.33 fold the monoculture control (Fig. 6B). Fibroblastic CAV1 protein levels after non-contact co-culture with MDA-MB-231 and THPD were 1.80 ± 0.81, and with MDA-MB-231 and THPT were 2.13 ± 0.91 fold the monoculture control. Figure 6C shows a representative Western image, and Figure 6D shows a schematic representation of the non-contact co-culture system.
Figure 6: Non-contact co-culture of human immortalised skin fibroblasts (hTERT-BJ) with a mildly invasive (MCF7) or a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 against control (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. Fibroblastic CAV1 mRNA levels were significantly increased after 3 days of co-culture with undifferentiated THP-1 cells (THPD). Results are representative of 4 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in fibroblastic CAV1 protein levels were detected after 3 days of coculture. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. (D) Schematic representation of the non-contact co-culture system. On the left, the monoculture control is shown, on the right, the non-contact co-culture is shown. BJ=hTERT-BJ, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1
In Fre85 fibroblasts co-cultured with THPT cells, CAV1 mRNA levels were 0.65 ± 0.14 fold the monoculture control, and in fibroblasts co-cultured with THPD cells, levels were 0.91 ± 0.05 fold the monoculture control (Fig. 7A). These changes were not significant. Fibroblastic CAV1 mRNA levels after non-contact co-culture with MCF7 and THPD were 0.74 ±0.12, and with MCF7 and THPT 0.56 ± 0.18, fold the monoculture control. When Fre85 was co-cultured with both MDA-MB-231 and THPT (0.44 ± 0.14), or MDA-MB-231 and THPD (0.59 ± 0.09), fibroblast CAV1 mRNA levels were lower than when co-cultured with MDA-MB-231 cancer cells alone, and significantly lower than the monoculture control (Fig. 5A, 7A). This same trend was seen for co-cultures of Fre85, MCF7 and THP-1 (Fig. 5A, 7A), but the decrease of fibroblastic mRNA in this triple-culture was not significant.

Fibroblastic CAV1 protein levels, similar to mRNA levels, but in contrast to hTERT-BJ CAV1 protein levels, showed a trend of decrease after 3 days of non-contact co-culture with breast cancer cells and TPA-treated THP-1 cells (Fig. 7B). Fibroblastic CAV1 protein levels after non-contact co-culture with THPD were 0.90 ± 0.16 fold the monoculture control, and after culture with THPT CAV1 levels were 0.62 ± 0.24 fold the monoculture control (Fig. 7B). CAV1 protein levels in Fre85 after co-culture with MCF7 or MDA-MB-231 cancer cells, and THPD (triple culture), were 1.16 ± 0.61 and 1.24 ± 0.62 fold the monoculture control, respectively, and with MCF7 or MDA-MB-231, and THPT were 0.85 ± 0.24 and 0.94 ± 0.32, respectively (Fig. 7B). These changes were not significant. Figure 7C shows a representative Western image.
Figure 7: Non-contact co-culture of human primary breast fibroblasts (Fre85) with a mildly invasive (MCF7) or a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 against control (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. Fibroblastic CAV1 mRNA levels were significantly decreased after 3 days of co-culture with MDA-MB-231 and THPD, and after co-culture with MDA-MB-231 and THPT. Results are representative of 4 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in fibroblastic CAV1 protein levels were detected after 3 days of co-culture. Results are representative of 4 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

Non-contact co-cultures showed high variability in CAV1 levels between experiments. Alternative reference genes were assessed for use in qRT-PCR for the determination of mRNA levels in fibroblasts in the co-culture system. Average expression levels were determined for reference genes after different co-culture conditions and the variability in expression levels was calculated as the standard
deviation from the average Ct. GAPDH had half the standard deviation as a percentage of the average Ct (% of AVE), compared to β-actin (1.51% versus 2.96%, Table 1), and was selected for continued use as a reference gene.

Table 1: Average Ct of housekeeping genes measured by qPCR in Fre85 human primary breast fibroblasts after non-contact co-culture. The average (AVE) and standard deviation (STDEV) were calculated from the Ct for different co-cultures. The standard deviation of the Ct was calculated as a percentage of the average Ct (%AVE). The average of 3 experiments is shown. The data show that GAPDH is more stable across the different co-culture conditions.

<table>
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<th>Housekeeping gene</th>
<th>AVE Ct</th>
<th>Average STDEV</th>
<th>Average STDEV as %AVE</th>
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<tr>
<td>GAPDH</td>
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<td>0.21</td>
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Confocal microscopy using fluorescently tagged antibodies was used to confirm changes in CAV1 protein levels seen with Western analysis. Interestingly, confocal microscopy showed lower CAV1 protein levels in Fre85 fibroblasts after 3 days of non-contact co-culture with breast cancer cells and THPT or THPD (triple culture), compared to co-culture with cancer cells alone (Fig. 8), which is consistent with the lower mRNA levels seen in Figure 7, however, the fibroblastic CAV1 levels after co-culture with cancer cells appeared higher compared to the monoculture control. CAV1 staining in Fre85 fibroblasts in the monoculture control (A), or after non-contact co-culture with MCF7 (B), MDA-MB-231 (C), THPD (D), THPT (E), MCF7 and THPD (F), MCF7 and THPT (G), MDA-MB-231 and THPD (H), and MDA-MB-231 and THPT (I), can be seen in Figure 8. Quantification of the CAV1 fluorescent signal is shown in Figure 8J.
Figure 8: Immunofluorescence microscopy of non-contact co-culture of human primary breast fibroblasts (Fre85) with a mildly invasive (MCF7) or a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line. After 3 days of co-culture, cells were fixed and immuno-labelled with antibodies against CAV1 (green). Nuclei were stained with ethidium bromide (red). Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) Mono-culture of human primary breast fibroblasts (Fre85). (B) Human primary breast fibroblasts (Fre85) were co-cultured with a mildly invasive human breast cancer cell line (MCF7). (C) Human primary breast fibroblasts (Fre85) were co-cultured with a highly invasive human breast cancer cell line (MDA-MB-231). (D) Human primary breast fibroblasts (Fre85) were co-cultured with carrier-treated THP-1 cells (THPD). (E) Human primary breast fibroblasts (Fre85) were co-cultured with TPA-treated THP-1 cells (THPT). Fre=Fre85, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1
Figure 8 continued: Immunofluorescence microscopy of non-contact co-culture of human primary breast fibroblasts (Fre85) with a mildly invasive (MCF7) or a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line. After 3 days of co-culture, cells were fixed and immuno-labelled with antibodies against CAV1 (green). Nuclei were stained with ethidium bromide (red). Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (F) Human primary breast fibroblasts (Fre85) were co-cultured with a mildly invasive human breast cancer cell line (MCF7) and carrier-treated THP-1 cells (THPD). (G) Human primary breast fibroblasts (Fre85) were co-cultured with a mildly invasive human breast cancer cell line (MCF7) and TPA-treated THP-1 cells (THPT). (H) Human primary breast fibroblasts (Fre85) were co-cultured with a highly invasive human breast cancer cell line (MDA-MB-231) and carrier-treated THP-1 cells (THPD). (I) Human primary breast fibroblasts (Fre85) were co-cultured with a highly invasive human breast cancer cell line (MDA-MB-231) and TPA-treated THP-1 cells (THPT). (J) Mean total CAV1 fluorescence of fibroblasts in co-culture, based on 1 experiment, 2 separate images, 6 separate cells. Fre=Fre85, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

To examine whether physical contact between fibroblasts and cancer cells and differentiated monocytes would affect nearby fibroblasts in the same system, human
primary breast fibroblasts (Fre85) were cultured for 3 days in the presence of, but physically separated from, a contact co-culture of Fre85, MDA-MB-231 breast cancer cells, and THPT cells. Non-contact co-culture of Fre85 with MDA-MB-231 resulted in decreased fibroblastic CAV1 mRNA levels (0.61 ± 0.05 fold monoculture control, p<0.05) (Fig. 9A), but this reduction in mRNA levels did not translate into lower fibroblastic CAV1 protein levels (0.99 ± 0.04 fold the monoculture control, Fig. 9B). The reduction of fibroblastic CAV1 mRNA and protein levels was enhanced in the presence of THPT cells (mRNA levels were 0.21 ± 0.07 fold the monoculture control (p<0.01), and protein levels were 0.52 ± 0.12 fold the monoculture control, Fig. 9A,B), compared to non-contact co-culture of Fre85 with MDA-MB-231 alone. The reduction of fibroblastic CAV1 mRNA and protein levels was also slightly enhanced in the presence of, but physically separate from, a contact co-culture of fibroblasts and breast cancer cells compared to non-contact co-culture with breast cancer cells alone, however this change was not statistically significant (Fig. 9A,B). Figure 9C shows a representative Western image, and Figure 9D shows a schematic representation of the non-contact co-culture system.
Figure 9: Non-contact co-culture of human primary breast fibroblasts (Fre85) with contact co-cultures of Fre85 with a mildly invasive (MCF7) or a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 ** p<0.01 (A) CAV1 mRNA levels, relative to GAPDH levels, were detected using qRT-PCR. Fibroblastic CAV1 mRNA levels were significantly decreased after 3 days of non-contact co-culture with all of the cell populations tested. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Fibroblastic CAV1 protein levels were significantly decreased after 3 days of non-contact co-culture with a mixed population of Fre85 and THPT. Fibroblastic CAV1 protein levels were also significantly lower after non-contact co-culture with a mixed population of MDA-MB-231 and THPT, compared to non-contact co-culture with MDA-MB-231 alone. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MDA=MDA-MB-231, THPT=TPA-treated THP-1
**Figure 9 continued: Non-contact co-culture of human primary breast fibroblasts (Fre85) with contact co-cultures of Fre85 with a mildly invasive (MCF7) or a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line.** (D) Schematic representation of the non-contact co-culture system. On the left, the monoculture control is shown, on the right, the non-contact co-culture is shown.

**Fibroblastic αSMA expression**

A commonly used marker for the identification of activated fibroblasts, or myofibroblasts, is the expression of αSMA. Levels of αSMA in fibroblasts were assessed after 3 days of non-contact co-culture with cancer cells and differentiated monocytes, or with a mixed culture of cancer cells and differentiated monocytes. Although these culture conditions led to significant changes in fibroblastic CAV1 protein levels, significant changes in fibroblastic αSMA protein levels were not observed. Fibroblastic αSMA levels after non-contact co-culture of primary human breast fibroblasts Fre85 with MDA-MB-231 breast cancer cells were $1.05 \pm 0.14$ fold the monoculture control (Fig. 10A,B). When these fibroblasts were cultured in the presence of, but physically separated from, a co-culture of Fre85 fibroblasts and MDA-MB-231 cancer cells, fibroblastic αSMA protein levels were $1.30 \pm 0.24$ fold the monoculture control (Fig. 10A,B). Fibroblastic αSMA protein levels after non-contact co-culture of Fre85 fibroblasts with THPT cells were $0.81 \pm 0.04$ fold the monoculture control, which was just outside of statistical significance (Fig. 10A,B,
p=0.05). Fibroblastic αSMA protein levels after non-contact co-culture with a mixed Fre85 and THPT population were 1.09 ± 0.23 fold the monoculture control (Fig. 10A,B). Fibroblastic αSMA protein levels after non-contact co-culture with a mixed population of MDA-MB-231 and THPT were 1.01 ± 0.14 fold the monoculture control, and with a mixed population of Fre85, MDA-MB-231, and THPT, levels were 0.93 ± 0.15 fold the monoculture control (Fig. 10A,B).

Figure 10: αSMA levels in non-contact co-cultures of human primary breast fibroblasts (Fre85) with mixed cultures of human primary breast fibroblasts, a highly invasive breast cancer cell line (MDA-MB-231) and a differentiated monocytic cell line (THPT). Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) Western analysis of α-smooth muscle actin (αSMA) protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in fibroblastic αSMA protein levels were detected after 3 days of co-culture. Results are representative of 3 independent experiments. (B) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MDA=MDA-MB-231, THPT=TPA-treated THP-1
Discussion

A correlation between low CAV1 levels in the tumour stroma and cancer progression has been reported in several studies (Bonuccelli et al., 2010; Simpkins et al., 2012; Sloan et al., 2009; Witkiewicz et al., 2009b), however the mechanism through which CAV1 is downregulated in CAFs is unclear. The results presented in this chapter show that non-contact co-culture with some cancer cells and immune cells affects fibroblastic CAV1 levels, suggesting the involvement of a secreted factor. Additionally, the results presented in this chapter point to a potential role for paracrine signalling between fibroblasts and immune cells in the reduction of CAV1 in fibroblasts.

The data presented in this chapter show that fibroblasts respond differently to co-culture with different cancer cell lines, with the more invasive cancer cell lines generally eliciting a stronger change in fibroblastic CAV1 levels. After co-culture with MDA-MB-231 breast cancer cells, hTERT-BJ CAV1 protein levels were significantly increased to 1.69 (± 0.27, p<0.05) fold that of control levels, whereas co-culture with the more epithelial-like MCF7 cells led to fibroblastic CAV1 protein levels of 1.38 (± 0.22) fold control levels. Furthermore, co-culture of murine mammary fibroblasts with the invasive murine cancer cell line 4T1.2 resulted in a significant reduction of fibroblastic CAV1 protein levels to 0.32 (± 0.04, p<0.05) fold control levels, whereas co-culture with less invasive cancer lines led to fibroblastic CAV1 protein levels between 1.05 and 1.26 fold the control. This is consistent with in vivo data that show that low stromal CAV1 levels are associated with advanced tumour stage and metastasis (Witkiewicz et al., 2009b).
The mouse system

The use of mice allows for extensive *in vivo* analysis of the role of CAV1 in the tumour stroma. This chapter evaluated the usefulness of an *in vitro* murine co-culture model. Results from co-culture of murine primary fibroblasts with murine cancer cells showed large variability between mice. As all murine fibroblasts used were isolated from BALB/c mice, genetic variability would be limited compared to the variability expected between breast cancer patients. Despite the large variability between mice, the results presented in this chapter show a strong and statistically significant (0.32 ± 0.04, p<0.05) reduction of fibroblastic CAV1 protein levels after non-contact co-culture with highly invasive murine cancer cell line 4T1.2. CAV1 mRNA levels measured at the same time-point did not show any changes compared to the control. This suggests either that mRNA levels may have returned to normal levels after an initial reduction, or that the reduction in CAV1 protein levels is not regulated by changes in transcription. A study by Martinez-Outschoorn et al. (2010b) similarly found that a reduction of CAV1 protein levels after co-culture of immortalised hTERT-BJ skin fibroblasts with MCF7 breast cancer cells was not accompanied by a reduction of CAV1 mRNA levels. The authors further reported that pre-treatment with lysosome-specific inhibitors rescued CAV1 levels that were otherwise found to be reduced after co-culture with MCF7 cancer cells. This suggests that CAV1 reduction after contact co-culture is mediated by autophagic/lysosomal degradation, rather than changes in transcription (Martinez-Outschoorn et al., 2010b).

The human system

The use of cell lines reduces genetic variability even further, compared to using individual mice from the same strain. This can be advantageous when a stable system
is required to investigate mechanisms of cell signalling. Fibroblasts and breast cancer cells from a number of different origins were co-cultured and evaluated for their usefulness in a co-culture system. Immortalised skin fibroblasts hTERT-BJ responded differently to non-contact co-culture with breast cancer cells and monocytes, compared to primary breast fibroblasts. Where Fre85 primary breast fibroblasts showed no change in CAV1 mRNA and protein levels after non-contact co-culture with MDA-MB-231 breast cancer cells, hTERT-BJ cells showed an increase in CAV1 protein levels after non-contact co-culture with MDA-MB-231. Similarly, small differences were observed when CAV1 levels of a primary human breast fibroblasts line (Fre85) co-cultured with cancer cells were compared to that of a primary human skin fibroblast line (800211) (data not shown). There may, therefore, be a difference in the response of fibroblasts originating from different tissues. Furthermore, immortalised fibroblasts may behave differently compared to primary fibroblasts. In studies on both immortalised retinal pigment epithelial (hTERT-RPE) cells (Alge et al., 2006) and immortalised skin fibroblasts (hTERT-BJ) (Lindvall et al., 2003), results showed significant differences in expression patterns between the immortalised cells and their primary counterparts.

Non-contact co-culture of human fibroblasts and breast cancer cells has been reported as having no effect on fibroblastic CAV1 levels (Martinez-Outschoorn et al., 2010b). Martinez-Outschoorn et al. reported that no changes in fibroblast CAV1 levels occur after non-contact co-culture of hTERT-BJ with MCF7. This is in line with the results presented in this chapter, which show no significant changes in hTER-BJ CAV1 levels after non-contact co-culture with MCF7. However, the results presented in this chapter show that non-contact co-culture with human breast cancer cell line MDA-
MB-231 did increase CAV1 protein levels in immortalised human skin fibroblasts (hTERT-BJ). This increase of CAV1 levels suggests that soluble factors secreted in the media mediate communication between cancer cells and fibroblasts, resulting in the regulation of CAV1 levels.

The role of differentiated monocytes

Macrophages are a well described component of the tumour microenvironment (Mantovani and Sica, 2010). Macrophages have numerous ways of communicating with, and altering the expression profiles of, cells in the local microenvironment. Alveolar macrophages have, for example, been shown to alter IL-8 expression levels in pulmonary fibroblasts in co-culture (Rolfe et al., 1991).

The results presented in this chapter indicate that CAV1 levels of primary human breast fibroblasts Fre85 were decreased after non-contact co-culture with cancer cells and differentiated monocytes. There was a trend towards lower fibroblastic CAV1 levels after co-culture with THPT alone, however there was no statistically significant effect. CAFs express a proinflammatory gene-signature in breast, skin, and pancreatic cancers, and promote macrophage recruitment (Erez et al., 2010). Thus recruitment of macrophages to the tumour microenvironment during cancer progression may account for the decrease in fibroblastic CAV1 levels, rather than interactions between fibroblasts and cancer cells alone.

The macrophages included in the co-culture system were THP-1 cells differentiated with TPA. Treatment with TPA differentiates THP-1 monocytes into a macrophage-like phenotype (Auwerx, 1991). TPA is a phorbol diester that activates protein

A potential mechanism, through which macrophages could reduce CAV1, is the production of ROS. Macrophages produce ROS at the tumour site (Comito et al., 2014). A study by Ishimoto et al. (2014) measured abundant ROS production by M1- and M2-polarised THP-1 macrophages, in vitro. ROS have been reported to drive CAV1 downregulation in CAFs. In a study by Martinez-Outschoorn et al. (2010a), using CM-H2DCFDA ROS staining for confocal microscopy, hTERT-BJ skin fibroblasts in contact co-culture with MCF7 breast cancer cells displayed signs of oxidative stress. CAV1 protein expression was restored in hTERT-BJ fibroblasts co-cultured with MCF7 breast cancer cells after treatment with ROS scavenger N-Acetyl-Cysteine (NAC) (Martinez-Outschoorn et al., 2010a).

The fibroblastic CAV1 protein downregulation in contact co-cultures of hTERT-BJ fibroblasts with MCF7 cancer cells, reported by Martinez-Outschoorn et al. (2010b), did not result in changes in CAV1 mRNA. In the co-cultures of Fre85 fibroblasts with cancer cells and differentiated monocytes described in this chapter, on the other hand, the decrease in CAV1 mRNA translated into a decrease in CAV1 protein levels. This consistency between CAV1 mRNA and CAV1 protein levels suggests that the decrease seen here, could be based on a different regulatory mechanism to the downregulation by autophagic/lysosomal degradation of CAV1 in the absence of mRNA changes, reported by Martinez-Outschoorn et al. (2010b).
The role of oxygen and distance
Physiological oxygen levels differ from the oxygen levels commonly used in cell culture. Oxygen levels in tissues vary, and are generally lower than those in cell culture conditions. The \( \text{pO}_2 \) in brain tissue is approximately 30-50 mmHg, and the \( \text{pO}_2 \) in superficial skin tissue is approximately 8 mmHg (Carreau et al., 2011). Oxygen levels in cell culture media cultured with 5% CO2 in air are approximately 18-20% or 140 mmHg (Newby et al., 2005). Cells cultured at 20% oxygen levels may show oxidative stress, DNA damage, and decreased genetic stability (Estrada et al., 2012). Cells require oxygen for the generation of energy in the form of ATP, however excess oxygen leads to the generation of ROS. High levels of ROS cause DNA damage and genetic instability (Cooke et al., 2003). When oxygen levels in co-culture conditions in the mouse system were reduced to physiological levels of 5%, the previously observed reduction of CAV1 protein levels was absent. This suggests that the model may require a degree of oxidative stress for changes in CAV1 levels to be detected. This oxygen effect was, however, not seen in the human cell culture system (data not shown).

Increased ROS in cancer cells is a well-described phenomenon. In addition to increased oxygen levels, the expression of oncogenes, dysfunctional mitochondria, and the loss of p53, are other potential causes of increased ROS levels that have been reported in cancer cells (Trachootham et al., 2009). As mentioned above, co-culture with human breast cancer cells has been shown to increase oxidative stress in human skin fibroblasts (Martinez-Outschoorn et al., 2010a). Co-cultures showed increased ROS and decreased CAV1 levels in fibroblasts and increased aneuploidy in breast cancer cells, whereas both cell types showed increased DNA damage (Martinez-Outschoorn et al., 2010a). Furthermore, CAV1 knockdown has been shown to lead to
oxidative stress and oxidative stress has in turn been shown to lead to CAV1
downregulation in fibroblasts (Martinez-Outschoorn et al., 2010a), indicating a
positive feedback mechanism involving CAV1 and oxidative stress.

Distance is a factor in the effectiveness of secreted signalling molecules inducing a
response in nearby cells. To examine this effect, the co-culture system, in which the
fibroblasts were separated from the breast cancer cells by a distance of approximately
1-2 mm, was adapted to decrease the distance between fibroblasts and breast cancer
cells, by culturing these cell types on either side of the membrane of a transwell filter
insert, with a thickness of 10 µm. The results suggested that CAV1 levels are not
altered when the distance between fibroblasts and breast cancer cells is decreased
(data not shown). This suggests that any signalling that is occurring in the non-contact
co-culture system is sustained over a larger distance.

