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M.Sc. Neşe Erdem

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ROLE OF STC1 IN THE ACTIVATION OF FIBROBLASTS IN THE CONTEXT OF BREAST CANCER

Referees: Prof. Dr. Stefan Wiemann
Prof. Dr. Frank Lyko

Declaration of Authorship

I hereby declare that the work presented in my dissertation was carried out between February 2015 and April 2019 under the supervision of Prof. Dr. Stefan Wiemann in the group of Molecular Genome Analysis at the German Cancer Research Center (DKFZ, Heidelberg, Germany).

If not stated differently and referenced within the text, the data described in my dissertation is original, has been gathered by myself and has not yet been presented as a part of a university examination. All main sources, as well as the work of joint cooperation have been referenced appropriately. I, as author, hereby declare no potential conflict of interest.

Heidelberg, _____

Neşe Erdem.

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SUMMARY

The tumor microenvironment (TME) is now recognized as an important factor in breast cancer progression and is crucial in determining the response to anti-cancer therapies. Fibroblasts are one component of the TME that are activated in the early stages of oncogenesis and remodel the TME as an early tissue repair response. In the later stages of tumor progression, these activated fibroblasts get transformed into cancer associated fibroblasts (CAFs) that promote tumor progression. The mechanisms underlying this transformation of fibroblasts from normal activated fibroblasts to CAFs remain largely unknown. To address this, bone marrow derived mesenchymal stromal cells (MSCs) that are known to be a significant source of CAFs were treated with conditioned media of a breast cancer cell line, namely, MDA-MB-23, to induce a CAF-like - phenotype. Activation of MSCs was confirmed by assessing their contractility and expression of CAF markers. Gene expression and secretome analysis was performed to identify differentially expressed genes and differentially secreted factors upon activation. Stanniocalcin 1 (STC1), a CAF-secreted protein implicated in metastasis and therapy resistance, was found to be upregulated upon conditioned media treatment. Therefore, the aim of this study was to understand the mechanism of STC1 upregulation and its involvement in the activation of fibroblasts. STC1 knockdown using RNAi both in conditioned media treated MSCs and patient derived activated CAFs showed inhibitory effects on activation status of these cells. Moreover, this down-regulation resulted in a decrease in the expression of several proteins such as NOTCH3, NOTCH4 and β-catenin suggesting possible mechanisms by which STC1 regulates the activation of fibroblasts. Furthermore, the upregulation of STC1 upon conditioned media treatment was found to be dependent on RELA and RELB transcription factors indicating that NF-κB signaling might be involved in its transcriptional regulation. In conclusion, this study demonstrates STC1 as an important factor in the activation of CAFs. Investigating the mechanism of the involvement of STC1 in the unabated activation of CAFs will enable a deeper understanding of the tumor microenvironment and its role in the tumor progression.

ZUSAMMENFASSUNG

Die Tumormikroumgebung (tumor microenvironment, TME) wird heute als ein bedeutender Faktor bei der Progression von Brustkrebs betrachtet und spielt eine zentrale Rolle beim Ansprechen auf Krebstherapien. Fibroblasten sind einer der Bestandteile der TME. Sie werden zu einem frühen Zeitpunkt der Onkogenese aktiviert und formen die TME im Rahmen einer Reaktion der Gewebereparatur um. Im Laufe der Tomorprogression werden diese aktivierten Fibroblasten zu Krebs-assoziierten Fibroblasten (cancer-associated fibroblasts, CAFs), die das Voranschreiten der Krankheit zusätzlich begünstigen. Die Mechanismen, die die Transformation von normalen, aktivierten Fibroblasten zu CAFs begründen, sind bislang größtenteils unbekannt.

Um diese Mechansimen zu untersuchen wurden mesenchymale Stammzellen (mesenchymal stem/stromal cells, MSCs) aus dem Knochenmark, die als bedeutende Quelle von CAFs in Patienten gelten, mit konditioniertem Medium einer Brustkrebszelllinie, MDA-MB-231, behandelt, um einen CAF-ähnlichen Phänotyp zu erzeugen. Die Aktivierung der MSCs wurde bestätigt, indem ihre Kontraktilität und die Expression von CAF-Markern bestimmt wurden. Genexpressions- und Sektretom-Analysen wurden durchgeführt, um nach der Aktivierung differentiell exprimierte Gene und differentiell sezernierte Proteine zu identifizieren.