Direct physical contact between fibroblasts and breast cancer cells or macrophages
could potentially induce the secretion of signalling molecules, which in turn could
alter CAV1 expression in nearby, but physically separated, fibroblasts. To test this, a
cell culture system was set up, in which a mixed population of fibroblasts and breast
cancer cells was cultured together with, but physically separated from, a monoculture
of fibroblasts. The results presented in this chapter suggest that contact co-culture of
fibroblasts and breast cancer cells may lower CAV1 protein levels in a nearby
monoculture of fibroblasts, however the effect was small and outside of statistical
significance. No difference was seen between non-contact co-culture of fibroblasts
with differentiated monocytes in the presence and absence of direct contact with
fibroblasts.
In contrast, co-culture of hTERT-fibroblasts stably expressing eNOS with control hTERT fibroblasts resulted in CAV1 downregulation in both types of fibroblasts, suggesting a ‘field effect’ driven by oxidative stress (Martinez-Outschoorn et al., 2010a). In this study co-cultures of hTERT-BJ with MCF7 showed evidence of increased fibroblastic ROS and NO production and it was proposed that CAFs activate neighbouring fibroblasts through the production of ROS and nitric oxide (NO) species (Martinez-Outschoorn et al., 2010a).

**CAF marker expression**
Loss of fibroblastic CAV1 levels has been proposed to drive fibroblast activation and induce a myofibroblast phenotype, characterised by increased αSMA expression (Martinez-Outschoorn et al., 2010b). A study by Martinez-Outschoorn et al. (2010b) reported increased fibroblastic αSMA expression after contact co-culture of immortalised skin fibroblasts with breast cancer cells, as well as in CAV1 siRNA transfected fibroblasts. In contrast, a study by Kunz-Schughart et al. (2001) showed that breast cancer cells were not capable of inducing αSMA expression in normal skin fibroblasts after co-culture.

The results presented in this chapter do not show a correlation between low fibroblastic CAV1 levels and high αSMA expression. Neither non-contact co-culture with cancer cells, nor non-contact co-culture with differentiated monocytes, resulted in altered fibroblastic αSMA levels. Interestingly, the highest fibroblastic αSMA levels were measured after non-contact co-culture with a mixed population of breast cancer cells and fibroblasts, although these levels were not significantly higher than the control. This trend may provide a further indication that close contact between
fibroblasts and cancer cells resulted in the activation of physically separated fibroblasts through a paracrine effect. It is important to note, however, that not all CAFs express αSMA and CAFs that do not express αSMA may also play a role in cancer progression (Sugimoto et al., 2006).

**Variability in CAV1 levels between experiments**
The effect on fibroblastic CAV1 levels that is seen after non-contact co-cultures of fibroblasts with breast cancer cells and differentiated monocytes is subject to large variability between replicates. This variability may be in part due to instability of the reference gene β-actin. Indeed, the level of β-actin mRNA across co-culture conditions was found to be more variable compared to GAPDH mRNA levels.

Figures 7 and 9 both show fibroblastic CAV1 levels after co-culture of Fre85 fibroblasts with MDA-MB-231 breast cancer cells and differentiated monocytes. The fibroblastic CAV1 mRNA levels from co-cultures shown in Figure 7 were measured against the β-actin reference gene. As part of the cytoskeleton, β-actin mediates internal cell motility. Caveolae are closely associated with actin fibres and CAV1 is thought to physically link to actin fibres through filamin A (Muriel et al., 2011). Both caveolae lateral migration and internalisation are facilitated by the actin cytoskeleton, and its associated proteins (Parton and del Pozo, 2013). It is therefore possible that changes in CAV1 levels have an effect on β-actin levels in the cell and *vice versa.*

In addition to a different reference gene used to assess CAV1 mRNA levels, the Fre85 fibroblasts used in the experiment shown in Figure 7 were from different batches of frozen stocks. In the experiment shown in Figure 9, the same batch of fibroblasts was
used for the replicate co-cultures, and a strongly significant reduction of fibroblastic CAV1 mRNA levels, relative to the more stable reference gene GAPDH, was seen in the presence of breast cancer cells and differentiated monocytes. The experiment shown in Figure 7 included a batch of fibroblasts that showed higher fibroblastic CAV1 protein levels, but not mRNA levels, after co-culture with cancer cells and differentiated monocytes (single experiment, data not shown). Interestingly, after 16 days and 2 passages in culture, that same batch of cells showed lower fibroblastic CAV1 protein levels after co-culture with cancer cells and differentiated monocytes (single experiment, data not shown). This variability in response to co-culture contributed to the variability in the data seen in Figure 7. The apparent switch in the response of Fre85 fibroblasts to co-culture, was further observed in one other batch of Fre85 fibroblasts (single experiment, data not shown), and suggests that changes in the phenotype of the cells, occurring over the culture period, for example epigenetic changes, may play a role in the regulation of fibroblastic CAV1 levels.

**Conclusion**

The results shed light on the potential role of a soluble factor/factors in the reduction of CAV1 in CAFs, relative to normal fibroblasts. Although the study suggests that breast cancer cells have a minimal influence on CAV1 levels in human fibroblasts in a non-contact fashion in this in vitro setting, the study shows for the first time that the involvement of differentiated monocytes does significantly reduce fibroblastic CAV1 levels in a non-contact co-culture. This points to the possibility that the reduced levels of CAV1 levels observed in the tumour stroma from a population of cancer patients with significantly worse outcomes, as found by multiple independent studies (El-Gendi et al., 2012; Qian et al., 2011; Simpkins et al., 2012; Sloan et al., 2009;
Witkiewicz et al., 2009b), may be caused by the recruitment of macrophages to the tumour site.

The cell lines included in this research were embryonic mouse fibroblasts, mouse primary mammary fibroblasts, human primary skin fibroblasts, human immortalized skin fibroblasts, and human primary breast fibroblasts (chapters 3-6). Unfortunately, access to further relevant cell lines and human tumour tissue was not available. Further research is needed to determine the effect of monocytes/macrophages on fibroblastic CAV1 levels in different cell lines, and to evaluate the findings from the in vitro system presented in this chapter in vivo.

A significant finding of the experiments was that CAV1 levels in fibroblasts appear unstable, with relatively large variability between experiments and between batches of fibroblasts. Others have found that CAV1 levels in CAFs can either be lower (8 out of 11 invasive ductal carcinoma (IDC) patients), the same (1 out of 11 IDC patients), or higher (2 out of 11 IDC patients), than in nearby unaffected fibroblasts (Mercier et al., 2008). In hTERT-BJ and MCF7 contact co-cultures described by Martinez-Outschoorn et al. (2010b), after 7 days of co-culture with MCF7 breast cancer cells, some sections of the co-culture were found to contain fibroblasts that showed higher levels of CAV1 protein. Furthermore, in the co-culture system reported in this chapter, the increase in CAV1 protein in hTERT-BJ fibroblasts after non-contact co-culture with MDA-MB-231 breast cancer cells was not accompanied by a concurrent increase in CAV1 mRNA levels, whereas the reduction of CAV1 levels in Fre85 fibroblasts after non-contact co-culture with MDA-MB-231 breast cancer cells and TPA-treated THP-1 cells was evident at both the mRNA and protein level. Together,
this suggests the involvement of additional regulatory mechanisms of CAV1 expression in fibroblasts. Further research is required to determine the mechanism(s) of CAV1 regulation in Fre85 fibroblasts.
Chapter 4: Caveolin-1 expression in fibroblasts in contact co-culture with cancer cells
Introduction

The previous chapter found that non-contact co-culture between Fre85 fibroblasts, and MCF7 or MDA-MB-231 breast cancer cells, did not lead to significant changes in fibroblastic caveolin-1 (CAV1) levels; however, the inclusion of differentiated monocytes did result in a significant decrease in CAV1 levels in Fre85 fibroblasts. The current chapter will investigate the effect of contact co-culture on fibroblastic CAV1 levels.

In breast cancer, most tumours develop as a lesion in the ductal or lobular epithelium. In this situation, the tissue architecture is largely intact, and the cancer cells are contained in the epithelial compartment by the basement membrane (Bissell and Hines, 2011). Interaction between the cancer cells and stromal cells occurs through the extracellular matrix (ECM), rather than through direct cell contact (Joyce and Pollard, 2009).

As the cancer progresses, and once the basement membrane is compromised, the cancer cells make contact with the stromal ECM. The interaction of the cancer cells with the stromal ECM results in the upregulation of MMPs and invasion and metastasis promoting signalling (Bissell and Hines, 2011). At this stage, the neoplastic growth may break out of the epithelial compartment, and physical contact between cancer cells and fibroblasts can occur.

In non-contact co-cultures, the signalling between the physically separated cell populations is limited to paracrine (e.g., cytokines or exosomes), and autocrine, interactions. In addition, the distance between the cell populations in the co-culture
system potentially limits paracrine signalling. In contact co-culture systems, juxtacrine interactions (e.g., membrane proteins-ligands or gap junctions) are possible, in addition to paracrine and autocrine signalling.

In a study by Martinez-Outschoorn et al. (2010b), hTERT-BJ immortalised human skin fibroblasts were grown together with MCF7 human breast cancer cells in a contact co-culture system. When cancer cells were seeded at low numbers in relation to fibroblast numbers (i.e., a 5:1 fibroblast to cancer cell ratio), MCF7 showed better proliferation, compared to being seeded in the absence of fibroblasts. The fibroblasts showed an altered shape and orientation in co-culture with MCF7 cancer cells, compared to monoculture, as well.

In this study, a reduction in CAV1 protein expression by fibroblasts was seen after 5 days of co-culture with MCF7 breast cancer cells by confocal microscopy (Martinez-Outschoorn et al., 2010b). This downregulation of CAV1 was reported to be mediated through lysosomal degradation, as treatment with lysosomal inhibitor chloroquine, but not with proteasomal inhibitor MG-132, rescued CAV1 expression (Martinez-Outschoorn et al., 2010b). Interestingly, after 7 days of co-culture with MCF7 breast cancer cells, some sections of the co-culture were found to contain fibroblasts that showed an upregulation of CAV1 protein (Martinez-Outschoorn et al., 2010b).

The current chapter investigates whether the results reported by Martinez-Outschoorn et al. (2010b) can be replicated with primary human breast fibroblasts Fre85, and using qPCR and Western analysis to determine CAV1 levels. In addition, the role of
THP-1 monocytes in fibroblast and cancer co-cultures, and their effect on fibroblastic CAV1 levels is examined.

Aims

The main aim of this study was to determine whether contact co-culture of tumour cells and fibroblasts influences the expression of CAV1 in fibroblasts.

The specific aims were:

• To determine whether an effect of breast cancer cells on fibroblastic CAV1 levels was tissue-specific; i.e., whether breast fibroblasts respond differently compared to skin fibroblasts.

• To determine whether an effect of breast cancer cells on fibroblastic CAV1 levels was dependent on the invasiveness of the cancer cells.

• To investigate a potential role of (differentiated) monocytes in influencing fibroblastic CAV1 levels.

Results

Co-cultures of immortalised human skin fibroblasts and breast cancer cells

The data presented in the previous chapter showed no significant changes in hTERT-BJ CAV1 levels after non-contact co-culture with breast cancer cells. To investigate whether there is an effect on fibroblastic CAV1 levels in contact co-cultures, CAV1 levels in hTERT-BJ were analysed in contact co-cultures with MCF7 using confocal
microscopy on immunofluorescently labelled cells. No significant changes in fibroblastic CAV1 levels were observed after 5 days of contact co-culture with MCF7 (Fig 1A-C). Quantification of the immunofluorescent signal showed CAV1 levels in hTERT-BJ in co-culture to be $1.16 \pm 0.14$ fold the levels in hTERT-BJ monocultures for co-cultures with a 2 to 1 fibroblast to cancer cell ratio (Fig. 1D analysis based on 1 experiment, 2 separate images, 6 individual cells). For a 6 to 1 fibroblast to cancer cell ratio, the CAV1 fluorescent signal was $2.05 \pm 0.14$ fold the levels in hTERT-BJ monocultures (Fig. 1D analysis based on 1 experiment, 2 separate images, 6 individual cells).
**Figure 1:** Immunofluorescence microscopy of contact co-culture of immortalised human skin fibroblasts (hTERT-BJ) with a mildly invasive (MCF7) breast cancer cell line. After 5 days of contact co-culture, cells were fixed and immuno-labelled with antibodies against CAV1 (green) and cytokeratin 8 (red). Nuclei were stained with Draq5 (blue). (A) Monoculture of immortalised human skin fibroblasts (hTERT-BJ). (B) Immortalised human skin fibroblasts (hTERT-BJ) were co-cultured with a mildly invasive human breast cancer cell line (MCF7) at a 6:1 fibroblast to cancer cell ratio. (C) Immortalised human skin fibroblasts (hTERT-BJ) were co-cultured with a mildly invasive human breast cancer cell line (MCF7) at a 2:1 fibroblast to cancer cell ratio. (D) Mean total CAV1 fluorescence of fibroblasts in co-culture, based on 1 experiment, 2 separate images, 6 separate cells. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. BJ=hTERT-BJ, MCF=MCF7

In addition to confocal microscopy on immunofluorescently labelled co-cultures, CAV1 levels in fibroblasts were also measured using Western analysis and qPCR. Triplicate contact co-culture experiments showed lower CAV1 mRNA and protein levels in co-cultures of hTERT-BJ immortalised human skin fibroblasts and MCF7 human breast cancer cells (Fig. 2). CAV1 levels were measured in the mixed cell population of fibroblasts and cancer cells, after 48 hours of co-culture. Figure 2A shows lower CAV1 mRNA levels after co-culture of hTERT-BJ with MCF7 (0.37 ±
0.08 fold), compared to monocultures of hTERT-BJ. Likewise, CAV1 protein levels are reduced (0.39 ± 0.18 fold) in hTERT-BJ and MCF7 co-cultures, compared to the monoculture control (Fig. 2B,C). As can be seen from the MCF7 monoculture control in Figure 2, MCF7 human breast cancer cells did not express detectable levels of CAV1 mRNA or protein (Fig. 2), thus causing a dilution of CAV1 to occur in mixed populations of fibroblasts and MCF7 cells. The reduction in CAV1 mRNA and protein seen in the co-cultures is, therefore, at least in part due to the presence of MCF7 cells that are CAV1 negative.

Figure 2: Contact co-culture of immortalised human skin fibroblasts (hTERT-BJ) with a mildly invasive (MCF7) breast cancer cell line. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean.

* p<0.05  (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. CAV1 mRNA levels were significantly decreased after 48 hours of co-culture with MCF7. CAV1 mRNA levels in MCF7 monocultures were significantly lower than in hTER-BJ monocultures. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. CAV1 protein levels in MCF7 monocultures were significantly lower than in hTER-BJ monocultures. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. BJ=hTERT-BJ, MCF=MCF7
In addition to the mildly invasive MCF7 breast cancer cells, that were reported by others to cause a reduction in hTERT-BJ CAV1 levels after contact co-culture, the effect of the presence of highly invasive MDA-MB-231 breast cancer cells in contact co-culture was analysed in this study. Triplicate 48-hour contact co-culture experiments of HTERT-BJ with MDA-MB-231 resulted in fibroblastic CAV1 mRNA levels that were $1.57 \pm 0.26$ fold the monoculture control (Fig. 3A) and fibroblastic CAV1 protein levels that were $1.14 \pm 0.39$ fold the monoculture control (Fig. 3B). Baseline CAV1 protein levels differed significantly between experiments, resulting in large error bars that may have obscured changes (Fig. 3B). Figure 3C shows a representative Western image.
Figure 3: Contact co-culture of immortalised human skin fibroblasts (hTERT-BJ) with a highly invasive (MDA-MB-231) breast cancer cell line. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. No significant changes in CAV1 mRNA levels were detected after 48 hours of co-culture. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in CAV1 protein levels were detected after 48 hours of co-culture. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. BJ=hTERT-BJ, MDA=MDA-MB-231

CAV1 levels in hTERT-BJ fibroblasts after contact co-culture with MDA-MB-231 were not analysed using confocal microscopy of immunofluorescently labelled cells, as these two cell types display great resemblance, both in cell shape and size, and in the cellular markers they express (e.g., CK8, αSMA, and fibroblast surface antigen (SFA), data not shown), thus being difficult to distinguish using this technique (see Figure 4 for the appearance of MCF7 (A) and MDA-MB-231 (B) in immunofluorescence microscopy).
Figure 4: Immunofluorescence microscopy of human breast cancer cells. Cells were fixed and immuno-labelled with antibodies against CAV1 (green) and cytokeratin 8 (red). Nuclei were stained with Draq5 (blue). (A) Mildly invasive human breast cancer cell line MCF7. (B) Highly invasive human breast cancer cell line MDA-MB-231.

**Co-cultures of primary human breast fibroblasts with breast cancer cells**

In the previous chapter human primary breast fibroblasts (Fre85) were shown to respond differently, compared to immortalised hTERT-BJ fibroblasts, to non-contact co-culture with breast cancer cells. In the case of contact co-culture of Fre85 fibroblasts with MCF7 or MDA-MB-231 breast cancer cells, confocal microscopy of fluorescently labelled cultures showed no significant changes in fibroblastic CAV1 levels (Fig. 5A-C), which was similar to the response seen in hTERT-BJ fibroblasts, as described above and shown in Figure 1.

Contact co-culture of Fre85 with MCF7 for 5 days, at a 6:1 fibroblast to cancer cell ratio, resulted in a fibroblastic fluorescent signal from CAV1 that was $1.65 \pm 0.17$ fold the signal from the monoculture control (Fig. 5D). Duplicate contact co-culture experiments of Fre85 with MCF7, at a 2:1 fibroblast to cancer cell ratio, resulted in a fibroblastic fluorescent signal from CAV1 that was $1.14 \pm 0.14$ fold the signal from the monoculture control (Fig. 5D, analysis based on 2 experiments, 4 separate images, 12 individual cells).
Figure 5: Immunofluorescence microscopy of contact co-cultures of immortalised human skin fibroblasts (hTERT-BJ) with a mildly invasive (MCF7) breast cancer cell line. After 5 days of co-culture cells were fixed and immuno-labelled with antibodies against CAV1 (green) and cytokeratin 8 (red). Nuclei were stained with Draq5 (blue). (A) Monoculture of primary human breast fibroblasts (Fre85). (B) Primary human breast fibroblasts (Fre85) were co-cultured with a mildly invasive human breast cancer cell line (MCF7) at a 6 fibroblasts to 1 cancer cell ratio. (C) Primary human breast fibroblasts (Fre85) were co-cultured with a mildly invasive human breast cancer cell line (MCF7) at a 2 fibroblasts to 1 cancer cell ratio. (D) Mean total CAV1 fluorescence of fibroblasts in co-culture, based on 2 experiments, 4 separate images, 12 separate cells. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. Fre=Fre85, MCF=MCF7

As MCF7 cells were shown to have undetectable CAV1 levels, leading to dilution of overall levels in the co-culture, 48-hour triplicate co-cultures of Fre85 fibroblasts with MDA-MB-231 cells were analysed using qPCR and Western analysis. In contact co-cultures of Fre85 with MDA-MB-231, the CAV1 mRNA levels were $1.21 \pm 0.25$ fold the CAV1 mRNA levels in Fre85 monocultures (Fig. 6A). CAV1 protein levels in co-cultures of Fre85 fibroblasts with MDA-MB-231 cancer cells were $1.65 \pm 0.55$ fold
the CAV1 protein levels in monoculture controls (Fig. 6B). There was a high degree
of variability in CAV1 levels measured between experiments and none of the changes
were statistically significant.

Figure 6: Contact co-culture of primary human breast fibroblasts (Fre85) with a highly
invasive (MDA-MB-231) breast cancer cell line. Results for the co-cultures are relative to
the fibroblast monoculture control. Error bars represent the standard error of the mean. (A)
CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. No significant
changes in CAV1 mRNA levels were detected after 48 hours of co-culture. Results are
representative of 2 independent experiments. (B) Western analysis of CAV1 protein levels,
relative to total protein levels, as detected by Ponceau staining. No significant changes in
CAV1 protein levels were detected after 48 hours of co-culture. Results are representative of
3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a
loading control. Fre=Fre85, MDA=MDA-MB-231

Co-cultures of primary human breast fibroblasts with monocytes

As there was significant variability in CAV1 mRNA and protein levels after 3-day co-
cultures of fibroblasts with cancer cells, CAV1 levels were assessed over the course
of 10 days, to determine whether a time point could be found at which CAV1 levels
were more consistent between experiments. Triplicate contact co-cultures of Fre85
with MDA231, as well as triplicate monocultures of MDA-MB-231, showed large variability in CAV1 protein levels over the course of 10 days (Fig. 7A).

Previous experiments showed a reduction of fibroblastic CAV1 levels after the inclusion of THP-1 cells in non-contact co-cultures of Fre85 and MDA-MB-231. To investigate whether THP-1 monocytes influence the expression of CAV1 in fibroblasts upon contact co-culture in a similar way, Fre85 fibroblasts were cultured together with THP-1 monocytes. The inclusion of THP-1 in this contact co-culture model resulted in the decrease of CAV1 levels in the mixed cell populations of fibroblast and monocyte (Fig. 7B).