Stanniocalcin 1 (STC1), ein von CAFs sezerniertes Protein mit Implikationen auf Metastasierung und Therapieresistenz, war nach der Behandlung mit konditioniertem Medium stärker exprimiert und in größeren Mengen sezerniert. Aus diesem Grund war das Ziel dieser Studie, den Mechanismus der Hochregulation von STC1 sowie die Beteiligung dieses Proteins an der Aktivierung von Fibroblasten zu verstehen.

Knockdown von STC1 mithilfe von RNAi verringerte sowohl bei MSCs, die mit konditioniertem Medium behandelt worden waren, als auch bei konstitutiv aktivierten CAFs aus Brustkrebspatientinnen den Aktivierungsstatus der Zellen. Weiterhin führte der Knockdown zu einer verminderten Expression mehrerer Proteine, wie beispielsweise NOTCH3, NOTCH4 und β-Catenin. Hieraus können Hypothesen für potentielle Mechanismen abgeleitet werden, die der Regulation der Aktivierung von Fibroblasten durch STC1 zugrunde liegen. Darüber hinaus konnte gezeigt werden, dass die Hochregulierung von STC1 nach der Behandlung mit konditioniertem Medium von den

Transkriptionsfaktoren RELA und RELB abhängig war, was eine Beteiligung von NFκB an der transkriptionellen Regulation von STC1 nahelegt.

Zusammenfassend etabliert diese Studie STC1 als einen bedeutenden Faktor in der Aktivierung von CAFs. Die weitere Aufklärung der zugrundeliegenden Mechanismen wird zu einem vertieften Verständnis der Tumormikroumgebung und ihrer Rolle in der Tumorprogression beitragen.

1. INTRODUCTION

1.1. Breast Cancer

Breast cancer, the most common cancer type in women, ranks as the second leading cause of cancer mortality after lung cancer and represents 14% of all cancer related deaths in women worldwide (American Cancer Society, 2018). It is a heterogeneous disease with multiple subgroups that differ in their molecular features, response to therapies and prognosis which makes the breast cancer diagnosis and treatment a challenging task.

1.1.1. Molecular subtypes

Using immunohistochemistry techniques, breast cancer traditionally has been classified into three major types; hormone receptor positive tumors that express estrogen receptor (ER) and/or progesterone receptor (PR), human epidermal growth factor 2 receptor (HER2) positive tumors and triple negative breast tumors which are negative for the expression of ER, PR and HER2 (Carlson et al., 2009). High throughput gene expression profiling and hierarchical clustering of breast tumors have further divided these initial three major types into several distinct molecular subtypes of breast cancer including luminal A, B, HER2enriched, basal, and claudin-low which differentially express ER, PR and HER2 together with the genes regulated under the control of these receptors (Kittaneh et al., 2013). Luminal subtypes of breast cancer, namely luminal A and luminal B, have expression profiles that are similar to the luminal epithelial component of the normal breast (Perou et al., 2000). Luminal A subtype is the most common subtype that corresponds to around half of the total cases of breast cancer (Fan et al., 2006). Luminal A tumors are ER positive with overexpression of ER regulated genes such as *LIV1* and *CCND1* but they lack the expression of ERBB2 receptor and have low levels of expression of proliferation related genes (Yersal & Barutca, 2014). They have the best prognosis (Z. Hu et al., 2006) and the lowest recurrence rates among all the subtypes (Metzger-Filho et al., 2013). Luminal B subtype corresponds to around 20% of all cases of breast cancer (Sorlie et al., 2001). It has a similar expression pattern to that of luminal A. However, it has higher variability in the expression of ER and higher expression of proliferative genes, e.g., *Ki67*, that might be associated with mutations in *TP53* and genomic instability (Alcalá-Corona et al., 2017). Compared to luminal A tumors, luminal B ones have poorer prognosis (Haque et al., 2012).

The HER2-enriched subtype of breast cancer is characterized by overexpression of the ERRBB2 receptor which is mostly accompanied by chromosomal level amplification of the gene locus (Burstein, 2005). 40-80% of these tumors have mutations in *TP53* and they have poorer prognosis and higher relapse risk compared to the luminal subtypes (Yang et al., 2011). Tumors of the basal subtype of breast cancer have a gene expression profile that is similar to the basal mammary epithelium (Alcalá-Corona et al., 2017) with high expression of basal markers such as keratin 5, 6, 14, and 17 and EGFR (Dai et al., 2015). 20% of all breast cancer cases corresponds to this subtype and the majority of triple negative tumors that are lacking expression of ER, PR and HER2 belong to the basal subtype (Alcalá-Corona et al., 2017). Triple negative tumors generally have *TP53* mutation (O'Brien et al., 2010), low BRCA1 expression (Abd El-Rehim et al., 2005) and they are associated with higher genetic instability. Therefore, they are the most aggressive tumors with poorest 5-year survival rates (Alcalá-Corona et al., 2017). Another subtype that also has triple negative tumors is claudin-low which has low expression of genes involved in tight junctions and cell-cell adhesions including claudin 3, 4 and 7, occluding and E-cadherin. Like the other triple negative tumors, claudin low tumors also have poor clinical outcome (Yersal & Barutca, 2014).