In contrast to Fre85 co-cultures with cancer cells, CAV1 protein levels in triplicate Fre85 co-cultures with monocytes were significantly lower after 10 days. CAV1 levels were 0.58 ± 0.22 fold the monoculture control after 3 days, 0.53 ± 0.25 fold the monoculture control after 5 days, 0.42 ± 0.19 fold the monoculture control after 7 days, and 0.54 ± 0.07 fold the monoculture control (p<0.05 after 10 days (Fig. 7B). THP-1 is a non-adherent cell line that does not express high levels of CAV1. A small portion of the THP-1 cells were observed to adhere to the culture substrate and, therefore, contributed to overall CAV1 levels measured in the mixed populations.
Figure 7: Non-contact co-culture of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line over 10 days. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Results are representative of 3 independent experiments. (B) Presenting the Fre85 and THP-1 co-cultures only. Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. CAV1 protein levels were significantly lower after 10 days of co-culture of Fre85 and THP-1 compared to a Fre85 monoculture control. Results are representative of 3 independent experiments. Fre=Fre85, MDA=MDA-MB-231, THP=THP-1
The reduction in CAV1 levels seen in contact co-cultures of Fre85 fibroblasts with THP-1 monocytes using Western analysis was not corroborated with confocal microscopy of fluorescently labelled 5-day co-cultures. A monoculture of Fre85 (Fig. 8A), a contact co-culture of Fre85 with THP-1 (Fig. 8B) and a triple culture of Fre85, MCF7 and THP-1 (Fig. 8C), were immunofluorescently labelled with antibodies to CAV1 and cytokeratin 8. Analysis of the fluorescent signal from the confocal images showed that fibroblastic CAV1 levels in contact co-cultures of Fre85 and THP-1 were 1.05 ± 0.11 fold levels in the monoculture control (Fig. 8D). Analysis of confocal images did, however, show a trend towards a reduction (0.41 ± 0.15 fold, analysis based on 2 experiments, 4 separate images, 12 individual cells) of fibroblastic CAV1 levels after contact co-culture of Fre85 fibroblasts with MCF7 cancer cells and THP-1 monocytes combined (triple culture).
Figure 8: Immunofluorescence microscopy of contact co-culture of primary human breast fibroblasts (Fre85) with a mildly invasive (MCF7) breast cancer cell line and a monocytic cell line (THP-1). After 5 days of culture, cells were fixed and immuno-labelled with antibodies against CAV1 (green) and cytokeratin 8 (red). Nuclei were stained with Draq5 (blue). (A) Mono-culture of primary human breast fibroblasts (Fre85). (B) Primary human breast fibroblasts (Fre85) were co-cultured with a monocytic cell line (THP-1) at a 2 fibroblasts to 1 monocyte ratio. (C) Primary human breast fibroblasts (Fre85) were co-cultured with a mildly invasive human breast cancer cell line (MCF7) and a monocytic cell line (THP-1) at a 2 fibroblasts to 1 cancer cell and 1 monocyte ratio. (D) Mean total CAV1 fluorescence of fibroblasts in co-culture, based on 2 experiments, 4 separate images, 12 separate cells. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. Fre=Fre85, MCF=MCF7, THP=THP-1

To investigate whether this trend towards a reduction in fibroblastic CAV1 levels was specific to Fre85 fibroblasts, hTERT-BJ fibroblasts were similarly cultured in the presence of MCF7 cancer cells and THP-1 monocytes, and cultures were labelled for confocal microscopy (Fig. 9A,B). Analysis of the fluorescent signal from CAV1 antibody labelling of cultures revealed a trend towards an increase of fibroblastic
CAV1 levels after contact co-culture (1.79 fold ± 0.40) (Fig. 9C, analysis based on 1 experiment, 2 separate images, 6 individual cells).

Figure 9: Immunofluorescence microscopy of contact co-culture of primary human breast fibroblasts (Fre85) with a mildly invasive (MCF7) breast cancer cell line and a monocytic cell line (THP-1). After 5 days of culture, cells were fixed and immuno-labelled with antibodies against CAV1 (green) and cytokeratin 8 (red). Nuclei were stained with Draq5 (blue). (A) Mono-culture of human immortalised skin fibroblasts (hTERT-BJ). (B) Human immortalised skin fibroblasts (hTERT-BJ) were co-cultured with a mildly invasive human breast cancer cell line (MCF7) and a monocytic cell line (THP-1) at a 2 fibroblasts to 1 cancer cell and 1 monocyte ratio. (C) Mean total CAV1 fluorescence of fibroblasts in co-culture, based on 1 experiment, 2 separate images, 6 separate cells. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. BJ=hTERT-BJ, MCF=MCF7, THP=THP-1

Fluorescence Activated Cell Sorting

To provide further support for data showing changes in CAV1 levels after co-culture, the fibroblast compartment and the cancer cell compartment were analysed separately,
using Fluorescence Activated Cell Sorting (FACS). In order to be able to sort cells using FACS, cell populations had to be made distinguishable through fluorescent labelling. In initial experiments, MCF7 cells labelled with tdTomato fluorescent protein were used in the co-cultures. FACS analysis of tdTomato-labelled MCF7 cells, however, showed the presence of unlabelled cells (20% of viable cells), thus MCF7 cells could not be reliably identified in the co-cultures during FACS.

To resolve the issue of contamination of the fibroblast population with unlabelled cancer cells, positive sorting (i.e., sorting for labelled cells), instead of negative sorting (i.e., sorting for unlabelled cells), was performed. Fre85 fibroblasts were labelled with CFDA-SE, and successfully removed from the mixed population of the co-culture system by FACS. The effect of the labelling procedure, and the preparation of cells for FACS, on cell viability was analysed and found to be insignificant (data not shown).

Analysis after cell sorting showed large inter-experimental variability in fibroblastic CAV1 mRNA and protein levels, after 5 days of co-culture with human breast cancer cells (Fig. 10, 11 and 13), and changes were not significant. Triplicate contact co-cultures of Fre85 primary human fibroblasts with MCF7 breast cancer cells resulted in a 1.02 fold ± 0.22 change in fibroblastic CAV1 mRNA levels (Fig. 10A), and a 1.06 fold ± 0.53 change in fibroblastic CAV1 protein levels (Fig. 10B,C). Triplicate contact co-cultures of Fre85 primary human fibroblasts with MDA-MB-231 breast cancer cells resulted in a 1.20 fold ± 0.53 increase in fibroblastic CAV1 mRNA levels (Fig. 11A), and a 1.04 fold ± 0.60 change in fibroblastic CAV1 protein levels (Fig. 11B,C).
Figure 10: Fibroblastic CAV1 levels in contact co-cultures of primary human breast fibroblasts (Fre85) with a mildly invasive (MCF7) breast cancer cell line after FACS. CAV1 levels in fibroblasts were measured after cell sorting. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. No significant changes in fibroblastic CAV1 mRNA levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in fibroblastic CAV1 protein levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MCF=MCF7
Figure 11: Fibroblastic CAV1 levels in contact co-cultures of primary human breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) breast cancer cell line after FACS. CAV1 levels in fibroblasts were measured after cell sorting. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. No significant changes in fibroblastic CAV1 mRNA levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in fibroblastic CAV1 protein levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MDA=MDA-MB-231

In contrast, triplicate contact co-cultures with THP-1 monocytes showed a more consistent change in fibroblastic CAV1 levels, and resulted in fibroblastic CAV1 mRNA levels that were 0.41 fold ± 0.20 fold the control (Fig. 12A, p=0.1), and in a 0.36 fold ± 0.11 (p<0.05) reduction in fibroblastic CAV1 protein levels (Fig. 12B,C), compared the monoculture control.
Figure 12: Fibroblastic CAV1 levels contact co-cultures of primary human breast fibroblasts (Fre85) with a monocytic cell line (THP-1) after FACS. CAV1 levels in fibroblasts were measured after cell sorting. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 against control (A) CAV1 mRNA levels, relative to β-actin levels, were detected using qRT-PCR. No significant changes in fibroblastic CAV1 mRNA levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Fibroblastic CAV1 protein levels were significantly lower after 5 days of contact co-culture with THP-1. Results are representative of 3 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, THP=THP-1

Analysis of fibroblastic CAV1 protein levels of FACS sorted hTERT-BJ fibroblasts after 5 days of contact co-culture with MCF7 breast cancer cells did not show a significant change compared to levels in hTERT-BJ monocultures (1.07 fold ± 0.30) (Fig. 13A,B).
Discussion

CAV1 downregulation in fibroblast has been proposed as a marker of cancer progression (Sloan et al., 2009; Witkiewicz et al., 2009b). This study examined the effect of contact co-culture of fibroblasts with cancer cells and monocytes on fibroblastic CAV1 levels. The results show that contact co-culture of primary human breast fibroblasts with monocytes resulted in a decrease of CAV1 protein levels in the fibroblasts. This decrease in fibroblastic CAV1 levels was not seen in co-cultures of primary human breast fibroblasts with breast cancer cells, nor was a decrease in CAV1 protein levels seen in immortalised skin fibroblasts after contact co-culture with breast cancer cells, as has been reported previously (Martinez-Outschoorn et al., 2010a; Martinez-Outschoorn et al., 2010b; Martinez-Outschoorn et al., 2010c).
**Contact co-culture of fibroblasts with breast cancer cells**

Other reports have described contact co-culture of hTERT-BJ with MCF7 resulting in a reduction of fibroblastic CAV1 protein levels, as seen in confocal microscopy of antibody-labelled cells (Martinez-Outschoorn et al., 2010a; Martinez-Outschoorn et al., 2010b). This reduction was, however, not shown by Western analysis and solely shown by immunofluorescence.

**Confocal microscopy**

This study looked at the effects of contact co-culture of fibroblasts with breast cancer cells on fibroblastic CAV1 mRNA and protein levels, using immunofluorescent confocal microscopy, qPCR and Western analysis. For confocal microscopy, co-cultures of fibroblasts were labelled with green-fluorescent antibodies to CAV1 and red-fluorescent antibodies to cytokeratin 8 (CK8). Cell nuclei were labelled with a nuclear stain. The use of a fluorescently labelled antibody to CAV1 enabled the level of CAV1 protein in cells to be determined. The use of CK8 staining enabled epithelial-like cancer cells, expressing high levels of CK8, to be distinguished from fibroblasts, which express little CK8. As MDA-MB-231 breast cancer cells have lost many of the epithelial-like characteristics of their pre-cancerous origins, among which is CK8 expression, distinguishing MDA-MB-231 cells from fibroblasts was difficult.

Analysis of the fluorescent signal from CAV1 antibody staining showed no effect of contact co-culture of hTERT-BJ fibroblasts with MCF7 breast cancer cells on fibroblastic CAV1 levels. Likewise, contact co-culture of primary human breast fibroblasts Fre85 with MCF7 breast cancer cells showed no significant change in fibroblastic CAV1 levels.
**qPCR and Western analysis**

Contact co-culture of immortalised human skin fibroblasts (hTERT-BJ) with mildly invasive (MCF7) or highly invasive (MDA-MB-231) breast cancer cells did not significantly affect fibroblastic CAV1 mRNA or protein levels, as determined by qPCR and Western analysis of the different cell populations after FACS. Similarly, breast cancer cells did not significantly affect CAV1 levels in primary human breast fibroblasts (Fre85).

Co-cultures of hTERT-BJ fibroblasts and MCF7 breast cancer cells showed lower levels of CAV1 mRNA and protein levels compared to those in hTERT-BJ alone. However, as MCF7 cells did not express CAV1 to a detectable level, lower levels of CAV1 in co-cultures, as determined by qPCR and Western analysis, cannot be positively ascribed to reduced levels of CAV1 in hTERT-BJ, but could merely be an effect of diluting the pool of CAV1 expressing cells (hTERT-BJ) with CAV1 non-expressing cells (MCF7).

As MDA-MB-231 breast cancer cells express similar CAV1 mRNA and protein levels to hTERT-BJ fibroblasts, and slightly higher levels than Fre85 fibroblasts, a reduction in CAV1 levels after co-culture of fibroblasts with MDA-MB-231 breast cancer cells would be due to a reduction in CAV1 levels, rather than to dilution. For this reason, CAV1 levels were measured after contact co-culture of both hTERT-BJ, and Fre85, with MDA-MB-231, whereas contact co-culture with MCF7 was not duplicated in Fre85. No significant changes in CAV1 levels were found after contact co-culture of hTERT-BJ or Fre85 fibroblasts with MDA-MB-231 breast cancer cells. However, it cannot be determined whether any changes in this system would be due
to changes in the fibroblast compartment, or in the cancer cell compartment. Furthermore, it would be possible that any changes in the fibroblast compartment would, in this system, be masked by changes in the cancer cell compartment.

To overcome the issues caused by measuring CAV1 levels in the whole system, CAV1 levels were measured in the individual cell compartments. To determine the CAV1 levels in the fibroblast compartment of our system, fibroblasts were separated from cancer cells by fluorescence-activated cell sorting (FACS) the cell populations after co-culture. FACS was attempted with tdTomato fluorescent protein labelled MCF7 cells and unlabelled fibroblasts, however, a population of insufficiently labelled MCF7 cells was detected. To prevent contamination of the fibroblast population with insufficiently labelled cancer cells, fibroblasts were fluorescently labelled and pulled out of the mixed co-culture, using FACS. This way, any insufficiently labelled cells would be included in the cell population that would not be evaluated for CAV1 levels, and contamination of the fibroblast fraction would be minimised.

The fluorescent protein used for labelling the fibroblasts was 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFDA-SE). Concentrations of CFDA-SE above 5 µM caused cell death in Fre85 fibroblasts (data not shown). Confocal microscopy on Fre85 fibroblasts labelled with different concentrations of CFDA-SE showed a concentration of 2 µM CFDA-SE to have a minimal effect on cell proliferation, while generating sufficient fluorescence in Fre85 (data not shown). However, analysis of the cell populations using FACS suggested that populations of non-viable and insufficiently labelled cells were present. Non-viable and insufficiently labelled cells
were excluded from the fibroblast population and were not analysed for CAV1 levels. The presence of non-viable cells suggests that the CFDA-SE-labelling of fibroblasts increased the sensitivity of cells to cell death during the cell sorting process.

Analysis of fibroblastic CAV1 mRNA and protein levels in the fibroblastic compartment after FACS was performed using qPCR and Western analysis. CAV1 levels in both immortalised human skin fibroblasts (hTERT-BJ), and primary human breast fibroblasts (Fre85), were not changed after contact co-culture with breast cancer cells. These findings are in line with the observations of fluorescently labelled co-cultures using confocal microscopy. However, these findings do not corroborate results published by others, which suggest that contact co-culture of hTERT-BJ with MCF7 results in a reduction of fibroblastic CAV1 protein levels (Martinez-Outschoorn et al., 2010b).

Data from immunofluorescence analysis using confocal microscopy is to be interpreted with caution when assessing expression levels of proteins. Fluorescence intensity is dependent on many factors, such as bleaching by excitation, focus-plane position, and quenching due to the presence of nearby dye molecules, to name a few. Any observed changes in fluorescence intensity should be confirmed by assessing protein levels through other techniques. This study has been unable to find a significant reduction of hTERT-BJ CAV1 protein levels after co-culture with MCF7 by Western or qPCR, nor has it been able to reproduce the results described by Martinez-Outschoorn et al. (2010b) by confocal microscopy. This study, in contrast, shows that contact co-culture with breast cancer cells does not alter fibroblastic CAV1 levels, irrespective of the fibroblast cell line tested (hTERT-BJ or Fre85), the
cancer cell line included (MCF7 or MDA-MB-231) or the technique used for CAV1 measurement (qPCR, Western analysis or confocal microscopy).

**Contact co-culture of fibroblasts with differentiated monocytes**

Previous experiments using a non-contact co-culture system have shown that differentiated THP-1 monocytes downregulated CAV1 levels in Fre85 fibroblasts. THP-1 cells express CAV1 at a very low level. THP-1 cells are non-adherent and should, therefore, not be collected together with the fibroblasts. However, a small portion of THP-1 cells was seen to attach in the cell culture system and, therefore, this same principle of dilution with low-expressing cells could explain the downregulation seen in contact co-culture with THP-1.

To investigate whether there was a reduction in fibroblastic CAV1 levels in contact co-cultures of fibroblasts with monocytes, co-cultures were sorted into individual cell populations using FACS. Analysis of the fibroblasts collected after FACS of co-cultures, showed a reduction in CAV1 mRNA and protein levels, although changes in mRNA levels were just outside of statistical significance. This confirms that the changes that were seen prior to cell sorting were indeed caused by a reduction of CAV1 levels in fibroblasts, and not by dilution of overall CAV1 levels in the co-culture by low CAV1 expression in THP-1 monocytes.

In contrast to the reduction in fibroblastic CAV1 levels seen after contact co-culture with monocytes by Western analysis and qPCR, confocal microscopy of immunofluorescently labelled co-cultures did not show a change in fibroblastic CAV1 levels. It is possible that a difference in antibody sensitivity in immunofluorescent
labelling for confocal microscopy versus Western blotting is causing a potential reduction in CAV1 levels to be detected with Western blotting, but not with confocal microscopy.

Although fluorescently labelled contact co-cultures of Fre85 fibroblasts and THP-1 monocytes did not show a reduction in fibroblastic CAV1 levels by confocal microscopy, triple cultures of Fre85, MCF7 and THP-1, on the other hand, did show a trend towards reduced fibroblastic CAV1 levels by confocal microscopy. These results are in line with what was found in non-contact triple cultures with the same cell lines (previous chapter). It is possible that the presence of both cancer cells and immune cells results in a signalling dynamic that leads to reduced CAV1 levels in fibroblasts, or enhances the fibroblastic CAV1 reduction caused by immune cells alone. Further investigation is required to resolve the inconsistencies between immunofluorescence and Western blotting, where present.

Fibroblastic CAV1 protein levels were decreased in a subset of breast cancer patients, and lower stromal CAV1 levels have been linked to worse outcomes in breast cancer (El-Gendi et al., 2012; Qian et al., 2011; Simpkins et al., 2012; Sloan et al., 2009; Witkiewicz et al., 2009b). In this study, contact co-cultures of fibroblasts and breast cancer cells did not result in decreased fibroblastic CAV1 levels. Possibly, the decrease in fibroblastic CAV1 levels seen in certain patients is caused by the recruitment of monocytes to the tumour site. The recruitment of monocytes, and infiltration of tumours by macrophages, is a well-described part of tumour progression (Hanahan and Weinberg, 2011; Ueno et al., 2000).
Interaction between cancer cells and fibroblasts involves the production of a plethora of cytokines, some of which play a role in the recruitment and differentiation of monocytes. Both fibroblasts and cancer cells can secrete cytokines that recruit monocytes. Cancer-associated fibroblasts secrete stromal cell-derived factor 1 (SDF1), which recruits monocytes in a chemo-attraction assay, as does monocyte chemoattractant protein 1 (MCP1/CCL2) secreted by cancer cells (Comito et al., 2014). In addition, macrophages produce cytokines, such as macrophage inflammatory protein 1α (MIP-1α), a monocyte/macrophage chemoattractant and macrophage activator, upon contact with fibroblasts (Steinhauser et al., 1998).

There are several cytokines that have been shown to affect CAV1 expression. Macrophages express epidermal growth factor (EGF) and expression levels are, interestingly, increased in the presence of colony stimulating factor 1 (CSF-1), which is secreted by cancer cells (Goswami et al., 2005). EGF has been shown to downregulate CAV1 expression in human squamous carcinoma cells (Lu et al., 2003). GM-CSF secreted by fibroblasts increases CAV1 expression in monocytes and differentiates monocytes to macrophages (Fu et al., 2012). Further research is needed to investigate whether cytokines secreted in fibroblast and monocyte/macrophage co-cultures are responsible for the reduction in fibroblastic CAV1 levels that was seen.

**Conclusion**
The reduction of CAV1 levels in cancer-associated fibroblasts has been associated with decreased survival in breast cancer patients. This *in vitro* study did not show an effect of cancer cells alone on changing CAV1 levels in fibroblasts. The study indicates, however, that the presence of monocytes/macrophages reduces CAV1
levels in fibroblasts. This suggests that the recruitment and differentiation of monocytes may play an important role in the reduction of fibroblastic CAV1 observed in patients. Increased tumour growth and angiogenesis in nude mice injected with human breast cancer cells and CAV1 −/− fibroblasts (Bonuccelli et al., 2010), shows that CAV1 downregulation is not merely a marker of a progressive tumour stroma, but plays an active role in cancer progression. Therefore, understanding the mechanisms of CAV1 downregulation in fibroblasts may provide therapeutic targets for the treatment of a subset of breast cancer patients.
Chapter 5: Cytokines secreted by co-cultures of fibroblasts, breast cancer cells and (differentiated) monocytes
Introduction

The data presented in Chapters 3 and 4 showed that non-contact and contact co-culture of primary human fibroblasts with human breast cancer cells and (differentiated) monocytes alters fibroblastic caveolin-1 (CAV1) levels. In addition, unexplained variability in fibroblastic CAV1 levels between experiments was seen. This chapter will investigate factors that could affect variability in cell culture experiments.

A potential mechanism through which co-culture could affect CAV1 levels is the secretion of soluble factors. This chapter will investigate the secretion of cytokines in non-contact and contact co-cultures. Cytokine concentrations are ideally measured in serum-free cell culture medium and this chapter will investigate the effect of a serum replacement on fibroblastic CAV1 levels and variability.

Cytokines in co-cultures
As the cell populations are physically separated in non-contact co-culture systems, one way that the different cell populations can communicate and affect gene regulation, is through paracrine signalling (Holt et al., 2010). In contact co-culture, the signalling that may occur could be of a paracrine or juxtacrine nature. Paracrine signalling can occur through the secretion of soluble factors, such as growth factors, cytokines (e.g., interleukins, chemokines, tumour necrosis factors, and other cytokines), as well as Wnt and Hedgehog proteins. Examples of other signalling mechanisms that could play a role in non-contact and contact co-culture are exosome
transfer, and signalling through metabolic cues (Ata and Antonescu, 2017; Luga et al., 2012).

Growth factors and cytokines are secreted by a wide range of cells, including macrophages, monocytes, fibroblasts, and cancer cells. The tumour microenvironment, a location these cell types share, is known to contain increased levels of many cytokines and growth factors (Korkaya et al., 2011). Co-culture of stromal cells with cancer cells, in vitro, has similarly been shown to lead to increased levels of different cytokines and growth factors. For example, co-culture of mesenchymal cells with breast cancer cells resulted in the increased production of CXC chemokines: CXCL5, CXCL6, and CXCL1, and of interleukins IL-6 and IL-8, in a study by Liu et al. (2011). Co-culture of fibroblasts with macrophages led to the increased production of IL-6, monocyte chemoattractant protein-1 (MCP-1), and RANTES, in a study by Holt et al. (2010).

Examples of CAV1 regulation by cytokines and growth factors have been reported in several different cell types. Epithelial growth factor (EGF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon γ (IFN-γ), and MCP-1, alter CAV1 levels in human squamous carcinoma cells, monocytes, thyrocytes, and brain microvascular endothelial cells, respectively (Fu et al., 2012; Lu et al., 2003; Marique et al., 2014; Song et al., 2007).
In this chapter, the concentrations of several growth factors and cytokines in the conditioned medium from non-contact, and contact, co-cultures that show lowered fibroblastic CAV1 levels, was measured using a cytokine assay. The data from this assay could be used to select cytokine and growth factor candidates that can be investigated further for their potential ability to regulate CAV1 levels in fibroblasts.