1.1.2. Therapy

Various therapeutic approaches can be used for the management of breast cancer according to the stage and molecular subtype of the tumor. Surgery and radiotherapy are still the gold standards in the treatment of breast cancer. In some cases, radiotherapy or chemotherapy can be used as a neoadjuvant therapy prior to surgery aimed to shrink the tumor. Pathological and molecular classification come into the play in the planning of adjuvant therapy after the surgery to increase the chance of cure by eradicating micro-metastatic disease (Yeo et al., 2014).

Endocrine therapy is the main treatment option for hormone receptor positive luminal tumors. It works by blocking the effects of hormones, mostly of estrogen, that are involved in disease progression. Tamoxifen is a selective estrogen receptor modulator and is the most commonly used drug for the luminal subtype. It is an estrogen analog thereby it blocks the binding of estrogen to its receptor and inhibits proliferative activities of estrogen.

Adjuvant usage of tamoxifen for 5 years after surgery has been shown to reduce the risk of relapse by 41% and death from breast cancer by 31% making it the standard of care for premenopausal women (Yeo et al., 2014). Another class of endocrine therapeutic drugs is aromatase inhibitors such as letrozole, anastrozole and exemestane that inhibit conversion of androgen to estrogen thus leading to estrogen depletion. Aromatase inhibitors have been demonstrated to be superior to tamoxifen for postmenopausal women (Yeo et al., 2014).

Some patients of luminal breast cancer might have intrinsic or acquired resistance to endocrine therapy. Therefore, additional therapies have been developed to combine with different endocrine therapies. Cyclin-dependent kinase 4 and 6 inhibitors such as palbociclib, ribociclib and abemaciclib, for example, have been used in combination with hormonal therapy in patients with metastatic luminal tumors that lack HER2 expression (H. Xu et al., 2017). Other possible therapeutic options include PI3K inhibitors, mTOR inhibitors, histone deacetylase inhibitors and steroid sulfatase inhibitors (Tong et al., 2018).

For patients with HER2 overexpressing tumors, the main therapeutic approach is to utilize molecular agents targeting ERBB2 receptor, alone or in combination with chemotherapy. These molecular agents include trastuzumab and pertuzumab, directly targeting HER2, ado-trastuzumab, an antibody-cytotoxic agent conjugate and lapatinib, a dual tyrosine kinase inhibitor (Tong et al., 2018). It has been shown that trastuzumab administrated in combination with chemotherapy significantly improves overall survival of HER2 overexpressing breast cancer (Slamon et al., 2001). Lapatinib is used together with chemotherapy in the patients that do not benefit from trastuzumab therapy (Geyer et al., 2006).

Lack of expression of hormonal receptors and HER2 make triple negative breast cancer (TNBC) difficult to treat and despite the recent advances in targeted therapy field, chemotherapy still remains as the standard treatment for this type of cancer. TNBC has the highest complete response rate to chemotherapy compared to all the other subtypes however, recurrence and metastasis rates are also higher (Liedtke et al., 2008). Therefore, new novel therapy strategies are required for the treatment of TNBC. Most of the TNBC have deficiency in their homologous repair mechanism mostly due to *BRCA* mutations. Therefore, Poly (ADP-ribose) polymerase (PARP) inhibitors have been shown to have promising clinical outcomes in patients with mutations in *BRCA1*/*BRCA2* and/or other

genes involved in DNA damage response (Bryant et al., 2005). Clinical trials administrating anti-angiogenic agents like bevacizumab in combination with chemotherapy showed higher response rate to therapy in metastatic TNBC (Robert et al., 2011). Due to its promising outcomes in many different cancer types, Pembrolizumab, a monoclonal antibody against PD-1, has been subjected to several clinical trials also in TNCB. It has been demonstrated that Pembrolizumab has durable antitumor activity in some patients with heavily pretreated metastatic TNBC (Adams et al., 2017) and its effect is independent of PD-L1 expression (Nanda et al., 2016).

1.1.3. Role of the tumor microenvironment in breast tumorigenesis

There have been recent advances in the detection, therapy and understanding of the molecular basis of breast cancer. However, the recurrence of the disease and resistance to therapeutic drugs are still some of the major clinical problems. While the main focus of breast cancer research had been on the investigation of the molecular events in breast epithelial cells that lead to the formation of malignant tumors for long, the tumor microenvironment is now recognized as an important player in tumor development because of the structural, cellular, functional, genetic, and epigenetic changes in the tumor stroma that can influence breast cancer progression (Artacho-Cordón et al., 2012).

The normal breast duct is composed of luminal epithelial and myoepithelial cells surrounded by extracellular matrix (ECM), endothelial cells, fibroblasts, immune cells, adipocytes and other stromal cells. The stromal compartment in the breast microenvironment has been shown to affect the terminal differentiation of epithelia and be involved in the mammary duct morphogenesis via affecting the epithelial phenotype (Maller et al., 2010). In addition to its influence on the normal mammary development, the microenvironment has been also reported to regulate the growth and differentiation state of breast cancer cells via different mechanisms (Allinen et al., 2004).

Cancer cells evolve together with their microenvironment. As the tumor progresses and gains new characteristics, the abundance, histologic organization and phenotypic properties of the stromal cells also change and this enables the transformation of the tumor from primary to invasive and to metastatic tumors (Hanahan & Weinberg, 2011). Therefore, it

is crucial to identify each cell type in the tumor microenvironment that can have different functions in the different stages of tumor development (Figure 1).

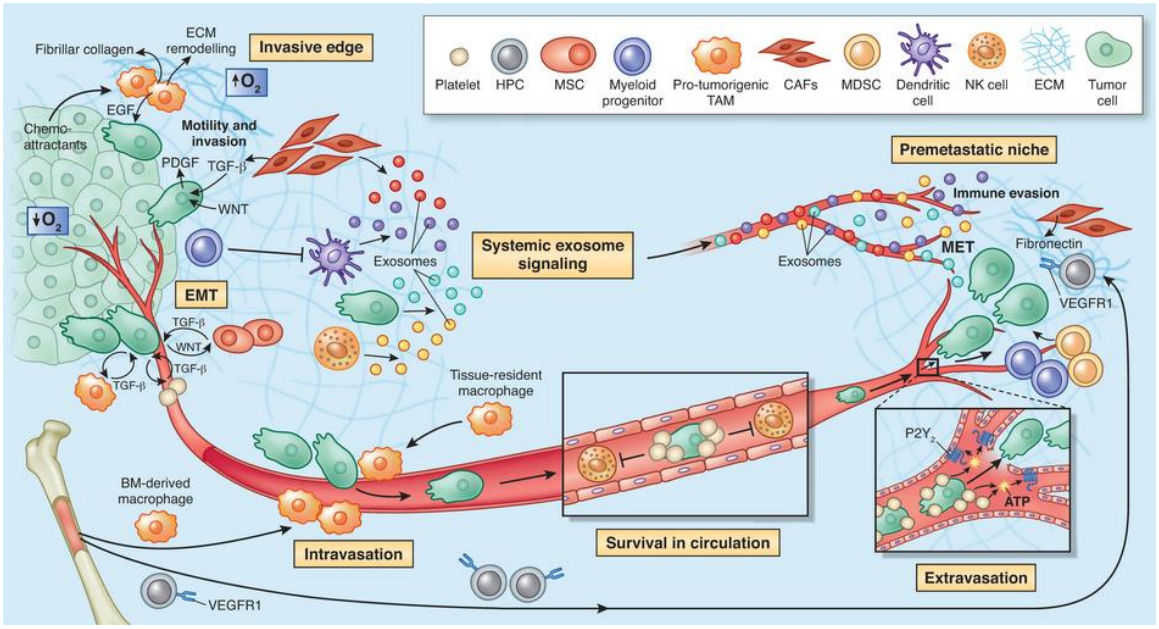

Figure 1 Role of the tumor stroma in cancer progression Stromal cells can be involved in each step of tumor progression. Tumor associated macrophages, cancer associated fibroblasts and pericytes contribute to epithelial-mesenchymal transition of the primary tumor cells and induce the transformation into a more invasive phenotype. With the help of tumor associated macrophages tumor cells can intravasate into the blood circulation. Once in the blood circulation, platelets escort tumor cells to the site of extravasation and help them to exit the circulation to enter secondary tissues. At the secondary sites, myeloid derived suppressor cells and natural killer cells create an immunosuppressive microenvironment and contribute to the re-colonization of cancer cells (image taken from Quail & Joyce, 2013)**.**