**The role of serum in cell culture**

It is important that the measurements of cytokines and growth factors are performed against a controlled background. The conditioned medium that is used to determine the concentrations of cytokines and growth factors secreted by cells in the co-culture system is derived from cell culture medium. The addition of growth factor containing supplements to cell culture medium is common practice in cell culture, with foetal bovine serum (FBS) being a commonly used additive. FBS contains growth and adhesion factors, hormones, lipids, and minerals, and, being a natural product, the exact composition is variable, depending on the source (Baker, 2016).

Alternatives to FBS as a supplement to cell culture medium have been developed, and implemented by researchers when factors in FBS are expected to interfere with experimental measurements or reproducibility (Van der Valk et al., 2010). For example, when cellular processes that are influenced by serum components (e.g., hormones, growth factors) are investigated, the use of serum is often problematic. Similarly, when studies require the measurement of secreted cellular products, the presence of serum proteins can complicate the measurements. This chapter will therefore investigate the effect of a chemically defined, serum-free cell culture
supplement (Serum Replacement 1, Sigma) on experimental outcomes and reproducibility in non-contact and contact co-cultures, preceding the measurement of cytokines in conditioned medium from the co-culture system.

**Aims**

The main aims of this study were to investigate the effect of factors, such as homogeneity in cell cycle phase, and the presence of serum, on experimental outcomes and reproducibility, and to measure cytokines in conditioned medium from co-cultures of fibroblasts, breast cancer cells, and (differentiated) monocytes.

The specific aims were:

- To investigate the effect of serum starvation on fibroblastic CAV1 levels after non-contact co-culture with highly invasive breast cancer cells (MDA-MB-231) and differentiated monocytes (THPT).

- To investigate the effect of serum concentration on fibroblastic CAV1 levels after non-contact co-culture with highly invasive breast cancer cells (MDA-MB-231) and THPT.

- To investigate the effect of Serum Replacement 1 on fibroblastic CAV1 levels after non-contact co-culture with highly invasive breast cancer cells (MDA-MB-231) and THPT.
To measure the concentrations of several cytokines in conditioned medium from non-contact and contact co-cultures with lowered fibroblastic CAV1 levels.

**Results**

Previous experiments, including both non-contact (chapter 3) and contact co-cultures (chapter 4) of fibroblasts with cancer cells and lymphocytes, showed unexplained variability in CAV1 protein levels between experiments. To explore whether the presence of FBS could account for CAV1 variability between experiments, non-contact co-cultures of Fre85 primary human breast fibroblasts with MDA-MB-231 human breast cancer cells, and differentiated human monocytes (THPT), were cultured with 10% FBS, 2% FBS, heat-inactivated FBS, and with a serum replacement.

**The effect of serum starvation on fibroblastic CAV1 protein levels in co-cultures**

An often-cited technique to standardize cell culture experiments is serum starvation. Serum starvation causes cells to withdraw from the cell cycle and arrest in the G0/G1 phase. The addition of serum after starvation causes cells to start dividing again, with all cells entering the cell cycle simultaneously, resolving any variability that may be caused by cells residency in different stages of the cell cycle. The comparison of 3-day non-contact co-cultures containing fibroblasts that were cultured with 0.2% FBS for 24 hours prior to co-culture (starved fibroblasts), with non-contact co-cultures containing fibroblasts that were not starved prior to co-culture, showed no difference in CAV1 protein levels, or variability, between experiments (Fig. 1). Non-contact co-
culture of Fre85 that had not been starved prior to co-culture, with MDA-MB-213 and THPT, led to CAV1 protein levels of 1.21 ± 0.30 fold the control levels (Fig. 1A,B). Non-contact co-culture of starved Fre85 with MDA-MB-213 and THPT in the presence of 10% FBS led to CAV1 protein levels of 1.15 ± 0.31 fold control (Fig. 1A,B). In addition, fibroblastic CAV1 protein levels were analysed after 24 hours of co-culture, and no significant changes were detected (data not shown).

Figure 1: Non-contact co-culture of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and a differentiated monocytic cell line (THPT) after serum starvation. Co-cultures using Fre85 that were serum-starved (0.2% FBS for 24 hours before co-cultures were set up) were compared with normal co-cultures. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in fibroblastic CAV1 protein levels were detected after 3 days of co-culture. Serum starvation did not result in changes in experimental outcome or variability. Results are representative of 4 independent experiments. (B) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MDA=MDA-MB-231, THPT=TPA-treated THP-1

The effect of serum reduction on fibroblastic CAV1 protein levels in co-cultures

The reduction of FBS from 10% to 2% led to a reduction in variability of CAV1 mRNA levels between experiments for 3 out of 5 conditions, and an increase for 2 out
5 conditions (Fig. 2). Non-contact co-culture of Fre85 with MDA-MB-231 led to CAV1 mRNA levels of 1.49 ± 0.29 (relative standard error (RSE) 20%) with 10% FBS (Fig. 2A, Table 1), and to levels of 0.91 ± 0.02 (RSE 2%) with 2% FBS (Fig. 2B, Table 2). Non-contact co-culture of Fre85 with THPT led to CAV1 mRNA levels of 1.05 ± 0.05 (RSE 5%) with 10% FBS (Fig. 2A, Table 1), and to levels of 0.88 ± 0.15 (RSE 17%) with 2% FBS (Fig. 2B, Table 2). Non-contact co-culture of Fre85 with undifferentiated THP-1 cells (THPD) led to CAV1 mRNA levels of 1.35 ± 0.14 (RSE 10%) with 10% FBS (Fig. 2A, Table 1), and to levels of 0.74 ± 0.15 (RSE 21%) with 2% FBS (Fig. 2B, Table 2). Non-contact co-culture of Fre85 with MDA-MB-231 and THPD led to CAV1 mRNA levels of 0.80 ± 0.53 (RSE 66%) with 10% FBS (Fig. 2A, Table 1), and to levels of 1.54 ± 0.54 (RSE 35%) with 2% FBS (Fig. 2B, Table 2). Non-contact co-culture of Fre85 with MDA-MB-231 and THPT led to CAV1 mRNA levels of 1.25 ± 0.82 (RSE 66%) with 10% FBS (Fig. 2A, Table 1), and to levels of 0.43 ± 0.09 (RSE 21%) with 2% FBS (Fig. 2B, Table 2).
Figure 2: Fibroblastic CAV1 mRNA levels after non-contact co-culture of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or a differentiated (THPT) monocytic cell line at different serum levels. Cultures at normal 10% FBS were compared with cultures at 2% FBS. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) CAV1 mRNA levels, relative to β-actin levels, after non-contact co-culture at 10% FBS, were detected using qRT-PCR. No significant changes in fibroblastic CAV1 mRNA levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. (B) CAV1 mRNA levels, relative to β-actin levels, after non-contact co-culture at 2% FBS, were detected using qRT-PCR. Fibroblastic CAV1 mRNA levels were significantly lower after 5 days of non-contact co-culture with MDA-MB-231 and with MDA-MB-231 and THPT. Results are representative of 3 independent experiments.
Table 1: Variability in CAV1 mRNA levels in non-contact co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or a differentiated (THPT) monocytic cell line, at 10% FBS. Relative standard error (RSE) is the standard error of the mean (SEM) calculated as a percentage of the average CAV1 mRNA levels for different 5-day non-contact co-cultures at 10% FBS. Fre=Fre85, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

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<td>Fre+MDA+THPT</td>
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Table 2: Variability in CAV1 mRNA levels in non-contact co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or a differentiated (THPT) monocytic cell line, at 2% FBS. Relative standard error (RSE) is the standard error of the mean (SEM) calculated as a percentage of the average CAV1 mRNA levels for different 5-days non-contact co-cultures at 2% FBS. Fre=Fre85, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

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<td>Fre+MDA+THPT</td>
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The reduction of FBS from 10% to 2% led to a decrease in variability for 1 out of 5 conditions, and an increase in variability of CAV1 protein levels for 4 out of 5 conditions (Fig. 3). Non-contact co-culture of Fre85 with MDA-MB-231 led to CAV1 mRNA levels of 1.27 ± 0.28 (RSE 22%) with 10% FBS (Fig. 3A, Table 3), and to levels of 2.91 ± 1.00 (RSE 34%) with 2% FBS (Fig. 3B, Table 4). Non-contact co-culture of Fre85 with THPT led to CAV1 mRNA levels of 0.60 ± 0.14 (RSE 23%) with 10% FBS (Fig. 3A, Table 3), and to levels of 2.60 ± 0.91 (RSE 35%) with 2% FBS (Fig. 3B, Table 4). Non-contact co-culture of Fre85 with THPD led to CAV1 mRNA levels of 0.80 ± 0.12 (RSE 15%) with 10% FBS (Fig. 3A, Table 3), and to levels of 2.61 ± 1.41 (RSE 54%) with 2% FBS (Fig. 3B, Table 4). Non-contact co-culture of Fre85 with MDA-MB-231 and THPD led to CAV1 mRNA levels of 1.19 ±
0.30 (RSE 26%) with 10% FBS (Fig. 3A, Table 3), and to levels of 0.47 ± 0.30 (RSE 64%) with 2% FBS (Fig. 3B, Table 4). Non-contact co-culture of Fre85 with MDA-MB-231 and THPT led to CAV1 mRNA levels of 0.74 ± 0.26 (RSE 35%) with 10% FBS (Fig. 3A, Table 3), and to levels of 1.03 ± 0.21 (RSE 20%) with 2% FBS (Fig. 3B, Table 4).

Figure 3: Fibroblastic CAV1 protein levels after non-contact co-culture of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or a differentiated (THPT) monocytic cell line at different serum levels. Cultures at normal 10% FBS were compared with cultures at 2% FBS. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) Western analysis of CAV1 protein levels, relative to total protein levels, after non-contact co-culture at 10% FBS, as detected by Ponceau staining. No significant changes in fibroblastic CAV1 mRNA levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, after non-contact co-culture at 2% FBS, as detected by Ponceau staining. No significant changes in fibroblastic CAV1 mRNA levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. Fre=Fre85, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1
Table 3: Variability in CAV1 protein levels in non-contact co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or a differentiated (THPT) monocytic cell line, at 10% FBS. Relative standard error (RSE) is the standard error of the mean (SEM) calculated as a percentage of the average CAV1 mRNA levels for different 5-day non-contact co-cultures at 10% FBS. Fre=Fre85, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

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Table 4: Variability in CAV1 protein levels in non-contact co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or a differentiated (THPT) monocytic cell line, at 2% FBS. Relative standard error (RSE) is the standard error of the mean (SEM) calculated as a percentage of the average CAV1 mRNA levels for different 5-day non-contact co-cultures at 2% FBS. Fre=Fre85, MCF=MCF7, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

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<td>Fre+MDA</td>
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The effect of heat-inactivated serum and serum replacement on fibroblastic CAV1 protein levels in co-cultures
To further explore the role of FBS in the regulation of CAV1 levels, 3-day non-contact co-cultures were set up to compare the addition of 10% FBS with the addition of 10% heat-inactivated FBS, and with the addition of a serum replacement. Non-contact co-culture of Fre85 with MDA-MB-231 and THPT in the presence of 10% FBS led to CAV1 protein levels of 1.21 ± 0.30 fold control. Non-contact co-culture of Fre85 with MDA-MB-231 and THPT in the presence of 10% heat-inactivated FBS led to CAV1 protein levels of 0.80 ± 0.22 fold control. Non-contact co-culture of Fre85 with MDA-MB-231 and THPT in the presence of serum replacement led to CAV1 protein levels of 0.65 ± 0.10 fold control (p<0.05, Fig. 4A,B). In addition,
fibroblastic CAV1 protein levels were assessed after 24 hours of co-culture, and no significant changes were detected (data not shown).

Figure 4: Non-contact co-culture of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and a differentiated monocytic cell line (THPT) using heat-inactivated serum or a serum replacement. Cultures at normal 10% FBS were compared with cultures at heat-inactivated FBS and Serum Replacement 1. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 against control (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Fibroblastic CAV1 protein levels were significantly lower after 3 days of co-culture with MDA-MB-231 and THPT using serum replacement. The use of a serum replacement resulted in changes in experimental outcome and variability compared to 10% FBS. Results are representative of 4 independent experiments. (B) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1, HI=heat-inactivated, SR=Serum Replacement 1

As the replacement of FBS with Serum Replacement 1 reduced the variability between experiments in triple cultures with fibroblasts (Fre85), cancer cells (MDA-MB-231), and differentiated monocytes (THPT), the effect of using Serum Replacement was tested for the other non-contact co-cultures assayed previously in chapter 3. Using serum replacement improved the reproducibility of 3-day co-culture effects on CAV1 levels across the co-culture system, compared to previously
described results (chapter 3). Non-contact co-culture of Fre85 with MDA-MB-231 resulted in fibroblastic CAV1 protein levels of 0.61 ± 0.16 fold the monoculture control. Non-contact co-culture of Fre85 with THPD resulted in fibroblastic CAV1 protein levels of 0.59 ± 0.12 fold the monoculture control. Non-contact co-culture of Fre85 with THPT resulted in fibroblastic CAV1 protein levels of 0.43 ± 0.12 fold the monoculture control (p<0.05). Non-contact co-culture of Fre85 with MDA-MB-231 and THPD resulted in fibroblastic CAV1 protein levels of 0.52 ± 0.07 fold the monoculture control (p<0.05). Non-contact co-culture of Fre85 with MDA-MB-231 and THPT resulted in fibroblastic CAV1 protein levels of 0.71 ± 0.10 fold the monoculture control (Fig. 5A,B).
Figure 5: Non-contact co-culture of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line using Serum Replacement 1. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. * p<0.05 against control (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Fibroblastic CAV1 protein levels were significantly lower after 3 days of co-culture with THPT alone or with MDA-MB-231 and THPD, or with MDA-MB-231 and THPT. Results are representative of 3 independent experiments (7 independent experiments for Fre+MDA-MB-231+THPT. (B) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

Similarly, the use of serum replacement in the contact co-culture system led to higher reproducibility of CAV1 protein levels compared to previously described results (chapter 4). Contact co-culture of Fre85 with MDA-MB-231 led to fibroblastic CAV1 protein levels of 1.67 ± 0.21 fold the monoculture control. Contact co-culture of Fre85 with THPD led to fibroblastic CAV1 protein levels of 0.72 ± 0.11 fold the
monoculture control. Contact co-culture of Fre85 with THPT led to fibroblastic CAV1 protein levels of 0.83 ± 0.27 fold the monoculture control. Contact co-culture of Fre85 with MDA-MB-231 and THPD led to fibroblastic CAV1 protein levels of 1.70 ± 0.61 fold the monoculture control. Contact co-culture of Fre85 with MDA-MB-231 and THPT led to fibroblastic CAV1 protein levels of 2.02 ± 0.86 fold the monoculture control. CAV1 protein levels in the MDA-MB-231 monoculture control were 2.59 ± 0.44 fold the monoculture control (Fig. 6A,B).

Figure 6: Contact co-culture of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line using Serum Replacement 1. Results for the co-cultures are relative to the fibroblast monoculture control. Error bars represent the standard error of the mean. (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in CAV1 levels were detected after 5 days of co-culture. Results are representative of 3 independent experiments. (B) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1
Cytokines secreted by co-cultures

To determine the levels of several cytokines and chemokines produced in the non-contact and contact co-cultures, 3-day non-contact, and 5-day contact, co-cultures were set up in the presence of serum replacement. Conditioned medium was collected from the fibroblast compartment of non-contact co-cultures. Conditioned medium from the first set of replicates from figure 5 was assayed for the concentrations of 17 different cytokines.

CAV1 levels

Fibroblastic CAV1 protein levels from the Fre85 and MDA-MB-231 non-contact co-culture were 0.37 fold the monoculture control (Fig. 7A,B). Fibroblastic CAV1 protein levels from the Fre85 and THPD non-contact co-culture were 0.36 fold the monoculture control (Fig. 7A,B). Fibroblastic CAV1 protein levels from the Fre85 and THPT non-contact co-culture were 0.21 fold the monoculture control (Fig. 7A,B). Fibroblastic CAV1 protein levels from the Fre85 and MDA-MB-231 and THPD non-contact co-culture were 0.39 fold the monoculture control (Fig. 7A,B). Fibroblastic CAV1 protein levels from the Fre85 and MDA-MB-231 and THPT non-contact co-culture were 0.46 fold the monoculture control (Fig. 7A,B).

For the contact co-culture system, conditioned medium was collected, cells were sorted on the FACS, and CAV1 protein levels were determined in the fibroblast compartment. Fibroblastic CAV1 protein levels from the Fre85 and MDA-MB-231 contact co-culture were 1.22 fold the monoculture control (Fig. 7C,D). Fibroblastic CAV1 protein levels from the Fre85 and THPD contact co-culture were 0.53 fold the
monoculture control (Fig. 7C,D). Fibroblastic CAV1 protein levels from the Fre85 and THPT contact co-culture were 0.70 fold the monoculture control (Fig. 7C,D). Fibroblastic CAV1 protein levels from the Fre85 and MDA-MB-231 and THPD contact co-culture were 0.77 fold the monoculture control (Fig. 7C,D). Fibroblastic CAV1 protein levels from the Fre85 and MDA-MB-231 and THPT contact co-culture were 0.79 fold the monoculture control (Fig. 7C,D).

Figure 7: Co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line for cytokine assay. Results for the co-cultures are relative to the fibroblast monoculture control. Conditioned medium from these co-cultures was used in a cytokine assay. (A) Non-contact co-culture. Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. (B) Contact co-culture. Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Cultures were sorted using FACS. (C) Western analysis image of non-contact co-culture. β-actin is shown as a loading control. (D) Western analysis image of contact co-culture. β-actin is shown as a loading control. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1
**Cytokine concentrations**

The conditioned medium from the 3-day non-contact, and 5-day contact, co-cultures, described in Figure 7, was screened using a cytokine assay, and the concentration of a number of interleukins, chemokines, and growth factors, was measured. Cytokine concentrations were measured in duplicate from a single (representative) sample. This study was unable to measure triplicate samples due to monetary constraints.

Interleukins

The interleukins detected by the assay were IL-1β, IL-1RA, IL-6, IL-8, IL-10 and IL-13. IL-1β was secreted in concentrations that were below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 1 pg/mL, in the 3-day monocultures of primary human fibroblasts Fre85, highly invasive human breast cancer cells MDA-MB-231, undifferentiated monocytes THPD and differentiated monocytes THPT. In the corresponding 3-day non-contact co-cultures, Fre85 cultured with THPT resulted in IL-1β levels of 2.63 ± 0.08 pg/mL and Fre85 cultured with MDA-MB-231 and THPT resulted in IL-1β levels of 2.44 ± 0.25 pg/mL in the conditioned medium (Fig. 8A). IL-1β levels in 5-day contact co-cultures were all below the threshold of 1 pg/mL, with the exception of the THPT monoculture (4.06 ± 0.15 pg/mL, Fig. 8B).

The highest levels of IL-1RA were measured in the THPT monocultures: 205 ± 10 pg/mL in conditioned medium from the 3-day monoculture (Fig. 8C), and 1476 ± 65 pg/mL in conditioned medium from the 5-day monoculture (Fig. 8D). Non-contact co-cultures containing THPT also had high levels of IL-1RA, with 201 ± 15 pg/mL in
conditioned medium from Fre85 cultured with THPT alone, and 116 ± 0.46 pg/mL in conditioned medium from Fre85 cultured with both THPT and MDA-MB-231 (Fig. 8C). The levels measured in contact co-cultures were 81.5 ± 3.35 pg/mL in conditioned medium from Fre85 cultured with THPT alone and 218 ± 4.55 pg/mL in conditioned medium from Fre85 cultured with both THPT and MDA-MB-231 (Fig. 8D).

IL-6 was detected at high concentrations in the conditioned medium from some of the cultures. In fact, conditioned medium from selected cultures had to be diluted and rescreened in order to reliably measure IL-6 concentrations. Unfortunately, not all co-cultures were rescreened, due to limitations of the assay, and the IL-6 concentrations measured in the non-contact co-culture of Fre85 with THPT may be an underestimation.

IL-6 levels were highest in non-contact co-cultures of Fre85 with both MDA-MB-231 and THPT (triple culture), with a concentration of 21,865 ± 5,665 pg/mL (Fig. 8E). IL-6 levels in non-contact co-cultures of Fre85 with THPT (7,371 ± 2,289 pg/mL), and of Fre85 with MDA-MB-231 and THPD (3,784 ± 310 pg/mL), were also elevated, compared with the relevant 3-day monoculture of Fre85 (2,480 ± 242 pg/mL, Fig. 8E). Monocultures of MDA-MB-231, THPD, and THPT, had, in comparison, minimal IL-6 concentrations of 2.0-8.6 pg/mL (Fig. 8E).
After 5 days, the IL-6 concentration in the Fre85 monoculture had increased to 9,037 ± 188 pg/mL IL-6 (Fig. 8F). Levels of IL-6 in 5-day contact co-cultures of Fre85 with MDA-MB-231 (3,653 ± 577 pg/mL), of Fre85 with THPD (8,499 ± 350 pg/mL), and of Fre85 with THPT (11,383 ± 1,677 pg/mL), were also higher, compared to the respective non-contact co-cultures, however the increase was not as strong as in the Fre85 monoculture (Fig. 8F). The 5-day contact triple cultures of Fre85 with MDA-MB-231 and THPD (3,075 ± 328 pg/mL), and of Fre85 with MDA-MB-231 and THPT (7,038 ± 901 pg/mL), showed a decrease in IL-6 concentrations, compared to the respective 3-day non-contact co-cultures, and compared to the 5-day Fre85 monoculture. Contact co-culture of Fre85 with THPD (8,499 ± 350 pg/mL) or THPT (11,383 ± 1,677 pg/mL) alone led to higher concentrations of IL-6 in the conditioned medium, compared to contact co-culture of Fre85 with MDA-MB-231 alone (3,653 ± 577 pg/mL), or triple culture with THPD (3,075 ± 328 pg/mL) or THPT (7,038 ± 901 pg/mL, Fig. 8F).