The first step in breast cancer development is the transformation of a single cell due to the accumulation of genetic and epigenetic changes. After epithelial hyperproliferation, the tumor progresses to in situ, invasive and metastatic carcinomas (Polyak, 2007). In a gene profiling study comparing normal epithelial cells with non-invasive primary tumors, it has been found that in addition to the genes regulating cell growth and differentiation, a significant fraction of secreted proteins is differentially expressed. This suggests the activation of aberrant autocrine and paracrine regulatory loops that would lead to the identified alterations in the tumor microenvironment (Allinen et al., 2004). In non-invasive breast carcinoma stage, it has been observed that there is an increase in the number of infiltrated leukocytes and fibroblast. These secrete growth factors, cytokines, chemokines,

and matrix metalloproteinases (MMPs) thus jointly promoting tumor growth and progression (Place et al., 2011). The transition from non-invasive to invasive and metastatic carcinomas is also accompanied by changes in the tumor microenvironment. Several genes associated with the components of the ECM such as MMPs have been reported to be differentially expressed between non-invasive and invasive or metastatic tumors (Schäfer & Werner, 2008). The ECM is one of the components of tissues together with cells and vascular space and it is composed of approximately 300 proteins. The ECM is one of the major components of the tumor microenvironment and it is involved in cancer development and progression. Tumors and stromal cells can remodel their surrounding ECM by increasing deposition and crosslink of ECM proteins that can facilitate cancer cell proliferation, migration and invasion (Xiong & Xu, 2016). For example, it has been shown that crosslinked and oriented collagen in breast cancer correlates with poor survival (Conklin et al., 2011).

One of the hallmarks of cancer is tumor promoting inflammation (Hanahan & Weinberg, 2011). Immune cells of both adaptive and innate immunity infiltrate the tumor site and create an inflammatory tumor microenvironment. Every neoplastic lesion contains immune cells at different densities because of the attempt of the immune system to clear tumor cells (Pagès et al., 2010). However, tumors can evade immune responses and even make use of the presence of the immune cells as it has been shown repeatedly that chronic inflammation promotes tumor growth and progression (Gonda et al., 2009). Tumor infiltrating lymphocytes (TILs), mainly consisting of T cells, are among the important players of the breast tumor microenvironment. It has been demonstrated that different breast cancer subtypes have different quantities of infiltrating T cells (Miyan et al., 2016) with TNBC having the highest number of TILs in their tumor microenvironment (Stanton & Disis, 2016). The population of infiltrating T cells consists of CD8+ cytotoxic T cells, CD4+ helper T cells and CD4+ regulatory T cells (Tregs). CD8+ cytotoxic T cells are the main T cell type with anti-tumor immunity properties, therefore, the number of infiltrating CD8+ T cells reversely correlates with ER and PR expression (Mahmoud et al., 2011). In the early phases of tumor development, T cells act as anti-tumorigenic entities. However, as the tumor progress, they differentiate into Tregs with tumor promoting properties. It has been shown that TNBC has higher numbers of Tregs compared to the other subtypes (Stanton &

Disis, 2016). Tumor-associated macrophages (TAMs) are another major factor in the tumor stroma of breast cancer. They are recruited to the tumor site via CCL2 that is produced by the tumor and get activated via CSF-1 which is also secreted by breast cancer cells. As a result they secrete EGF which promotes cancer growth (Qian et al., 2011). TAMs have also been reported to produce significant amounts of MMPs which are crucial factors in the degradation of the ECM thus facilitating local invasion of breast cancer cells (Qian & Pollard, 2010). Another component of the tumor microenvironment is the tumor-associated vasculature, comprised of endothelial cells. Under the influence of the tumor microenvironment, they can undergo an angiogenic switch, which is important for the tumor to exceed a certain size and metastasize to secondary organs. In addition to immune cells and endothelial cells, there is another cell type in the tumor microenvironment, namely fibroblasts that are discussed in detail in the following chapter.

1.2. Fibroblasts

Fibroblasts are first described as elongated cells in connective tissue that synthesize collagen (Virchow, 1858). Today, it is known that they are the most common cell type in connective tissue in animals and can be defined as non-epithelial and non-immune cells that produce structural proteins of the extracellular matrix. In normal tissues, they present as single cells in a quiescent or resting state and are located in the interstitial space or near capillaries (Kalluri, 2016).

Fibroblasts are mesenchymal cells that are derived from embryonic mesoderm tissue. They are found in every tissue in the body and depending on their anatomical site, their morphologies might differ. Fibroblasts located in different sites of body, have been also shown to have different gene expression patterns (Sorrell & Caplan, 2009) that might suggest that they take up their relative position in the body during development. However, the influences of different microenvironments on fibroblast characteristics cannot be excluded (Tschumperlin, 2013).