Similar to IL-6, IL-8 was detected in high concentrations in some cultures. Concentrations of IL-8 may be underestimated in non-contact co-cultures of Fre85 with MDA-MB-231, of Fre85 with THPD, of Fre85 with THPT, and of Fre85 with MDA-MB-231 and THPD (triple culture). In addition, the 3-day monoculture of THPT, and 5-day contact co-cultures of Fre85 with MDA-MB-231 and THPD (triple culture), and of Fre85 with MDA-MB-231 and THPT (triple culture), may have IL-8 concentrations that are underestimated in the cytokine assay, due to a limited capacity for rescreening diluted samples.
The non-contact co-culture of Fre85 with MDA-MB-231 and THPT (triple culture) resulted in an IL-8 concentration in the conditioned medium that was higher than the monoculture controls; 44,618 ± 6,012 pg/mL, compared to 6,370 ± 675 pg/mL (Fre85), 295 ± 11 pg/mL (MDA-MB-231), and 3,141 ± 47 pg/mL (THPT, Fig. 8G). The remaining non-contact co-cultures had IL-8 concentrations below the concentration measured in the triple culture of Fre85, MDA-MB-231 and THPT, but the concentrations measured are likely underestimated and may lie between 6000-44000 pg/mL.

After 5 days, the IL-8 concentration in the monoculture of Fre85 was 11,513 ± 157 pg/mL, the IL-8 concentration in the monoculture of THPD was 61.3 ± 3.90 pg/mL, and the IL-8 concentration in the THPT monoculture was 78,659 ± 41,389 pg/mL (Fig. 8H). The contact co-cultures of Fre85 with MDA-MB-231 (4,802 ± 298 pg/mL), with THPD (5,671 ± 191 pg/mL), and with THPT (22,175 ± 2,331 pg/mL), were below the average IL-8 concentrations measured in the respective monoculture controls (Fig. 8H). The triple cultures with Fre85, MDA-MB-231, and THPD or THPT, had IL-8 concentrations between 2000-4000 pg/mL, but these are likely underestimated.

The concentrations of IL-10 measured in conditioned medium from non-contact co-cultures, and the corresponding 3-day monoculture controls, were below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 1.6 pg/mL, with the exception of the co-cultures of Fre85 with THPT.
(14.5 ± 0.77 pg/mL), and of the triple culture of Fre85 with MDA-MB-231 and THPT (16.1 ± 0.31 pg/mL, Fig. 8I).

In the 5-day monoculture controls corresponding to the contact co-cultures, concentrations of IL-10 were below the threshold of 1.6 pg/mL, with the exception of THPT (1.83 ± 0.42 pg/mL, Fig. 8J). The highest IL-10 concentrations measured in the contact co-cultures resulted from Fre85 cultured with THPT (4.48 ± 0.29 pg/mL), and from Fre85 cultured with MDA-MB-231 and THPT (triple culture, 4.78 ± 0.59 pg/mL, Fig. 8J). The remaining contact co-cultures had IL-10 levels that were higher than the corresponding monoculture controls.

Similar to the concentrations found for IL-10, the IL-13 concentration was highest in the non-contact co-cultures of Fre85 with THPT (2.19 ± 0.25 pg/mL), and of Fre85 with MDA-MB-231 and THPT (triple culture, 3.2 ± 0.69 pg/mL, Fig. 8K). The remaining non-contact co-cultures and corresponding 3-day monocultures had concentrations of IL-13 that were below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 1.9 pg/mL (Fig. 8K).

IL-13 concentrations in the contact co-cultures, and their corresponding 5-day monoculture controls, were similarly highest in the cultures of Fre85 with THPT (2.9 ± 1.0 pg/mL), and of Fre85 with MDA-MB-231 and THPT (triple culture, 4.13 ± 3.2 pg/mL), and were below the threshold in the remaining co-cultures and monoculture controls (Fig. 8L).
Figure 8: Interleukin levels measured in conditioned medium from co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The minimum detectable concentration plus 2 standard deviations is indicated with a red line, unless all measured concentrations exceed this limit. (A) IL-1β levels measured in the conditioned medium after 3 days of non-contact co-culture. (B) IL-1β levels measured in the conditioned medium after 5 days of contact co-culture. (C) IL-1RA levels measured in the conditioned medium after 3 days of non-contact co-culture. (D) IL-1RA levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1.
Figure 8 continued: Interleukin levels measured in conditioned medium from co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The maximum concentration that can be measured accurately is indicated with a purple line, unless all measured concentrations are below this limit. (E) IL-6 levels measured in the conditioned medium after 3 days of non-contact co-culture. (F) IL-6 levels measured in the conditioned medium after 5 days of contact co-culture. (G) IL-8 levels measured in the conditioned medium after 3 days of non-contact co-culture. (H) IL-8 levels measured in the conditioned medium after 5 days of contact co-culture. # The IL-8 levels measured in the non-contact co-culture and the contact triple cultures are underestimated and are likely to be between 5,000 pg/mL and 80,000 pg/mL. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1.
Figure 8 continued: Interleukin levels measured in conditioned medium from co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The minimum detectable concentration plus 2 standard deviations is indicated with a red line, unless all measured concentrations exceed this limit. (I) IL-10 levels measured in the conditioned medium after 3 days of non-contact co-culture. (J) IL-10 levels measured in the conditioned medium after 5 days of contact co-culture. (K) IL-13 levels measured in the conditioned medium after 3 days of non-contact co-culture. (L) IL-13 levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

Chemokines

The chemokines detected by the assay were MCP-1 (CCL2), macrophage inflammatory protein-1α (MIP-1α/CCL3), and RANTES (CCL5). MCP-1 concentrations were high in some of the undiluted samples, and the concentration measured in the Fre85 non-contact co-culture with THPT, and the Fre85 contact co-culture with MDA-MB-231 and THPD or THPT (triple cultures), are likely to be an
underestimation. MCP-1 levels were elevated in all non-contact co-cultures, compared to the corresponding monoculture controls, with the highest concentrations measured in the non-contact co-culture of Fre85 with MDA-MB-231 and THPT (triple culture, 15,322 ± 1,230 pg/mL), and Fre85 with THPT (8,303 ± 135 pg/mL, possible underestimation, Fig. 9A). MCP-1 concentrations in the contact co-cultures of Fre85 with MDA-MB-231 (17,732 ± 1,634 pg/mL), and of Fre85 with THPT (23,585 ± 151 pg/mL), were above the corresponding monoculture controls (Fig. 9B). The remaining co-cultures had lower MCP-1 levels, however the concentrations in the triple cultures are likely to be an underestimation.

MIP-1α concentrations were highest in conditioned medium from non-contact co-cultures of Fre85 with THPT (1,656 ± 13 pg/mL), Fre85 with MDA-MB-231 and THPT (triple culture, 484 ± 12 pg/mL), and the 3-day monoculture of THPT (1,138 ± 6.6 pg/mL, Fig. 9C). Concentrations in the remaining non-contact co-cultures, and the corresponding monocultures, were below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 6.2 pg/mL.

The conditioned medium from 5-day contact co-cultures of Fre85 with THPT, and Fre85 with MDA-MB-231 and THPT (triple culture), had lower MIP-1α concentrations than their 3-day non-contact counterparts; 68.9 ± 0.11 pg/mL and 55.5 ± 1.62 pg/mL respectively (Fig. 9D). The 5-day monoculture of THPT, on the other hand, showed a higher concentration of 4,139 ± 660 ng/mL MIP-1α, compared to the 3-day monoculture (Fig. 9D). The 5-day THPD monoculture had a concentration of 7.06 ± 0.54 pg/mL (Fig. 9D). Concentrations in the remaining contact co-cultures, and
the 5-day MDA-MB-231 monoculture, were below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 6.2 pg/mL.

The concentration of RANTES in the 3-day non-contact co-cultures of Fre85 with MDA-MB-231 (6.8 ± 0.78 pg/mL), of Fre85 with THPD (168 ± 3.8 pg/mL), and of Fre85 with MDA-MB-231 and THPD (triple culture, 171 ± 7.3 pg/mL), was higher than the corresponding 3-day monocultures of Fre85 (2.45 ± 0.00 pg/mL), MDA-MB-231 (below threshold of 1.9 pg/mL), and THPD (90.4 ± 2.16 pg/mL, Fig. 9E). The 3-day monoculture of THPT, on the other hand, was 906 ± 2.9 pg/mL, which was higher than the concentrations measured in the co-cultures of Fre85 with THPT (330 ± 2.2 pg/mL), and of Fre85 with MDA-MB-231 and THPT (triple culture, 335 ± 0.00 pg/mL, Fig. 9E).

A similar pattern was seen in the 5-day contact co-cultures. The contact co-culture of Fre85 with MDA-MB-231 resulted in increased RANTES concentrations in the conditioned medium (435 ± 7.2 pg/mL), compared to the 5-day monoculture controls of Fre85 (17.3 ± 0.00 pg/mL), and MDA-MB-231 (48.5 ± 2.78 pg/mL, Fig. 9F). Co-cultures of Fre85 with THPD (422 ± 28.5 pg/mL) had similar concentrations to the 5-day monoculture of THPD (458 ± 21.3 pg/mL), however the triple culture of Fre85 with MDA-MB-231 and THPD (792 ± 6.1 pg/mL) had increased concentrations of RANTES, compared with the monoculture controls (Fig. 9F). The concentrations were the highest in the 5-day monoculture of THPT (3,531 ± 122 pg/mL), and this was higher than the concentrations in the co-cultures of Fre85 with THPT (701 ± 28.7
pg/mL), and of Fre85 with MDA-MB-231 and THPT (triple culture, 1,055 ± 20.8 pg/mL, Fig. 9F).

Figure 9: Chemokine levels measured in conditioned medium from co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. (A) MCP-1 levels measured in the conditioned medium after 3 days of non-contact co-culture. (B) MCP-1 levels measured in the conditioned medium after 5 days of contact co-culture. (C) MIP-1α levels measured in the conditioned medium after 3 days of non-contact co-culture. (D) MIP-1α levels measured in the conditioned medium after 5 days of contact co-culture. # The MCP-1 levels measured in the Fre85+MDA+THPT contact co-culture are underestimated and are likely to be between 15,000 pg/mL and 25,000 pg/mL. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1
Figure 9 continued: Chemokine levels measured in conditioned medium from cocultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. (E) RANTES levels measured in the conditioned medium after 3 days of non-contact co-culture. (F) RANTES levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

Growth factors

The growth factors measured by the assay were EGF, basic fibroblast growth factor (FGF-2), platelet-derived growth factor (PDGF) –AA and –AB/BB, and vascular endothelial growth factor (VEGF). The threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of EGF is 4.6 pg/mL. The concentrations in the conditioned medium from the monocultures, and cocultures, were below this threshold, with the exception of the 3-day non-contact coculture of Fre85 with THPT (4.84 ± 0.78 pg/mL), the 5-day contact co-culture of Fre85 with THPT (5.7 ± 0.88 pg/mL), and the 5-day monoculture of THPT (5.9 ± 0.52 pg/mL, Fig. 10A,B). The average concentration in the 3-day monoculture of MDA-MB-231 (6.6 ± 4.1 pg/mL) was also above the threshold, however the standard deviation for this measurement was very large (Fig. 10A).
The concentration of FGF-2 measured in the non-contact co-cultures of Fre85 with MDA-MB-231 (38 ± 1.1 pg/ml), of Fre85 with THPD (56 ± 0.95 pg/mL), and of Fre85 with MDA-MB-231 and THPD (triple culture, 31 ± 1.9 pg/mL), was higher than in the 3-day monocultures of Fre85 (22 ± 2.3 pg/mL), MDA-MB-231, and THPD (the latter two were below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 11.8 pg/mL, Fig. 10C). Non-contact co-cultures containing THPT had a FGF-2 concentration close the concentration in the 3-day Fre85 monoculture; the concentration of FGF-2 in conditioned medium from the co-culture of Fre85 with THPT was 18 ± 3.4 pg/mL, and 20 ± 9.0 pg/mL in conditioned medium from the triple culture of Fre85 with MDA-MB-231 and THPT (Fig. 10C). The 3-day monoculture of THPT did not have a FGF-2 concentration above the threshold. Conversely, in the 5-day contact co-cultures the only concentration of FGF-2 detected above the threshold was 15 ± 2.8 pg/mL in the triple culture of Fre85 with MDA-MB-231 and THPT (Fig. 10D).

High concentrations of PDGF-AA were detected in the 3-day monocultures of MDA-MB-231 (73 ± 4.6 pg/mL), and THPT (175 ± 2.5 pg/mL), compared to the non-contact co-cultures (Fig. 10E). The non-contact co-culture of Fre85 with THPD (4.5 ± 0.17 pg/mL) had a PDGF-AA concentration slightly above the concentration in the corresponding 3-day monoculture of Fre85 (2.6 ± 0.18 pg/mL), and THPD (3.9 ± 0.52 pg/mL, Fig. 10E). The remaining non-contact co-cultures had a concentration below the average of the concentrations in the corresponding 3-day monoculture controls.
The PDGF-AA concentration in the 5-day monocultures of Fre85 (5.33 ± 0.40 pg/mL), MDA-MB-231 (590 ± 13.1 pg/mL), THPD (22.0 ± 2.55 pg/mL), and THPT (596 ± 33.8 pg/mL) was higher compared to the 3-day monocultures (Fig. 10F). This was, however, the case to a much lesser extend in the contact co-cultures of Fre85 with MDA-MB-231 (13.4 ± 0.69 pg/mL for the 3-day non-contact co-culture, compared to 19.0 ± 0.49 pg/mL for the 5-day contact co-culture), and the triple cultures of Fre85 with MDA-MB-231 and THPD (9.31 ± 0.71 pg/mL, compared to 28.5 ± 0.66 pg/mL) or THPT (10.5 ± 0.21 pg/mL, compared to 23.5 ± 0.55 pg/mL, Fig. 10F). Conversely, the remaining 5-day contact co-cultures showed similar or lower PDGF-AA concentrations, compared to the corresponding 3-day non-contact co-cultures; The PDGF-AA concentration in Fre85 non-contact co-culture with THPD was 4.49 ± 0.17 pg/mL versus 4.85 ± 0.09 pg/mL in the contact co-culture, and the concentration of PDGF-AA in non-contact co-culture of Fre85 with THPT was 11.6 ± 0.59 pg/mL versus 6.97 ± 0.25 pg/mL in the contact co-culture (Fig. 10F).

For the non-contact co-cultures, the only PDGF-AB/BB levels measured above the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 2.7 pg/mL were found in the cultures of Fre85 with THPT (10.9 ± 4.61 pg/mL), and of Fre85 with MDA-MB-231 and THPT (11.8 ± 4.96 pg/mL, Fig. 10G). The PDGF-AB/BB concentration in the 3-day monoculture of THPT was 4.59 ± 0.49 pg/mL, and the concentration in the 3-day monocultures of Fre85 and THPD was below the threshold. The average concentration in the 3-day monoculture of MDA-MB-231 was above the threshold, but had a very large standard deviation (27.0 ± 32.0 pg/mL).
PDGF-AB/BB levels in the contact co-cultures were the highest in the cultures containing THPT. The culture of Fre85 with THPT had a PDGF-AB/BB concentration of 18.5 ± 1.54 pg/mL, and the concentration in the triple culture of Fre85 with MDA-MB-231 and THPT was 14.1 ± 6.44 pg/mL (Fig. 10H). The highest concentration was, however, found in the 5-day monoculture of MDA-MB-231 (31.6 ± 2.63 pg/mL), whereas the concentration in the 5-day monoculture of THPT was 10.9 ± 2.93 pg/ml (Fig. 10H). The concentration in the 5-day monoculture of Fre85 was 5.62 ± 3.85 pg/mL, and the 5-day monoculture of THPD did not have a concentration above the threshold (Fig. 10H). Interestingly, the contact co-culture of Fre85 with MDA-MB-231 resulted in PDGF-AB/BB levels in the conditioned medium (3.88 ± 1.55 pg/mL) below concentrations measured in the corresponding monocultures (Fig. 10H). Conversely, the concentrations in contact co-culture of Fre85 with THPD (9.41 ± 6.96 pg/mL), and of Fre85 with THPT (18.5 ± 1.54 pg/mL), were higher than the corresponding controls (Fig. 10H).

VEGF levels were higher in all non-contact co-cultures, compared to the corresponding 3-day monoculture controls. The highest concentration was measured in the conditioned medium from the triple culture of Fre85 with MDA-MB-231 and THPT (2,792 ± 53.4 pg/mL, Fig. 10I). The concentrations in the 3-day monocultures were highest in Fre85 (1,575 ± 27.0 pg/mL), whereas 3-day monocultures of MDA-MB-231 (226 ± 1.8 ng/mL), THPD (14.5 ± 4.71 pg/mL), and THPT (178 ± 16.3 pg/mL), had comparatively low concentrations of VEGF (Fig. 10I).
The 5-day monocultures of MDA-MB-231 (2,932 ± 104.6 pg/mL), and THPD (242.1 ± 11.9 pg/mL), showed a higher VEGF concentration, compared to the corresponding 3-day monoculture (226.18 ± 1.76 pg/mL and 14.52 ± 4.71 pg/mL, respectively, Fig. 10J). The 5-day monoculture of THPT showed a concentration of 699.0 ± 3.1 pg/mL, compared to 178.04 ± 16.3 pg/mL in the 3-day monoculture, and the monoculture of Fre85 showed a concentration of 1,168 ± 14.4 pg/mL, compared to 1,575.22 ± 27.0 pg/mL in the 3-day monoculture. The contact co-cultures containing THPD had higher concentrations of VEGF than contact co-cultures containing THPT; the VEGF concentration in the culture of Fre85 with THPD was 1,541 ± 143.8 pg/mL, Fre85 with THPT was 860.2 ± 11.4 pg/mL, triple culture of Fre85 with MDA-MB-231 and THPD was 1,831 ± 28.6 ng/mL and triple culture of Fre85 with MDA-MB-231 and THPT was 1,358 ± 87.5 pg/mL (Fig. 10J). The VEGF concentration in the 5-day monoculture of THPD (242.1 ± 11.9 pg/mL) was, however, lower than the VEGF concentration in the 5-day monoculture of THPT (699.0 ± 3.1 ng/mL, Fig. 10J).
Figure 10: Growth factor levels measured in conditioned medium from co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The minimum detectable concentration plus 2 standard deviations is indicated with a red line, unless all measured concentrations exceed this limit. (A) EGF levels measured in the conditioned medium after 3 days of non-contact co-culture. (B) EGF levels measured in the conditioned medium after 5 days of contact co-culture. (C) FGF-2 levels measured in the conditioned medium after 3 days of non-contact co-culture. (D) FGF-2 levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1
Figure 10 continued: Growth factor levels measured in conditioned medium from cocultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The minimum detectable concentration plus 2 standard deviations is indicated with a red line, unless all measured concentrations exceed this limit. (E) PDGF-AA levels measured in the conditioned medium after 3 days of non-contact co-culture. (F) PDGF-AA levels measured in the conditioned medium after 5 days of contact co-culture. (G) PDGF-AB/BB levels measured in the conditioned medium after 3 days of non-contact co-culture. (H) PDGF-AB/BB levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fré85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1
Figure 10 continued: Growth factor levels measured in conditioned medium from cocultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The minimum detectable concentration plus 2 standard deviations is indicated with a red line, unless all measured concentrations exceed this limit. (I) VEGF levels measured in the conditioned medium after 3 days of non-contact co-culture. (J) VEGF levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

Other cytokines

The cytokine assay measured the concentrations of a further 3 cytokines, a colony stimulating factor (GM-CSF), an interferon (IFN-γ) and a tumour necrosis factor (TNF-α). The highest concentrations of GM-CSF were measured in the monocultures of MDA-MB-231, and the co-cultures that contain MDA-MB-231. The concentration of GM-CSF in the 3-day monoculture of MDA-MB-231 was $89.2 \pm 3.49$ pg/mL, the concentration in the non-contact co-culture of Fre85 with MDA-MB-231 was $31.4 \pm 1.74$ pg/mL GM-CSF, and the concentrations in the triple cultures of Fre85 with MDA-MB-231 and THPD, and Fre85 with MDA-MB-231 and THPT, were $21.5 \pm 1.02$ and $56.4 \pm 1.84$ pg/mL, respectively (Fig. 11A). Interestingly, the non-contact co-culture of Fre85 with THPT ($48.8 \pm 0.93$ pg/mL GM-CSF), resulted in a higher concentration than either of the corresponding 3-day monocultures; the concentration
in the monoculture of THPT was 17.3 ± 0.21 pg/mL and the concentration in the monoculture of Fre85 was below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 15.0 pg/mL.

The concentration of GM-CSF in the conditioned medium from the 5-day monoculture of MDA-MB-231 was 2,621 ± 33.2 pg/mL, compared to 89.18 ± 3.49 pg/mL in the 3-day monoculture (Fig. 11B). The concentration of GM-CSF in the contact co-culture of Fre85 with MDA-MB-231 was 144.5 ± 4.9 pg/mL, the concentrations in the triple cultures of Fre85 with MDA-MB-231 and THPD, or THPT, were 58.4 ± 5.13 pg/mL and 147.0 ± 1.8 pg/mL GM-CSF respectively (Fig. 11B). In contrast to the effect seen in the 3-day non-contact co-cultures, the 5-day contact co-culture of Fre85 with THPT resulted in GM-CSF concentrations that were below the average concentration of the corresponding monocultures; conditioned medium from a contact co-culture of Fre85 with THPT was 12.8 ± 0.11 pg/mL, conditioned medium from a 5-day monoculture of Fre85 was 8.77 ± 0.30 pg/mL and of THPT was 69.5 ± 1.25 pg/mL GM-CSF.
Figure 11: Colony stimulating factor levels measured in conditioned medium from co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The minimum detectable concentration plus 2 standard deviations is indicated with a red line, unless all measured concentrations exceed this limit. (A) GM-CSF levels measured in the conditioned medium after 3 days of non-contact co-culture. (B) GM-CSF levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

The concentrations of IFN-γ measured in the non-contact co-cultures, and their corresponding 3-day monoculture controls, were below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 1.1 pg/mL, with the exception of the culture of Fre85 and THPT (7.92 ± 0.30 pg/mL), and of the triple culture of Fre85 with MDA-MB-231 and THPT (5.99 ± 0.90 pg/mL, Fig. 12A).

Similar to what was seen in the non-contact co-cultures, the highest concentrations of IFN-γ were measured in the conditioned medium from 5-day contact co-cultures containing Fre85 and THPT (4.17 ± 0.76 pg/mL), and Fre85, MDA-MB-231 and
THPT (2.35 ± 0.00 pg/mL), whereas the remaining cultures had concentrations below the threshold (Fig. 12B).

Figure 12: Interferon levels measured in conditioned medium from co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The minimum detectable concentration plus 2 standard deviations is indicated with a red line, unless all measured concentrations exceed this limit. (A) IFN-γ levels measured in the conditioned medium after 3 days of non-contact co-culture. (B) IFN-γ levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

The concentrations of TNF-α measured in the non-contact co-cultures were the highest in cultures containing THPT, and in the corresponding 3-day monoculture control; the concentration in conditioned medium from the culture of Fre85 with THPT was 83.4 ± 1.75 pg/mL, from the triple culture of Fre85 with MDA-MB-231 and THPT was 15.7 ± 0.45 pg/mL, and from the 3-day monoculture of THPT was 132.1 ± 0.13 pg/mL TNF-α (Fig. 13A). The concentrations in the conditioned medium from the remaining non-contact cultures and their corresponding 3-day monocultures
were below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 1.0 pg/mL.

The concentration of TNF-α measured in the 5-day monoculture of THPT was 494.7 ± 0.46 pg/mL (Fig. 13B). In contrast, the 5-day contact co-cultures containing THPT showed concentrations of TNF-α that were lower than the concentrations measured in the corresponding 3-day non-contact co-cultures; the concentration in conditioned medium from the culture of Fre85 with THPT was 2.22 ± 0.08 pg/mL, and from the triple culture of Fre85 with MDA-MB-231 and THPT was 1.65 ± 0.48 pg/mL TNF-α (Fig. 13B). The conditioned medium from the remaining contact co-cultures, and their corresponding 5-day monocultures, had concentrations of TNF-α below the threshold of the manufacturer-provided minimum detectable concentration plus 2 standard deviations of 1.0 pg/mL, with the exception of the 5-day monoculture of MDA-MB-231, which had a TNF-α concentration of 2.58 ± 0.09 pg/mL (Fig. 13B).
Figure 13: Tumour necrosis factor levels measured in conditioned medium from co-cultures of human primary breast fibroblasts (Fre85) with a highly invasive (MDA-MB-231) human breast cancer cell line and an undifferentiated (THPD) or differentiated (THPT) monocytic cell line, using serum replacement. Concentrations were measured using a Milliplex MAP human cytokine/chemokine magnetic bead panel. Error bars represent the standard deviation. The minimum detectable concentration plus 2 standard deviations is indicated with a red line, unless all measured concentrations exceed this limit. (A) TNF-α levels measured in the conditioned medium after 3 days of non-contact co-culture. (B) TNF-α levels measured in the conditioned medium after 5 days of contact co-culture. Fre=Fre85, MDA=MDA-MB-231, THPD=carrier-treated THP-1, THPT=TPA-treated THP-1

Table 5 and Table 6 show the differentially secreted cytokines in conditioned medium from non-contact, and contact, co-cultures, respectively.
Table 5: Differentially secreted cytokines in 3-day non-contact co-cultures, compared to the 3-day Fre85 monoculture control. Fre = Fre85 primary human breast fibroblasts, MDA = MDA-MB-231 invasive human breast cancer cells, THPD = undifferentiated monocytes, THPT = differentiated monocytes. + indicates that the concentration is slightly higher, ++ moderately higher, and +++ much higher than in the Fre85 monoculture. - indicates that the concentration is slightly lower, -- moderately lower, and --- much lower than in the Fre85 monoculture. * Likely underestimation of concentration.

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Table 6: Differentially secreted cytokines in 5-day contact co-cultures, compared to the 5-day Fre85 monoculture control. Fre = Fre85 primary human breast fibroblasts, MDA = MDA-MB-231 invasive human breast cancer cells, THPD = undifferentiated monocytes, THPT = differentiated monocytes. + indicates that the concentration is slightly higher, ++ moderately higher, and +++ much higher than in the Fre85 monoculture. - indicates that the concentration is slightly lower, -- moderately lower, and --- much lower than in the Fre85 monoculture. * Likely underestimation of concentration.

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Discussion

The results from this study show that the inclusion of differentiated monocytes in non-contact and contact co-cultures of fibroblasts with breast cancer cells leads to increased concentrations of cytokines that are known to play a role in CAF-tumour cell interactions. Additionally, the co-culture of fibroblasts with cancer cells and differentiated monocytes resulted in increased concentrations of cytokines associated with ‘alternatively’ activated (M2) macrophages.

The conditioned medium from co-cultures that resulted in low fibroblastic CAV1 levels contained several cytokines in increased concentrations. These potential CAV1-regulating cytokines include RANTES, MCP-1, VEGF, and GM-CSF. This study provides several candidates for the further investigation of fibroblastic CAV1 regulation through secreted cytokines. Additionally, this study shows that FBS affects fibroblastic CAV1 levels in co-cultures, which is an important consideration for future investigations into the regulation of CAV1, using cell culture methods.

Serum conditions

The addition of FBS to cell culture medium provides an, often necessary, source of growth and adhesion factors, hormones, lipids, and minerals, to the cells in culture. Many cell lines require the addition of serum to grow, and many more perform less optimally without serum. A downside to using serum in cell culture is the introduction of unknown variables. There can be significant variability from one batch of serum to the next, significantly impacting experimental outcomes (Baker, 2016; Khodabukus...
and Baar, 2014; Sikora et al., 2016). Additionally, many components of serum are unstable over time, when used in routine cell culture.

As is common practice in many labs, the same batch of serum was used in all the experiments for the duration of this study, thereby eliminating batch-to-batch variability. However, serum contains many unstable proteins, such as growth factors and cytokines, which can lose activity over time. Manufacturers of recombinant growth factors and other cytokines recommend long-term storage in lyophilized form, and short-term storage at -80 °C. Similarly, manufacturers advise that foetal bovine serum be stored at -80 °C. Routine cell culture techniques, such as the thawing out of serum, storing and handling of serum-containing medium, including warming up serum-containing medium, can, therefore, potentially introduce changes to serum activity.

Reducing, or removing, the serum content in cell culture media can improve the reproducibility of experiments (Van der Valk et al., 2010). To investigate whether the addition of FBS to co-cultures contributed to inter-experimental variability, this study compared co-culture of fibroblasts, cancer cells, and differentiated monocytes, at conventional serum levels to reduced serum levels.

The comparison of non-contact co-culture of fibroblasts with cancer cells and lymphocytes at 10% serum conditions, with 2% serum conditions, showed a trend towards an increased variability in fibroblastic CAV1 protein levels, and no change in
the variability in fibroblastic mRNA levels, with a lower serum concentration. Interestingly, decreasing the FBS levels from 10% to 2% led to drastically different average fibroblastic CAV1 protein levels after co-culture. This suggests that FBS affects the fibroblastic CAV1 protein response during co-culture.

Many cell lines do not respond well to culture without serum, or with reduced serum. Primary cells in particular, may rely on the presence of serum to be able to continue growing after having been removed from the extracellular matrix provided in the body (Baker, 2016). Removing serum from cultures may cause cells to become more fragile or stressed (Van der Valk et al., 2010), which is a possible explanation for seeing changed cell behaviour. Additionally, cells that are fragile, or behave differently, due to serum conditions that are suboptimal for their growth, may respond more strongly to variability in the serum.

Heat-inactivation is commonly used to inactivate heat-labile complement in serum. The practice of heat-inactivation of serum is controversial, as the benefits of heat-inactivation that once existed may no longer outweigh the disadvantages, as the content of heat-labile complement is significantly reduced today, and is no longer likely to have any measurable effect on most experimental applications. Inactivation of mycoplasma, another benefit of heat-inactivation, is likewise obsolete, due to standard filtration practices of manufacturers.
The disadvantage of heat-inactivation is that the procedure may inactivate proteins in the serum that are required for optimal cell growth. To further investigate the effect of serum components on inter-experimental variability, the use of heat-inactivated serum in co-cultures of fibroblasts, cancer cells, and differentiated monocytes, was compared with normal serum. No significant changes in inter-experimental variability, and CAV1 protein levels, were observed with the use of heat-inactivated serum. However, a trend towards lower average CAV1 protein levels after non-contact co-culture with cancer cells and differentiated monocytes, using heat-inactivated FBS, compared to using standard FBS, was observed. Heat-inactivation may, correspondingly, lead to changed cell behaviour, due to altered serum content.

As serum reduction and inactivation were shown to have an effect on CAV1 expression levels after co-culture, and did not improve inter-experimental variability, the use of a serum replacement was examined. Different serum replacement formulations are available from several manufacturers. This study used Serum Replacement 1 from MilliporeSigma (Darmstadt, Germany). All cell lines used in the co-culture system continued to proliferate, and no increase in cell death was observed, however, cells were unable to adhere to the growth surface in the absence of normal serum. For this reason, cells were seeded in the presence of FBS, and the medium was changed to serum-replacement medium, after 18-20 hours.

Non-contact co-culture of primary breast fibroblasts with highly invasive breast cancer cells and differentiated monocytes, in the presence of serum replacement, resulted in significantly lower fibroblastic CAV1 protein levels, compared to a
fibroblast monoculture control. Parallel co-cultures in the presence of 10% FBS did not show this reduction in CAV1 levels, and co-cultures in the presence of FBS, or heat-inactivated FBS, showed larger variability between experiments.

Serum replacement is a synthetic formulation, rather than a natural product like FBS, and is deficient of growth factors, steroid hormones, glucocorticoids, cell adhesion factors, detectable immunoglobulin and mitogens (manufacturer data). This study shows that the use of Serum Replacement 1 reduces inter-experimental variability in co-cultures, possibly due to the absence of labile factors in serum that influence CAV1 expression.

**Cytokine secretion profiles in co-cultures of fibroblasts, cancer cells and (differentiated) monocytes**

For the cytokine experiments, conditioned medium from co-cultures in the presence of serum replacement, rather than FBS, was used. The reasons for this were, firstly, to reduce inter-experimental variability, as experiments using Serum Replacement 1 showed less variability than those using FBS, and, secondly, FBS can contain varying levels of cytokines, and the presence of FBS in conditioned medium could mask changes in concentrations, as measured in the assay.

Fibroblastic CAV1 levels were measured in all the co-cultures that corresponded to the conditioned medium used in the cytokine array. Non-contact co-culture in the presence of Serum Replacement 1 led to a significant reduction in fibroblastic CAV1,
when primary breast fibroblasts were cultured with differentiated monocytes (double culture), or with both highly invasive breast cancer cells and (differentiated) monocytes (triple culture). A representative sample of conditioned medium from each of these conditions was screened in the cytokine assay.

Similarly, CAV1 levels were measured in contact co-cultures in the presence of Serum Replacement 1. The conditioned medium for the cytokine assay was derived from co-cultures that were sorted into fibroblastic and non-fibroblastic cell populations by FACS, to be able to determine CAV1 levels in the fibroblastic compartment. The conditioned medium screened in the cytokine assay came from a set of co-cultures that showed lower fibroblastic CAV1 levels after co-culture with (differentiated) monocytes (double culture), or with highly invasive breast cancer cells and (differentiated) monocytes (triple culture).

The cytokine assay has determined the concentration of a number of interleukins, chemokines, and growth factors, in conditioned medium from non-contact and contact co-cultures. The cytokines present in the screening panel were selected based on previous implications in fibroblast and cancer cell co-culture, fibroblast and macrophage co-culture, or for being secreted by cancer-associated fibroblasts, as reported in the literature, and within the limitations of the assay.
Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) stimulate cancer proliferation, motility, angiogenesis, and inflammation (Erez et al., 2010). The co-culture of Fre85 fibroblasts with MDA-MB-231 breast cancer cells led to the differential secretion of several cytokines, compared to Fre85 monoculture controls. Some of these cytokines were secreted by MDA-MB-231 monocultures, while others were secreted by the fibroblast and cancer cell co-culture only.

The cytokine MCP-1 was increased in both non-contact, and contact, fibroblast and cancer cell co-cultures, compared to the fibroblast, and cancer cell, monoculture controls. The same is true for FGF-2 and VEGF in non-contact co-cultures, and IL-10 and RANTES in contact co-cultures. This suggests that the interaction between fibroblasts and cancer cells resulted in the increased secretion of these cytokines.

MCP-1 is a chemoattractant of monocytes that is secreted in increased amounts by CAFs (Yang et al., 2016). The results reported here are in line with those of others, who have found increases in MCP-1 secretion in fibroblast and cancer cell co-cultures (Li et al., 2014). When differentiated monocytes were introduced into the co-culture of fibroblasts and cancer cells, the secretion of MCP-1 was further increased. MCP-1 regulates the infiltration of monocytes/macrophages, and is produced by many cells, including fibroblasts, and monocytes/macrophages (Deshmane et al., 2009). Expression levels of MCP-1 are increased in breast cancer tissues from patients (Soria et al., 2011). MCP-1 levels are strongly correlated to decreased survival rates in breast cancer (Ueno et al., 2000), and characteristic of a tumorigenic microenvironment.
The increased MCP-1 secretion found in fibroblast, cancer cell, and differentiated monocyte, co-cultures suggests that the interaction of all three of these tumour microenvironment components plays an important role in the high levels of MCP-1 that are characteristic of breast cancer.

VEGF and FGF-2 are other cytokines that are secreted in increased amounts by CAFs (De Francesco et al., 2013; Strand et al., 2014). VEGF and FGF-2 play a role in tumorigenesis by promoting angiogenesis. Similar to what was seen with the secretion profile of MCP-1, VEGF and FGF-1 concentrations were higher in the co-cultures of fibroblast with cancer cells and differentiated monocytes, compared to the fibroblast and cancer cell co-cultures.

Another cytokine that was increased in fibroblast and cancer cell contact co-cultures, compared to fibroblast, and cancer cell, monocultures, is RANTES. RANTES was secreted in high concentrations by differentiated, and undifferentiated, monocyte monocultures, but not by cancer cell monocultures. As concentrations were higher after co-culture, compared to either of the fibroblast, or cancer cell, monocultures, the secretion of RANTES was increased upon the interaction of fibroblasts and cancer cells. In addition, RANTES was secreted in increased concentrations in 5-day contact co-cultures, but not in 3-day non-contact co-cultures. Therefore, either direct cell contact is required for the secretion of RANTES to be increased, or the secretion of RANTES after fibroblast and cancer cell interaction increases over time. Both RANTES and MCP-1 are key chemokines responsible for the recruitment of macrophages to the tumour site (Deshmane et al., 2009; Soria and Ben-Baruch, 2008),
and increased levels of MCP-1 and RANTES are found in breast cancer tissue (Svensson et al., 2015). The expression of RANTES in stage 2 breast cancer patients is a predictor of disease progression (Yaal-Hahoshen et al., 2006), and inactivation of RANTES secreted in adipocyte and MDA-MB-231 co-cultures resulted in lower cancer cell motility (D’Esposito et al., 2016).

PDGF-AA concentrations were higher in the fibroblast and cancer cell co-culture, compared to the fibroblast monoculture control. PDGF promotes cell division and motility in fibroblasts (Heldin and Westermark, 1999). The monoculture controls of MDA-MB-231, and THPT, secrete higher levels of PDGF-AA than any of the co-cultures. It is possible that the higher concentrations of PDGF-AA in the fibroblast and cancer cell co-cultures is due to the presence of cancer cells, and not due to the interaction of the different cell populations. However, the increased levels of PDGF-AA may contribute to the generation of a CAF-like environment in fibroblast and cancer cell co-cultures.

Proinflammatory cytokines IL-1β, IL-6, and MIP-1α, and growth factors FGF-2 and VEGF, are highly expressed by CAFs (Erez et al., 2010; Giulianelli et al., 2008; Shiga et al., 2015). In addition, sustained low levels of TNF-α play an important role in the activation of CAFs (Landskron et al., 2014; Shiga et al., 2015). Interestingly, IL-1β and TNF-α were measured in THPT containing co-cultures only, and were undetectable in other co-cultures. Furthermore, MIP-1α (CCL3) and IL-6 were increased in THPT containing co-cultures, and not in cancer only co-cultures. This, in addition to the increased concentrations of MCP-1, VEGF, and FGF-2, in triple
cultures, compared to fibroblast and cancer cell co-cultures, suggests that the interaction of Fre85 fibroblasts with THPT is required for CAF-like crosstalk between Fre85 and MDA-MB-231 to occur.

Tumour-associated macrophages

Macrophages respond to, and generate, a broad range of stimuli, including many cytokines. Cues from the microenvironment stimulate macrophages to assert different functions, ranging from tissue repair and matrix remodelling, to proinflammatory immunity (Williams et al., 2016). The cytokine profiles in both the non-contact and contact co-culture showed an increase in the number of differentially expressed cytokines in the presence of macrophages (THPT), compared to co-cultures with monocytes (THPD), or co-cultures with cancer cells. The cytokines that were detected in higher concentrations in co-cultures containing THPT include macrophage recruiting chemokines (MCP-1, RANTES), macrophage activating cytokines (IL-1β, GM-CSF, IFN-γ), and cytokines secreted by activated macrophages (IL-6, MIP-1α, TNF-α, VEGF).

Tumour-associated macrophages display a functionally distinct phenotype, belonging to a subset of macrophages known as M2 macrophages (Mantovani and Sica, 2010). M1 stimuli include GM-CSF, IFN-γ and other proinflammatory cytokines (e.g., IL-1β, IL-6, TNF), whereas M2 stimuli include IL-10 and IL-13 (Martinez and Gordon, 2014).
Crosstalk between macrophages and cancer cells can lead to M2 polarization. The co-culture of fibroblasts with differentiated monocytes resulted in higher concentrations of M1 stimuli IFN-γ and TNF-α, compared to fibroblast co-culture with cancer cells and differentiated monocytes. Non-contact co-culture of fibroblasts with cancer cells and differentiated monocytes, on the other hand, resulted in slightly higher concentrations of M2 stimuli IL-10 and IL-13. This is in line with a study by Müller-Quernheim et al. (2012), where the co-culture of monocyte-derived macrophages with A549 cancer cells resulted in an increase in the M2 phenotype marker CCL18.

The co-culture of fibroblasts and cancer cells resulted in increased concentrations of the monocyte chemoattractants MCP-1 and RANTES. These concentrations were further increased when differentiated monocytes were introduced into the co-cultures, resulting in the co-culture of all three cell populations. Interestingly, a study by Li et al. (2015) found that, by blocking the interaction of MCP-1 with its receptor via a CCLR antagonist, the M2 macrophage polarization, and the secretion of M2 cytokines, decreased in mouse bone marrow-derived macrophages. The high levels of MCP-1 in the THPT-containing co-cultures presented here could drive M2 polarization in THPT cells.

**Cytokines and CAV1 expression by fibroblasts**

Several of the cytokines measured in the assay have been shown by others to affect CAV1 expression levels in certain contexts. The objective of the cytokine assay was to take inventory of the cytokines that are differentially expressed in co-cultures that
have decreased fibroblastic CAV1 expression, and to identify potential CAV1 regulating cytokines for further investigation.

CAV1 protein levels were decreased in all non-contact, and contact, co-cultures assayed for cytokine secretion, with the exception of the fibroblast and cancer cell contact co-culture. The only cytokines that were measured in increased concentrations in all but one of the co-cultures with decreased CAV1 protein levels were IL-1RA, RANTES and VEGF. IL-1RA and RANTES concentrations were unchanged in Fre85 and MDA-MB-231 non-contact co-cultures, whereas VEGF concentrations were slightly lower in Fre85 and THPT contact co-cultures. In addition, concentrations of MCP-1 and GM-CSF were increased in all but two of the co-cultures with reduced CAV1 levels. MCP-1 concentrations were moderately lower in THPD containing contact co-cultures and GM-CSF concentrations were unchanged in Fre85 and THPD non-contact and contact co-cultures.

As discussed above, MCP-1 and RANTES are chemoattractants of monocytes, and important regulators of the tumour microenvironment. RANTES secretion has been shown to be affected by altered CAV1 expression. CAV1 expression suppressed RANTES secretion in stably transfected RAW264.7 mouse macrophages, compared to vector-transfected controls (Wang et al., 2009). MCP-1 on the other hand, has been shown to reduce CAV1 levels in brain microvascular endothelial cells in vitro (Song et al., 2007).
Two cytokines that have been found by others to increase CAV1 levels in certain contexts are GM-CSF and VEGF. GM-CSF is secreted by macrophages, cancer cells, and fibroblasts, and differentiates and activates macrophages. Mesenchymal-like breast cancer cell lines, such as MDA-MB-231, secrete increased amounts of GM-CSF (Su et al., 2014). Indeed, the levels of GM-CSF in the monoculture controls were high, as were the GM-CSF levels in co-cultures containing MDA-MB-231. In addition to being secreted in increased amounts, GM-CSF alone was found, in the study mentioned above by Su et al. (2014), to activate macrophages in a similar way to conditioned medium from MDA-MB-231 cancer cells. A study by Fu et al. (2012) showed that GM-CSF treatment (50 ng/mL, 7 days) simultaneously increased CAV1 and CD68 (a macrophage marker) mRNA expression in mouse peripheral blood mononuclear cells. Fu et al. (2012) further demonstrated that CAV1 is an important regulator of monocyte-macrophage differentiation. As discussed above, VEGF is expressed by CAFs, and plays an important role in angiogenesis and tumorigenesis. VEGF treatment (50 ng/mL) upregulated CAV1 mRNA and protein levels, and phosphorylated CAV1 at Y14, in mesenchymal stem cells (Cipriani et al., 2014).