The main role of fibroblasts is the production and secretion of components of the ECM. They produce structural extracellular protein fibers that form an interconnecting meshwork. These structures can form lamina separating different cell types or interstitial regions connecting cells with common features. Different tissues require different type of

structural ECM proteins, depending on their biological functions. For example, tissues that are different in morphology in terms of degree of flexibility vs. rigidity, have different expression levels of collagen and elastin proteins thus providing rigidity or elasticity, respectively. Another protein type that is produced by fibroblasts is adhesive ECM proteins such as fibronectin and laminin that are important to connect cells with the ECM. Both are involved in interaction with integrin receptors. Fibroblasts also produce the ground substance of the ECM by secreting proteoglycans. The latter form a cell free medium that is essential for cell migration of immune cells, fibroblasts and endothelial cells. In addition to providing a surface for cell migration, proteoglycans that are post-translationally modified by the addition of glycosaminoglycans, are also involved in cell signaling (Kendall & Feghali-Bostwick, 2014). In addition to their main role in extracellular matrix production and maintenance, fibroblasts are also involved in physiological processes such as inflammation and wound healing.

1.2.1. Wound healing

In case of an injury, integrity and homeostasis has to be restored immediately. Wound healing is a multistep, complex biological process in which various pathways need to be activated. Three overlapping phases of wound healing are inflammation, new tissue formation or proliferation and remodeling (Gonzalez et al., 2016).

Inflammation occurs directly after tissue damage. Coagulation cascade, inflammatory pathways and immune system are activated at this stage to prevent ongoing blood and fluid losses, to remove dead tissues and to prevent infection (Gurtner et al., 2008) . Aggregation of thrombocytes followed by formation of fibrin matrix creates a scaffold for infiltrating cells. First neutrophils infiltrate the lesion that activated by pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ. These cytokines induce the expression of several adhesion molecules that are LFA-1, MAC-1, gp150/95 and CD11d/CD18 that are involved in extravasation (Eming et al., 2007). Upon recruitment to the wound, neutrophils are involved in several processes such as killing invading microorganisms, resolution of fibrin clot, promoting angiogenesis and re-epithelization (Shaw & Martin, 2009). The second inflammatory cell type that is recruited to the injury side is monocytes that differentiate into macrophages. Macrophages clear up matrix, cell debris and also neutrophils. They are

also important for recruiting other inflammatory cells by producing chemotactic factors (Eming et al., 2007).

In the second phase of wound healing that is proliferation, the aim is the closure of wound gap and replenishment of lost tissue by contraction, fibroplasia, angiogenesis and re-epithelialization (Gonzalez et al., 2016). At this stage, fibroblasts, recruited to the wound via chemokines secreted by macrophages. They contribute to the formation of new stroma (granulation tissue) that replaces the fibrin clot by involving in synthesizing, bundling and alignment of type III collagen fibers that are the main components of connective tissue scar (Boris Hinz, 2007). Moreover, activated fibroblasts (see section 1.2.2.) accumulated on the wound borders, contract and bring the edges of the wound toward the center. Contraction of activated fibroblasts can be calcium-calmodulin-myosin light chain kinase or Rho-ROCK-myosin light chain phosphatase dependent (Bochaton-Piallat et al., 2016). Large focal adhesion complexes transmit the contractile forces generated by fibroblast to ECM that results in pulling and compacting the surrounding matrix into scar tissue (Carthy, 2018a).

The last phase of wound healing is remodeling. The main aim of this phase is to obtain maximum tensile strength of the wound by reorganization, degradation and re-synthesis of the ECM (Gonzalez et al., 2016). Type III collagen is replaced with type I collagen that has more tensile strength. There is a reduction in the number of new blood vessels and blood flow. Moreover, the inflammatory response to injury resolves and neutrophils are cleared up (Landén et al., 2016).

1.2.2. Activation of fibroblasts

Activated fibroblasts were first described in the context of wound healing as cells that that might be involved in wound contraction (Gabbiani et al., 1971). These cells were later called myofibroblasts because of both their resemblance to smooth muscle cells and their alpha-smooth muscle actin (αSMA) expression (Gabbiani et al., 1978; Skalli et al., 1986). They take part in proliferation phase of wound healing (see section 1.2.1.) and once the wound closes, they disappear through apoptosis (Desmoulière et al., 1995). Activated

fibroblasts are also present in fibrotic diseases such as scleroderma, liver, kidney and lung fibrosis and in repairing heart after myocardial infarction (Bochaton-Piallat et al., 2016).