IL-1RA may have an indirect effect on CAV1 levels, by inhibiting the action of IL-1β. A study by Takizawa et al. (2013) showed that TNF-α (10 ng/mL) and IL-1β (1 ng/mL) treatment of periodontal ligament cells for 24 hours increased CAV1 mRNA levels, and CAV1 secretion, in the culture medium. The results presented in this chapter show that relatively high levels of IL-1RA were secreted by monocultures of differentiated monocytes, and differentiated monocyte-containing co-cultures.
Some of the other cytokines that have been linked to CAV1 regulation include EGF, IFN-γ, and TNF-α. EGF treatment (100 ng/mL for 3-5 days) downregulated CAV1 protein levels in A431 human epidermoid carcinoma cells (Lu et al., 2003), and combined IL-1α (2 ng/mL) and IFN-γ (10 ng/mL) treatment downregulated CAV1 mRNA levels in human primary thyrocytes (Marique et al., 2014). However, EGF was secreted at relatively low concentrations in the monocultures and co-cultures, as shown by the results presented in this chapter.

The cytokine secretion profiles of the co-cultures containing MDA-MB-231 are markedly different from the secretions profiles of the co-cultures containing THPT. It is common for cytokines to exert their effect based on the context of other cytokines, and additional environmental cues. Examples of this include the action of IFN-γ in the presence or absence of LPS (Mayo and Stein, 2006), and IL-10’s dual role in anti-tumour immunity and tumour promoting immune suppression (Dennis et al., 2013). It is, therefore, possible that different CAV1 regulatory mechanisms play a role in the different contexts of fibroblast co-culture with cancer cells, and fibroblast co-culture with macrophages. The effect of the interaction between fibroblasts, cancer cells and macrophages in the co-culture system, as presented and discussed in this chapter, on the secretion of cytokines, and the proposed role of these cytokines in the tumour microenvironment, has been schematically represented in Figure 14.
Figure 14: Schematic overview of interactions between fibroblasts, breast cancer cells and macrophages. The interaction between human primary breast fibroblasts (Fre85), highly invasive human breast cancer cells (MDA-MB-231) and differentiated human monocytes (THPT) results in the secretion of a plethora of cancer-associated cytokines. MCP-1, VEGF and GM-CSF have been identified as potential CAV1 regulating cytokines in the literature. Blue boxes indicate effects of cytokines secreted in increased amounts after fibroblast-cancer cell interactions, red boxes indicate effects of cytokines secreted in increased amounts after fibroblast-macrophage interactions, black boxes indicate effects of cytokines secreted in increased amounts in monocultures. Fre=Fre85, MDA=MDA-MB-231, THPT=TPA-treated THP-1

Conclusion
Distinct profiles were seen in fibroblast and cancer cell versus fibroblast and differentiated monocyte co-cultures. Several cytokines, some of which have been previously implicated in the regulation of CAV1 expression, were secreted in
increased amounts in co-cultures with decreased fibroblastic CAV1 levels. The cytokine profiles generated by this study provide several candidates to be investigated further for their ability to regulate CAV1 levels in fibroblasts. Some of these candidates are MCP-1 (CCL2), RANTES (CCL5), VEGF, and GM-CSF.

The cytokine secretion profiles of fibroblast and cancer cell, and fibroblast and differentiated monocyte co-cultures, showed increased levels of inflammation and cancer related cytokines. The inclusion of cancer cells in fibroblast and differentiated monocyte co-cultures resulted in the increased secretion of cytokines that are known to drive the polarization of macrophages to a tumour-associated macrophage (TAM) phenotype. Additionally, the inclusion of differentiated monocytes in the fibroblast and cancer cell co-cultures resulted in the increased secretion of CAF-associated cytokines. This further supports the important role of fibroblast, cancer cell, and (differentiated) monocyte, interactions in the tumour microenvironment, and the potential role of these interactions for the regulation CAV1 expression.
Chapter 6: The effect of loss of caveolin-1 in fibroblasts on EMT and cancer stem cell markers in PMC42-LA breast cancer cells
Introduction

The previous chapters investigated the effect of fibroblast, cancer cell and (differentiated) monocyte interactions on fibroblastic caveolin-1 (CAV1) levels. The current chapter will investigate the effect of decreased fibroblastic CAV1 levels on breast cancer cells.

Epithelial-to-mesenchymal transition

An important step in the invasiveness and metastasis of carcinomas is a transition of epithelial cells to a less differentiated cell type, in a process known as epithelial-to-mesenchymal-transition (EMT) (Christiansen and Rajasekaran, 2006).

During EMT, a number of cellular processes occur, that result in a more motile phenotype. Epithelial cells associate with the basement membrane through their basal surface. With the progression to a mesenchymal phenotype, the epithelial cell undergoes changes in the expression of cell-surface proteins, the expression and organisation of cytoskeletal proteins, and the production of matrix metalloproteinases (MMPs) (Kalluri and Weinberg, 2009). Eventually, the cell disassociates from the degraded basement membrane and invades the stroma, an important hallmark in the progression of cancer. Common markers of EMT include the loss of E-cadherin, and the upregulation of vimentin (Sarrio et al., 2008).

Cancer stem cells

Cancer stem cells are cancer cells that display stem cell properties, and that can give rise to the different cell populations that are present in the tumour. Unlike other cancer cells, cancer stem cells are capable of sustaining the cancer, and may mediate
metastasis and contribute to treatment relapse. Studies in immunocompromised mice have shown that only certain populations of breast cancer cells are able to give rise to new tumours, and that these tumorigenic cancer cells can be distinguished by their expression of high levels of the cell surface protein CD44, and low levels of the cell surface protein CD24 (Al-Hajj et al., 2003). Cancer stem cells produce increased levels of aldehyde dehydrogenase (ALDH), and assays used to measure ALDH levels are used to identify cancer stem cells (Storms et al., 1999).

Some studies have found an overlap between cells that have undergone EMT, and cells that display cancer stem cell-like properties. The induction of EMT in immortalised human mammary epithelial cells resulted in the expression of mesenchymal markers, such as vimentin, and the loss of epithelial markers, such as E-cadherin, while simultaneously increasing the population of CD44+/CD24− expressing cells, in a study by Mani et al. (2008). The characterisation of cells isolated from primary breast tumours has revealed two overlapping populations of CD44+/CD24− expressing cells, displaying an EMT-phenotype, and of ALDH+ expressing cells, displaying a mesenchymal-to-epithelial transition (MET)-phenotype (Liu et al., 2014b).

**Cancer-associated fibroblasts**
The role of cancer-associated fibroblasts (CAFs) in inducing EMT in cancer cells has been studied widely. CAFs secrete soluble factors, such as transforming growth factor β (TGF-β) and stromal cell-derived factor 1 (SDF-1) (Kojima et al., 2010), that have been shown to induce EMT in cancer cells (Wendt et al., 2010; Yu et al., 2014). The conditioned medium from patient-derived CAFs in culture, has likewise been shown
to increase the migration of breast cancer cells (Lebret et al., 2007). CAFs are found in the tumour microenvironment and are often distinguished from normal fibroblasts by the expression of α-smooth muscle actin (αSMA).

Loss of CAV1 in mammary fibroblasts has been linked to the upregulation of fibroblastic αSMA (Sotgia et al., 2009), and the induction of EMT in mammary epithelial cells (Simpkins et al., 2012; Sotgia et al., 2009). CAV1 knockout mammary fibroblasts secrete the EMT-inducing hepatocyte growth factor (HGF), and conditioned medium from CAV1 knockout mammary fibroblasts induces the expression of αSMA in mammary epithelial cells (Sotgia et al., 2009). Similarly, treatment with conditioned medium from immortalised breast fibroblasts transfected with CAV1 siRNA enhances the invasiveness of breast cancer cells (Simpkins et al., 2012).

**PMC42-LA breast cancer cells**
The breast cancer cell line PMC42 is a heterogeneous cell line with cancer stem cell properties that has been shown to have the capacity to develop into eight distinct morphologic subtypes in culture (Whitehead et al., 1983). The PMC42-derived cell line PMC42-LA is a stable variant in which only 5-10% of the cells express vimentin, compared to 100% of PMC42 cells (Ackland et al., 2003). The cell line PMC42-LA has been shown to be inducible to EMT, and is an excellent cell culture model for the investigation of the relationship between fibroblastic CAV1 and breast cancer EMT (Ackland et al., 2003; Lebret et al., 2007).
Aims
The main aim of this study was to investigate the effect of decreased fibroblastic CAV1 levels on EMT and cancer stem cell populations in breast cancer cells.

The specific aims were:

• To reduce fibroblastic CAV1 levels through transfection with CAV1 siRNA.

• To investigate the effect of conditioned medium from CAV1 siRNA-transfected breast fibroblasts on the expression of EMT markers E-cadherin and vimentin in PMC42-LA breast cancer cells.

• To investigate the effect of conditioned medium from CAV1 siRNA-transfected breast fibroblasts on the expression of cancer stem cell markers CD24 and CD44 in PMC42-LA breast cancer cells.

Results
To investigate the effect of CAV1 downregulation in fibroblasts on the expression of EMT markers by PMC42-LA breast cancer cells, Fre85 primary human breast fibroblasts were transfected with CAV1 siRNA, and PMC42-LA cells were cultured in the presence of conditioned medium from these CAV1 siRNA-transfected fibroblasts.
**Transfection of primary human breast fibroblasts with CAV1 siRNA**

The optimal concentration of CAV1 siRNA, and optimal exposure time to CAV1 siRNA, were determined by measuring CAV1 protein levels in transfected Fre85 cells. After 24 hours of treatment with 25 nM CAV1 siRNA, fibroblastic CAV1 protein levels were 0.45 ± 0.09 fold the control (Fig. 1A). Exposure to a lower concentration of CAV1 siRNA (15 nM) did not result in fibroblastic CAV1 levels that were lower than control levels (Fig. 1A). CAV1 protein levels were at control levels 48 hours after transfection with both 15 nM and 25 nM CAV1 siRNA, and after 72 hours CAV1 protein levels in the transfection samples were 4-5 fold the control (Fig. 2, data from 2 independent experiments).

**Figure 1:** Transfection of Fre85 primary human breast fibroblasts with CAV1 siRNA for 24 hours. Samples were collected 24 hours after transfection. Results are relative to the transfection reagent-only control. Error bars represent the standard error of the mean. (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. CAV1 levels were lower after transfection with 25 nM CAV1 siRNA, but not with 15 nM CAV1 siRNA. Results are representative of 3 independent experiments. (B) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85
Figure 2: Transfection of Fre85 primary human breast fibroblasts with CAV1 siRNA for 48-72 hours. Samples were collected 48 and 72 hours after transfection. Results are relative to the transfection reagent-only control. Error bars represent the standard error of the mean. (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. No significant changes in average CAV1 levels were detected 48 hours after transfection with 15 nM CAV1 siRNA, or 25 nM CAV1 siRNA. Results are representative of 2 independent experiments. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Average CAV1 levels were 4-5 fold the control levels 72 hours after transfection with 15 nM CAV1 siRNA, or 25 nM CAV1 siRNA. Results are representative of 2 independent experiments. (C) Representative Western analysis image. β-actin is shown as a loading control. Fre=Fre85

The expression of αSMA by transfected fibroblasts
After CAV1 siRNA transfection, Fre85 fibroblasts were evaluated for αSMA protein expression, as a cancer-associated fibroblast marker. The fibroblasts showed no significant changes in expression of αSMA protein at 24 hours after transfection with CAV1 siRNA (Fig. 3A,B). In addition, no significant changes in αSMA protein levels were detected 48 hours and 72 hours after transfection with CAV1 siRNA (data not shown).
Figure 3: αSMA levels after transfection of Fre85 primary human breast fibroblasts with CAV1 siRNA. Samples were collected 24 hours after transfection. Results are relative to the transfection reagent-only control. Error bars represent the standard error of the mean. (A) Western analysis of α-smooth muscle actin (αSMA) protein levels, relative to total protein levels, as detected by Ponceau staining. No change in αSMA levels was detected. Results are representative of 3 independent experiments. (B) Representative Western analysis image. Fre=Fre85

The treatment of PMC42-LA with conditioned medium from transfected fibroblasts
Conditioned medium was collected from Fre85 cultures, after 24 hours of 25 nM CAV1 siRNA treatment, and used to treat PMC42-LA breast cancer cells. After treatment with conditioned medium, the PMC42-LA breast cancer cells were evaluated for several EMT markers. The conditioned medium used, came from Fre85 fibroblasts that showed CAV1 protein expression of 0.72 ± 0.05 fold the control (Fig. 4A, p<0.05). After 7 days of treatment with transfection conditioned medium, E-cadherin protein expression in PMC42-LA breast cancer cells was 0.49 ± 0.07 fold control (Fig. 4B, p<0.05), vimentin protein expression was 1.51 ± 0.12 fold control (Fig. 4C, p<0.05) and αSMA protein expression was similar to the control levels (data not shown). The evaluation of E-cadherin and vimentin protein levels in PMC42-LA after 3 days of treatment with conditioned medium from CAV1 siRNA-transfected fibroblasts showed similar results to 7-day treatments (data not shown).
Figure 4: PMC42-LA human breast cancer cells were cultured with conditioned medium (CM) from CAV1 siRNA-transfected human primary breast fibroblasts. Results are relative to the transfection reagent-only control. Error bars represent the standard error of the mean. * p<0.05  (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Fibroblastic CAV1 levels were significantly lower after transfection with 25 nM CAV1 siRNA. Results are representative of 3 independent experiments. (B) Western analysis of E-cadherin protein levels, relative to total protein levels, as detected by Ponceau staining. E-cadherin levels in PMC42-LA were significantly lower after treatment with CM from CAV1 siRNA-transfected fibroblasts. Results are representative of 3 independent experiments. (C) Western analysis of vimentin protein levels, relative to total protein levels, as detected by Ponceau staining. Vimentin levels in PMC42-LA were significantly higher after treatment with CM from CAV1 siRNA-transfected fibroblasts. Results are representative of 3 independent experiments. (D) Representative Western analysis image. β-actin is shown as a loading control.

In addition to the evaluation of EMT markers, PMC42-LA cells were also evaluated for the expression of cancer stem cell markers CD44 and CD24. PMC42-LA cells were treated with conditioned medium from CAV1 siRNA-transfected Fre85 fibroblasts, and their CD44/CD24 status was determined using flow cytometry. No changes in the percentage of CD44+/CD24− cells, as part of the PMC42-LA cell
population, were observed after 3-day treatment with conditioned medium from Fre85 cells transfected with CAV1 siRNA, compared to PMC42-LA cells treated with control conditioned medium (Fig. 5A-C). No changes in the percentage of CD44+/CD24- cells, as part of the PMC42-LA cell population, were observed after 7-day treatment with conditioned medium from Fre85 cells transfected with CAV1 siRNA, compared to PMC42-LA cells treated with control conditioned medium (Fig. 5D-F).

Figure 5: Flow cytometric analysis of PMC42-LA human breast cancer cells cultured with conditioned medium (CM) from CAV1 siRNA-transfected human primary breast fibroblasts. Cells were stained with CD24-PE and CD44-FITC antibodies. (A) Flow cytometric analysis of PMC42-LA after 3-day treatment with CM from control Fre85 cells. (B) Flow cytometric analysis of PMC42-LA after 3-day treatment with CM from Fre85 cells transfected with non-targeting siRNA. (C) Flow cytometric analysis of PMC42-LA after 3-day treatment with CM from Fre85 cells transfected with CAV1 siRNA. (D) Flow cytometric analysis of PMC42-LA after 7-day treatment with CM from control Fre85 cells. (E) Flow cytometric analysis of PMC42-LA after 7-day treatment with CM from Fre85 cells transfected with non-targeting siRNA. (F) Flow cytometric analysis of PMC42-LA after 7-day treatment with CM from Fre85 cells transfected with CAV1 siRNA.
**Optimization and controls**

To ensure that the effects measured in PMC42-LA cells were due to a reduction in fibroblastic CAV1 expression, rather than to the transfection procedure, a control sample was transfected with non-targeting siRNA. As can be seen in Fig. 6A, transfection with the non-targeting siRNA reduced CAV1 protein levels to a similar extent as CAV1 siRNA. Treatment of PMC42-LA with conditioned medium from these reduced CAV1 fibroblasts, transfected with non-targeting siRNA, resulted in epithelial E-cadherin and vimentin levels that were similar to those after treatment with medium from CAV1 siRNA-transfected fibroblasts (Fig. 6B,C). These results are based on two independent experiments for CAV1 and vimentin protein levels, and a single experiment for E-cadherin protein levels.
Figure 6: PMC42-LA human breast cancer cells were cultured with conditioned medium (CM) from CAV1 siRNA-transfected human primary breast fibroblasts and non-targeting siRNA-transfected human primary breast fibroblasts. Results are relative to the transfection reagent-only control. Error bars represent the standard error of the mean. (A) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Results are representative of 2 independent experiments. (B) Western analysis of E-cadherin protein levels, relative to total protein levels, as detected by Ponceau staining. Results are representative of 1 experiment. (C) Western analysis of vimentin protein levels, relative to total protein levels, as detected by Ponceau staining. Results are representative of 2 independent experiments. (D) Representative Western analysis image. Calnexin is shown as a loading control.

An alternative non-targeting siRNA from a different manufacturer (GE Dharmacon, Lafayette, CO, USA) was tested. Transfection with the Dharmacon non-targeting siRNA resulted in unstable CAV1 protein levels (Fig. 7C,D, single experiment), however CAV1 mRNA levels were closer to control levels (Fig. 7A,B, single experiment). In addition, Figure 8A and Figure 8B show an effect of transfection with Dharmacon non-targeting siRNA on fibroblastic CAV1 protein, but not mRNA levels (single experiment).
To investigate whether the transfection efficiency could be improved, a different transfection reagent was tested. CAV1 mRNA and protein levels were compared after transfection with CAV1 siRNA, using siPORT transfection reagent from Ambion, and RNAiMAX transfection reagent from LifeTechnologies. CAV1 mRNA and protein levels were strongly reduced after transfection with CAV1 siRNA when using the RNAiMAX transfection reagent (Fig. 7B,D, single experiment), but not when using the siPORT transfection reagent (Fig. 7A,C, single experiment).

Figure 7: Transfection of primary human breast fibroblasts (Fre85), using siPORT and RNAiMAX transfection reagents. Error bars represent the standard deviation. (A) CAV1 and lamin mRNA levels, relative to GAPDH levels, were detected using qRT-PCR. Results are representative of 1 experiment. (B) CAV1 and lamin mRNA levels, relative to GAPDH levels, were detected using qRT-PCR. Results are representative of 1 experiment. (C) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Results are representative of 1 experiment. (D) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Results are representative of 1 experiment.
The RNAiMAX transfection reagent, and non-targeting and CAV1 siRNAs from Dharmaco, were used to generate conditioned medium from CAV1 siRNA-transfected fibroblasts. This conditioned medium was used to treat PMC42-LA cells. The transfection of Fre85 fibroblasts with CAV1 siRNA resulted in a strong reduction in CAV1 mRNA (Fig. 8A), but a very small reduction in CAV1 protein levels (Fig. 8B). After treatment of PMC42-LA with conditioned medium from CAV1 siRNA-transfected fibroblasts, vimentin protein levels were 2.71 ± 1.11 fold control (Fig. 8C).
PMC42-LA human breast cancer cells were cultured with conditioned medium (CM) from CAV1 siRNA-transfected human primary breast fibroblasts and non-targeting siRNA-transfected human primary breast fibroblasts, using Dharmacon transfection reagent and siRNAs. Results are relative to the transfection reagent-only control. (A) CAV1 and lamin mRNA levels, relative to GAPDH levels, were detected using qRT-PCR. Results are representative of 1 experiment. Error bars represent the standard deviation. (B) Western analysis of CAV1 protein levels, relative to total protein levels, as detected by Ponceau staining. Results are representative of 1 experiment. (C) Western analysis of vimentin protein levels, relative to total protein levels, as detected by Ponceau staining. No significant difference between treatment with control CM and CM from non-targeting siRNA- and CAV1 siRNA-transfected fibroblasts was detected. Results are representative of 4 independent experiments. Error bars represent the standard error of the mean. (D) Representative Western analysis image. β-actin is shown as a loading control.

Discussion

EMT plays an important part in the progression of cancer from the in situ tumour to cancer metastasis (McCuaig et al., 2016). This study shows that the treatment of PMC42-LA breast cancer cells with conditioned medium from CAV1 siRNA-transfected Fre85 primary fibroblasts results in lower E-cadherin, and higher vimentin, protein levels in PMC42-LA cells. The reduction of E-cadherin expression,
and the increase of vimentin expression, are considered markers of epithelial-to-mesenchymal transition (EMT) (Mani et al., 2008).

Cancer-associated fibroblasts play a well-described role in cancer progression, including the promotion of EMT (Zeisberg et al., 2007). Studies have shown that CAFs isolated from patient breast cancer tumours induce EMT in cancer cells, through secreted factors, in vitro. Breast cancer cells cultured in conditioned medium from patient CAFs grown in culture, showed increased expression of EMT markers, and greater migration and invasion (Lebret et al., 2007; Soon et al., 2013; Yu et al., 2014). The effect of conditioned medium from CAV1 siRNA-transfected Fre85 fibroblasts on PMC42-LA breast cancer cells presented in this chapter suggests that CAV1 plays a role in the CAF-driven induction of EMT in breast cancer cells.

**The effect of conditioned medium on EMT and cancer stem cell markers**

While the treatment of PMC42-LA with conditioned medium from transfected Fre85 led to the increase of the mesenchymal marker vimentin, and the decrease of the epithelial marker E-cadherin, no changes in the cancer stem cell markers CD44+/CD24− were detected in the cancer cell population. Mesenchymal cancer stem cells express high levels of CD44, and low levels of CD24, in addition to increased levels of vimentin, and decreased levels of E-cadherin, compared to other cell populations (Liu et al., 2014b).

The cell line PMC42 consists of a heterogeneous cell population, with multiple subpopulations that are distinguishable based on morphology, and the expression of markers (e.g., vimentin) (Ackland et al., 2003). This heterogeneity suggest that
PMC42 may be derived from a cancer stem cell origin, or has acquired stem cell properties (Hugo et al., 2007). PMC42-LA cells showed no increase in cell numbers expressing high levels of CD44, and low levels of CD24, after treatment with conditioned medium from CAV1 siRNA-transfected Fre85. These results suggest that the increased vimentin levels, and decreased E-cadherin levels, in PMC42-LA, after treatment with CAV1 siRNA-transfected Fre85 conditioned medium, are not linked to an increase in the population of cancer stem cells. However, it is possible that an effect on CD24 and CD44 expression levels was not measurable due to the conditioned medium, used for the treatment of PMC42-LA, originating from fibroblasts that only showed a small reduction in CAV1 protein levels.

The expression of αSMA by CAV1 siRNA-transfected primary human breast fibroblasts

The most commonly used marker used to distinguish different fibroblast populations in the tumour microenvironment is α-smooth muscle actin (αSMA) (Drake and Macleod, 2014). Fre85 fibroblasts did not show altered αSMA protein levels after transfection with CAV1 siRNA. This may suggest that the knockdown of CAV1 alone is not sufficient to induce a CAF phenotype in Fre85 fibroblasts.

These results are in contrast to others, who have found αSMA expression to be higher in immortalised skin fibroblasts transfected with CAV1 siRNA (Martinez-Outschoorn et al., 2010b). However, Goetz et al. (2011) found lower αSMA levels in MEFS from CAV1 knockout mice compared to WT mice. Although the study by Goetz et al. (2011) also found that higher CAV1 expression in fibroblasts is positively related to tumour growth and metastasis, and this further confirms the relationship between
αSMA and CAF-like properties, these results do show that the relationship between CAV1 expression and αSMA expression is still unclear. Additionally, the aforementioned studies investigated αSMA levels in CAV1 knockdown/knockout fibroblasts from human skin and mouse embryos, while this study used primary human breast fibroblasts. Therefore, the discrepancies could also be explained by different fibroblast sources being studied.

Although there is a strong relationship between αSMA expression and CAF-like properties, not all CAFs display increased αSMA expression. A study by Sugimoto et al. (2006) on mesenchymal markers expressed in tumours originating from the inoculation of mouse mammary fat pads with human breast cancer cells, showed that CAFs are a heterogeneous cell population, and not all CAFs express αSMA. The transfection of primary human fibroblasts with CAV1 siRNA resulted in a small reduction in CAV1 protein levels. The absence of changes in fibroblastic αSMA expression after transfection with CAV1 siRNA can, therefore, be due to low transfection efficiency. It is, however, also possible that the transfected fibroblasts displayed CAF-like properties without an increase in αSMA expression.

The transfection efficiency of primary human breast fibroblasts with CAV1 siRNA

The knockdown of genes by transfection with siRNA can have significant off-target effects. These effects include the unintentional miRNA-like regulation of genes, and the triggering of innate immune responses, through proinflammatory sequences in siRNAs binding to toll-like receptors (Jackson and Linsley, 2010). A recommended control, to distinguish sequence-specific from non-specific effects of transfection with
siRNAs, is the inclusion of a non-coding siRNA in the transfections. This study used non-targeting siRNA from two different manufacturers as a control in the generation of conditioned medium from transfected fibroblasts.

When the expression of E-cadherin and vimentin in PMC42-LA treated with conditioned medium from CAV1 siRNA-transfected Fre85 was evaluated against PMC42-LA treated with conditioned medium from non-targeting siRNA-transfected Fre85, no changes were observed. This would suggest that the changes in E-cadherin and vimentin expression described in this study are a result from the transfection procedure in general, rather than from a specific reduction in CAV1 levels by transfection with CAV1 siRNA. However, when CAV1 levels were evaluated in non-targeting siRNA-transfected Fre85 cells, CAV1 protein levels were reduced to levels similar to those in CAV1 siRNA-transfected Fre85 cells, while CAV1 mRNA levels were unaffected. It is therefore unclear whether the changes in E-cadherin and vimentin expression found in the non-targeting control samples were due to off-target effects from transfection, or due to reduced CAV1 protein levels.

To address the potential miRNA-like, and immunological off-target effects, described above, the inclusion of a non-targeting control alone is insufficient. The CAV1 siRNA sequence used could potentially elicit miRNA-like regulation of genes, as well as contain proinflammatory sequences, while the non-targeting siRNA sequence does not. Additionally, several commercially available non-targeting siRNAs were shown to alter cytokine profiles and the inflammatory response in human fibroblasts (Baum et al., 2010). Therefore, care should be taken with the selection of controls for transfection experiments. Evidence that reintroduction of CAV1 after transfection
would reverse changes in E-cadherin and vimentin expression in PMC42-LA cells treated with conditioned medium, would address these concerns, and should be evaluated in future experiments.

Primary cell lines can be more resistant to transfection than continuous cell lines (Liu et al., 2007). The transfection of the primary human fibroblasts Fre85 with CAV1 siRNA resulted in a relatively small, and inconsistent, reduction in CAV1 protein levels. The CAV1 protein levels in the transfected Fre85 fibroblasts were on average 26% lower than the control. In order to optimise the transfection efficiency, different concentrations of CAV1 siRNA from different manufacturers were tested. CAV1 siRNA from both Santa Cruz Biotechnology (Dallas, TX, USA) and GE Dharmacon (Lafayette, CO, USA) resulted in strong CAV1 mRNA reductions, but variable CAV1 protein reductions.

In addition to different sources of CAV1 siRNA, different sources of transfection reagent were tested. A direct comparison of siPORT transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) with RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) found a stronger transfection efficiency, measured by CAV1 protein reduction, after the use of RNAiMAX. However, subsequent transfections using RNAiMAX continued to show variable transfection efficiency, when CAV1 protein levels were evaluated.

The primary human fibroblasts showed a strong increase in CAV1 protein levels, following an initial decrease, after transfection with CAV1 siRNA. CAV1 protein levels returned to control levels, 48 hours after transfection, and after 72 hours CAV1
protein levels were 4-5 times fold control levels. The cell often maintains the expression of essential proteins, through a negative feedback mechanism. This feedback can be mediated by mRNA levels or by protein levels. The presence of a particular protein can inhibit the transcription, or translation, of its corresponding gene and mRNA and, similarly, the presence of transcripts from a particular gene can inhibit further transcription, or translation, of that gene and transcript (Singh, 2011). The reduction of mRNA or protein levels can relieve that inhibition, and result in increased transcription, or translation, of the target. Another regulatory response the cell can utilise to maintain stable levels of essential proteins, is enhancing mRNA or protein stability, when transcription or translation is impaired (Nguyen et al., 2003; Schwanhäusser et al., 2011).

The recovery of CAV1 protein levels, 72 hours after CAV1 siRNA transfection, that was seen in this study, may indicate the presence of one or more of these regulatory mechanisms, and, as such, the importance of maintaining CAV1 protein levels in the cell. Likewise, the variable transfection efficiency of the primary human breast fibroblast with CAV1 siRNA may be due to a need of these cells to maintain CAV1 protein levels.

Conclusion

In conclusion, the results in this study indicate that the treatment of PMC42-LA cells with conditioned medium from Fre85 fibroblasts that express lower levels of CAV1 protein results in increased vimentin, and decreased E-cadherin protein expression. These preliminary findings suggest that the loss of CAV1 in fibroblasts may play a role in EMT in breast cancer cells. An increase in the transfection efficiency of
primary human fibroblasts with CAV1 siRNA is likely needed to properly measure the markers, and functional effects, of EMT, after treatment with conditioned medium from transfected fibroblasts. Further research is needed to rule out that off-target effects are responsible for the results presented in this study. To confirm whether fibroblastic CAV1 plays a role in driving breast cancer cell EMT, further markers of EMT, including measurements of cell proliferation and motility, will need to be evaluated.
Chapter 7: Summaries and conclusions
The data presented in this thesis contributes to the understanding of the role of caveolin-1 (CAV1) in the breast cancer microenvironment.

CAV1 is a structural protein of caveolae, which are cup- or bulb-shaped invaginations of the cell membrane (Schlormann et al., 2010). Caveolae play a role in endocytosis, cellular signalling, cholesterol homeostasis, and mechanosensing (Parton and del Pozo, 2013).

The role of CAV1 in breast cancer is complex. CAV1 overexpression was found to be correlated with the particularly aggressive basal-like breast cancer phenotype in some studies (Pinilla et al., 2009; Savage et al., 2007), while others have found that the expression of CAV1 by breast cancer cell lines was associated with reduced proliferation (Lee et al., 1998), and that the absence of CAV1 expression resulted in increased tumour burden and metastasis in a CAV1 -/- mouse model (Williams et al., 2004).

In recent years, the importance of the role of the tumour microenvironment in cancer progression has become clear. During the progression of cancer, the microenvironment undergoes many changes, including the remodelling of the extracellular matrix (ECM), the recruitment of immune cells, the presence of stromal cells with a cancer-associated phenotype, and the secretion of growth factors and cytokines (Bissell and Hines, 2011). Two important cell types that are well-studied
components of the tumour microenvironment are cancer-associated fibroblasts and tumour-associated macrophages (Kalluri, 2016; Williams et al., 2016).

Similar to the role of CAV1 expression in breast cancer cells, the role of CAV1 in the tumour microenvironment appears dichotomous. The evaluation of CAV1 expression in breast cancer patient biopsies has shown a correlation between the loss of stromal CAV1 expression, and reduced progression-free survival (El-Gendi et al., 2012; Sloan et al., 2009; Witkiewicz et al., 2009b). A study that specifically evaluated CAV1 expression in fibroblasts from breast cancer patient biopsies, confirmed the findings of the aforementioned studies, and pointed to cancer-associated fibroblasts (CAFs) as a key player (Simpkins et al., 2012). Additionally, the co-injection of CAV1-deficient fibroblasts and human breast cancer cells into nude mice has been shown to lead to increased tumour mass and angiogenesis, compared to co-injection of breast cancer cells with normal fibroblasts (Bonuccelli et al., 2010).

In an apparent contradiction to the findings that the loss of CAV1 expression in the tumour microenvironment is correlated with unfavourable prognostic outcomes for breast cancer patients, others have found that an increased expression of CAV1 in CAFs in biopsies of breast tumours was associated with an increased mortality risk for breast cancer patients (Goetz et al., 2011). In support of their findings in patient biopsies, Goetz et al. (2011) reported that CAV1-expressing mouse embryonic fibroblasts (MEFs) generated an ECM, in vitro, that displayed the parallel alignment of fibres and increased matrix stiffness that is characteristic of a tumour stroma.
In addition to the controversy around the role of CAV1 in breast cancer, the mechanisms that result in altered CAV1 expression in CAFs are not clear. The first aim of this thesis was to investigate whether the co-culture of fibroblasts with cancer cells results in altered CAV1 expression by fibroblasts. The data presented in this thesis show that the co-culture of primary human breast fibroblasts Fre85 with human breast cancer cells does not result in significantly altered fibroblastic CAV1 expression. Primary human breast fibroblasts Fre85 were cultured with the mildly invasive and the highly invasive human breast cancer cell lines MCF7 and MDA-MB-231, respectively, in a non-contact, and a contact, co-culture system. Neither non-contact co-culture, nor contact co-culture, resulted in significant changes in fibroblastic CAV1 mRNA or protein levels. In addition, the non-contact, and contact, co-culture of immortalised human skin fibroblasts hTERT-BJ with human breast cancer cells MCF7 did not result in altered fibroblastic CAV1 mRNA or protein levels.

A study by Martinez-Outschoorn et al. (2010b), found that the contact co-culture of immortalised human skin fibroblasts hTERT-BJ with MCF7 breast cancer cells resulted in reduced fibroblastic CAV1 protein levels, in the absence of significant changes in fibroblastic CAV1 mRNA levels. These findings do not correspond with the data presented in this thesis. An important difference between the findings presented by Martinez-Outschoorn et al. (2010b), and the findings presented in this thesis, is the methods used to evaluate CAV1 expression. Martinez-Outschoorn et al. (2010b) evaluated CAV1 protein expression in immuno-labelled co-cultures by confocal microscopy only, whereas the CAV1 protein data presented in this thesis are primarily based on Western analysis. However, the confocal analysis of immuno-
labelled contact co-cultures of hTERT-BJ fibroblasts with MCF7 breast cancer cells (Chapter 3, Fig. 4) corresponded with the results obtained with Western analysis. This suggests that changes in fibroblastic CAV1 expression after co-culture with breast cancer cells are context dependent, and not easily replicated from one culture system, or one laboratory, to another.

As the co-culture of fibroblasts with breast cancer cells alone did not result in consistently and significantly altered CAV1 levels, a potential role for differentiated monocytes was investigated. The data presented in this thesis show for the first time that the addition of differentiated monocytes to non-contact co-cultures of primary human fibroblasts Fre85 and human breast cancer cells MDA-MB-231 reduces fibroblastic CAV1 mRNA and protein levels. In addition, the contact co-culture of primary human breast fibroblasts Fre85 with human monocytes THP-1 alone resulted in a reduction of fibroblastic CAV1 protein levels.

Macrophages are an important part of the tumour microenvironment (Mantovani and Sica, 2010). Tumour-associated macrophages (TAMs) secrete a plethora of cytokines, and the crosstalk between TAMs and cancer cells, and between TAMs and CAFs, plays an important role in tumorigenesis (Comito et al., 2014; Su et al., 2014). It is possible that the reduction in fibroblastic CAV1 levels seen in tissue biopsies from breast cancer patients is in part due to the recruitment of macrophages to the tumour site. Further research is required to evaluate the role of macrophages in the regulation of fibroblastic CAV1 levels, in vivo.
The observation that non-contact co-culture of fibroblasts with breast cancer cells and differentiated monocytes lowered fibroblastic CAV1 levels, points to the involvement of a secreted factor in the regulation of CAV1 expression by fibroblasts. This thesis aimed to investigate the secretion of cytokines by co-cultures of fibroblasts, breast cancer cells and (differentiated) monocytes. A cytokine assay on conditioned medium from non-contact, and contact, co-cultures of Fre85 fibroblasts, MDA-MB-231 breast cancer cells, and TPA-treated THP1 cells, showed markedly different cytokine responses between monoculture controls, and co-cultures of fibroblasts with cancer cells, fibroblasts with differentiated monocytes, and fibroblasts with cancer cells and differentiated monocytes. The inclusion of differentiated monocytes in a fibroblast and cancer cell co-culture resulted in a more CAF-like response of the fibroblasts, compared to fibroblast and cancer cell co-culture alone. In addition, the inclusion of differentiated monocytes resulted in increased levels of cytokines that have been previously implicated in the regulation of CAV1. The data presented in this thesis provides several cytokine candidates that need to be investigated further, for a potential role in the regulation of CAV1 expression by CAFs.

Studies have shown that a lower CAV1 level in CAFs affects multiple aspects related to cancer progression, including tumour growth, angiogenesis, and epithelial-to-mesenchymal transition (EMT) (Bonuccelli et al., 2010; Simpkins et al., 2012). This thesis aimed to investigate the role of fibroblastic CAV1 in breast cancer cell EMT, through the treatment of breast cancer cells with conditioned medium from CAV1 siRNA-transfected fibroblasts. The breast carcinoma cell line PMC42-LA has been shown to be inducible to EMT (Ackland et al., 2003), and is an excellent cell culture model for the investigation of the role of fibroblastic CAV1 levels in cancer cell
EMT. The data presented in this thesis show that treatment of PMC42-LA cells with conditioned medium from Fre85 fibroblasts transfected with CAV1 siRNA results in increased vimentin protein levels and reduced E-cadherin protein levels, important markers of EMT. However, changes in the expression of cancer stem cell markers CD24 and CD44 were not detected.

Fre85 fibroblasts were highly resistant to transfection with CAV1 siRNA, and significant reductions in CAV1 protein levels were not consistently achieved. Remarkably, increased CAV1 protein levels, 72 hours after transfection, followed an initial decrease in CAV1 protein levels, 24 hours after transfection. In addition, non-targeting siRNA affected fibroblastic CAV1 protein levels, and vimentin levels in PMC42-LA cells treated with conditioned medium from fibroblasts transfected with CAV1 siRNA were not significantly different from PMC42-LA cells treated with conditioned medium from fibroblasts transfected with non-targeting siRNA. Therefore, technical limitations hindered the thorough investigation of the role of fibroblastic CAV1 in breast cancer cell EMT.

The data presented in this thesis show significant variability in fibroblastic CAV1 levels between experiments. Several factors that could contribute to variability in cell culture systems were investigated for a potential effect on fibroblastic CAV1 levels. The synchronisation of the cell cycle of the fibroblast population, by serum starvation prior to co-culture, did not result in a reduction in fibroblastic CAV1 protein level variability between replicates. The inclusion of Serum Replacement 1, a chemically defined, serum-free, cell culture additive, in lieu of foetal calf serum (FBS), did,
however, reduce the variability in fibroblastic CAV1 protein levels after co-culture with cancer cells and differentiated monocytes.

FBS is a source of growth- and adhesion factors, hormones, lipids, and minerals, and has been identified as a contributing factor to challenges with the replication of cell culture experiments between different laboratories (Khodabukus and Baar, 2014). Although the same batch of FBS was used in all co-culture experiments described in this thesis, it is possible that variability in FBS composition, due to the labile nature of many of the components of FBS, contributed to variability in fibroblastic CAV1 levels between experimental replicates. In addition, it is possible that FBS plays a role in the conflicting results from the hTERT-BJ and MCF7 contact co-cultures described in this thesis, and by Martinez-Outschoorn et al. (2010b).

The elimination of FBS from the co-culture system resulted in reduced variability between replicates, and the non-contact co-culture of fibroblasts with differentiated monocytes, with or without cancer cells, resulted in a significant reduction in fibroblastic CAV1 levels (Chapter 4, Fig. 5), confirming the findings in previous chapters that implicated differentiated monocytes in fibroblastic CAV1 regulation.

It is important to note, however, that the use of the same batch of Fre85 fibroblasts reduced CAV1 protein variability between experimental replicates, as is shown when comparing Fig. 7B and Fig. 9B in Chapter 2. Moreover, some batches of Fre85 fibroblasts responded to co-culture with cancer cells by showing higher fibroblastic
CAV1 protein levels after co-culture at earlier passage number, while co-culture using the same cells at higher passage number resulted in lower fibroblastic CAV1 levels.

Other studies have provided conflicting reports on the effect of fibroblast and cancer cell interactions on fibroblastic CAV1 expression, and the implications of altered fibroblastic CAV1 expression for the progression of cancer (Goetz et al., 2011; Martinez-Outschoorn et al., 2010b). The evaluation of CAV1 levels in CAFs from 11 breast cancer patients showed a significant downregulation in 8 patients, and a significant upregulation of CAV1 in 2 patients (Mercier et al., 2008). The data presented in this thesis show that the effect of fibroblast and cancer cell interactions, in vitro, on fibroblastic CAV1 expression is dependent on many factors, including fibroblast origin, the presence of differentiated monocytes, and the use of serum in cell culture. In addition, the observation that fibroblasts from the same batch, cultured under the same conditions, appear to switch their response to co-culture with cancer cells from higher CAV1 expression to lower CAV1 expression, suggests that changes in the phenotype of the cells occurring over the culture period, for example epigenetic changes, may play a role in the regulation of fibroblastic CAV1 levels. Combined, the data presented in this thesis and the data reported by others show that the regulation of CAV1 expression by CAFs is highly complex and context dependent. Further research is required to untangle the regulatory mechanisms involved. Understanding the regulation of CAV1 expression in fibroblasts may provide novel therapeutic targets for the treatments of a subset of breast cancer patients.
Bibliography


MURC/Cavin-4 and cavin family members form tissue-specific caveolar complexes. J
Cell Biol 185, 1259-1273.

123, 3831-3836.

Baum, P., Fundel-Clemens, K., Kreuz, S., Kontermann, R.E., Weith, A., Mennerich,
reveals important differences in the cytokine profile and inflammation response of


46-54.

transcription, cell cholesterol, and growth by a novel mechanism. Biochemistry 39,

Boelens, M.C., Wu, T.J., Nabet, B.Y., Xu, B., Qiu, Y., Yoon, T., Azzam, D.J.,
transfer from stromal to breast cancer cells regulates therapy resistance pathways.
Cell 159, 499-513.

Bonuccelli, G., Whitaker-Menezes, D., Castello-Cros, R., Pavlides, S., Pestell, R.G.,
(2010). The reverse Warburg effect: glycolysis inhibitors prevent the tumor
promoting effects of caveolin-1 deficient cancer associated fibroblasts. Cell Cycle 9,

Bosch, M., Mari, M., Herms, A., Fernández, A., Fajardo, A., Kassan, A., Giralt, A.,


Gudjonsson, T., Adriance, M.C., Sternlicht, M.D., Petersen, O.W., and Bissell, M.J.
(2005). Myoepithelial cells: their origin and function in breast morphogenesis and

the caveolin-1/AKT/mTOR pathway. Mol Cell Biochem 406, 111-119.

Cell 144, 646-674.

Hayer, A., Stoeger, M., Bissig, C., and Helenius, A. (2010). Biogenesis of caveolae:
stepwise assembly of large caveolin and cavin complexes. Traffic 11, 361-382.

from unfavorable prognostic markers in breast cancer: the role of E-cadherin. Cancer
Res 60, 298-304.

platelet-derived growth factor. Physiol Rev 79, 1283-1316.

Hill, M.M., Bastiani, M., Luetterforst, R., Kirkham, M., Kirkham, A., Nixon, S.J.,
conserved cytoplasmic protein required for caveola formation and function. Cell 132,
113-124.

cultures of macrophages and fibroblasts. Biomaterials 31, 9382-9394.

Hugo, H., Ackland, M.L., Blick, T., Lawrence, M.G., Clements, J.A., Williams, E.D.,
and Thompson, E.W. (2007). Epithelial—mesenchymal and mesenchymal—epithelial


between epithelial and mesenchymal states reflective of their normal counterparts. Stem Cell Reports 2, 78-91.


Role of hypoxia, HIF1 induction and NFkB activation in the tumor stromal microenvironment. Cell Cycle 9, 3515-3533.


MMP-9 as a potential biomarker for cancer invasion and metastasis. Anticancer Res 34, 1355-1366.


transcriptional activation of the antioxidant response element Degradation of Nrf2 by the 26 S proteasome. J Biol Chem 278, 4536-4541.