To better understand what an activated fibroblast is, it is crucial to define the properties of a quiescent fibroblast. Adult quiescent fibroblasts are found in the interstitial stromal areas between layers of functional parenchyma and detected as thin elongated cells that have extensions and spindle-like shape. Although there is no absolute marker for quiescent fibroblasts, fibroblast-specific protein 1 (FSP1) has been considered as the closest one (Strutz et al., 1995; Kalluri, 2016). They are defined as quiescent because of their ability to respond to stimuli to become activated. Apart from their activation ability, other possible functions of quiescent fibroblasts are still mainly unknown. Isolating fibroblasts from most tissues relays on the fact that upon digestion of the tissue, fibroblasts are the only population of cells that can easily adhere and be cultured on plastic surfaces. There is a lack of knowledge on the molecular properties of quiescent fibroblasts because upon isolation, they lose their quiescent state and become activated due to artificial culture conditions. Moreover, embryonic and developing tissues do not have fibroblasts but probably active mesenchymal cells of which most undergo apoptosis and a few revert to a quiescent state once development is completed. Therefore, all the knowledge of fibroblasts come from their activated phenotype (Kalluri, 2016).

Activated fibroblasts have been shown to be highly heterogeneous with distinct gene expression patterns depending on the tissue that they are isolated from (H. Y. Chang et al., 2002). When there is a stimulus that can be an injury, stress or inflammation, quiescent fibroblasts can be reversibly activated (Figure 2). These activated fibroblasts start expressing makers such αSMA and vimentin. As a result of cytoskeletal rearrangements, their morphology changes from spindle to stellate shape. They gain contractile and migratory phenotype that is required in wound healing process. They start proliferating and contribute to ECM remodeling by secreting various factors. When the wound healing is completed, this activation could be reverted by reprogramming or activated fibroblasts could be cleared up by apoptosis. When there is persisting and unabated stimuli as in the case of fibrosis or cancer, activated fibroblasts gain more secretory phenotypes, specialized ECM remodeling ability, robust autocrine activation and immunomodulatory signaling

functions (Kalluri, 2016). This last step might be irreversible through epigenetic regulations (Zeisberg & Zeisberg, 2013 ; Bechtel et al., 2010).

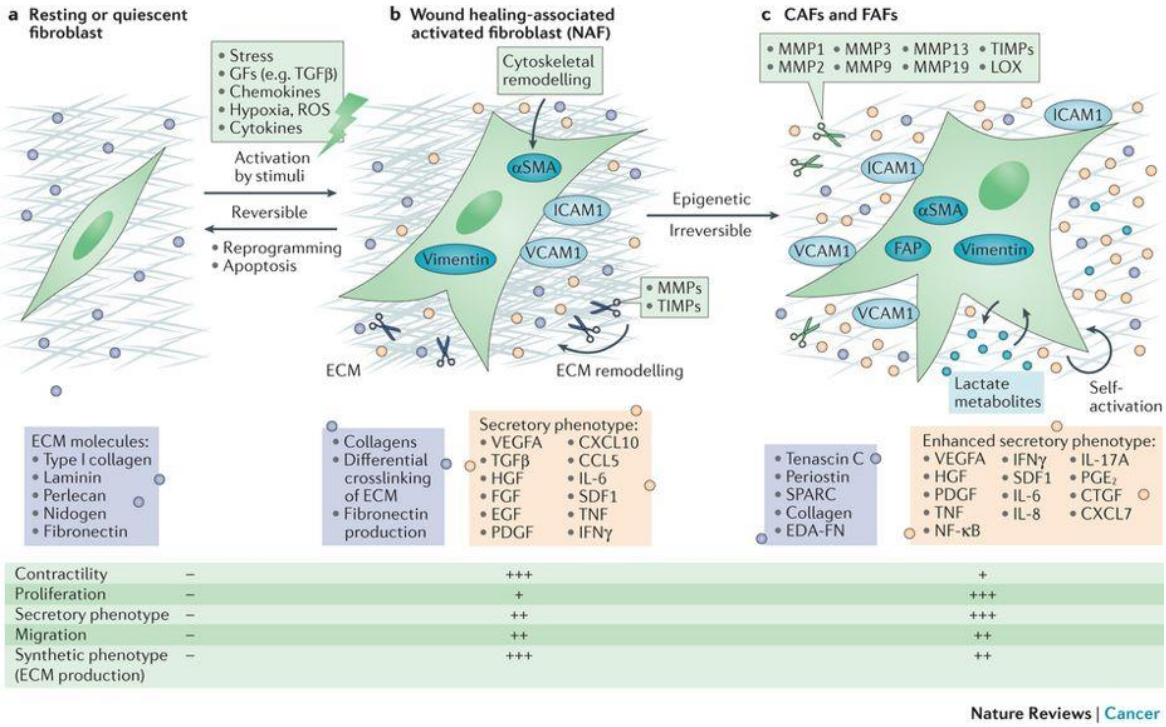

Figure 2 Multi-step activation of fibroblasts (Kalluri, 2016)

How this activation is regulated at molecular level is the subject of ongoing research. The most well-known inducer of fibroblast activation is TGFβ (Carthy, 2018a). TGFβ is secreted by the cells in its inactive form that needs to be activated in the ECM by proteolytic cleavage. Active TGFβ is free to bind to its receptor, TGFβR2. Upon ligand binding TGFβR2 phosphorylates TGFβR1 that induces phosphorylation of receptor activated Smad proteins which in turn translocate to the nucleus. In the nucleus, Smad proteins recognize specific Smad binding elements (SBE) in the enhancer or promoter regions of TGFβ pathway target genes. TGFβ can also activate non-Smad pathways including MAPK pathway, Rho-like GTPases and PI3K signaling (Carthy, 2018b). Involvement of TGFβ in the activation of fibroblasts has been shown for the first time in context of fibrosis using Smad3 knockout mouse models. Mice lacking Smad3 have been found to be protected against fibrosis in different disease models (Sato et al., 2003; Flanders et al., 2002). Later on, TGFβ signaling has been also associated with αSMA expression due to presence of

SBE in the proximal promoter region of ACTA2 gene (B. Hu et al., 2003). Wnt signaling is also involved in the activation of fibroblasts via crosstalk with TGFβ pathway. Upon activation of Wnt signaling, the effector of Wnt signaling pathway, namely, β-Catenin, translocates to nucleus and via formation of transcriptional complexes with TCF/LEF family transcription factors, it induces transcription of Wnt target genes (Clevers & Nusse, 2012). It has been demonstrated that β-catenin, that is activated as a result of TGFβ signaling, can form a complex with pSmad3 and the histone acetyltransferase CREB-bind protein (CBP) at the upstream SBE in the αSMA promoter (Zhou et al., 2012). In another study, it has been shown that induction of Wnt signaling by activated TGFβ pathway is required for TGFβ mediated fibrosis (Akhmetshina et al., 2012). Moreover, cancer cell derived Wnt7a has been reported to activate fibroblasts via TGFβ dependent manner (Avgustinova et al., 2016). Another pathway that is found to be associated with the activated fibroblasts is NF-κB pathway. As a transcription factor NF-κB is expressed in nearly all cell types and regulates the expression of a wide variety of genes with different functions. There are five members of this transcription factor family that share structural features and form homo- or heterodimers (Ghosh et al., 1998). When the dimers are in their inactive form, they are bound to the inhibitory molecules that belong to the IκB protein family. The activation of the pathway generally leads to the proteasomal degradation of the IκB molecules, which results in release and subsequent transportation of NF-κB to the nucleus where it acts as a transcription factor (Napetschnig & Wu, 2013). It is not clear if NF-κB pathway is required for the fibroblast activation. However, it is known that inflammatory features associated with activated fibroblasts are dependent on NF-κB signaling (Erez et al., 2010).

Activated fibroblasts are predominant stromal cells of human tumors. In the context of cancer, they are called cancer associated fibroblasts (CAFs) that are discussed in the next chapter.

1.2.3. Cancer Associated Fibroblasts

The term, cancer associated fibroblast, is often used to define a complex population of dynamically heterogeneous mesenchymal cells found in stroma of solid tumors (LeBleu & Kalluri, 2018). In tissue sections of various tumors, CAFs can be identified based on their

spindle shape and elongated cytoplasmic processes (Kalluri, 2016). In addition to their different morphology, another way to distinguish them from other cells types in the tumor is using an exclusion criterion that is lack of expression of endothelial, epithelial and immune cell markers.

Compared to not activated quiescent fibroblasts, CAFs have enhanced proliferation and biosynthetic activities such as production of components of ECM, enzymes that are involved in ECM remodeling, growth factors and cytokines (LeBleu & Kalluri, 2018). Therefore, CAFs are important components of tumor progression and contribute to wide range of fibrotic stromal programs of many different tumors (Kalluri, 2016).

1.2.3.1. Origin and heterogeneity

The origin of CAFs is still an open research question mainly due to their heterogeneity and lack of specific markers. However, it has been observed that a significant proportion of CAFs are likely derived from cells that have a mesoderm origin (Madar et al., 2013). When a solid tumor arises, cancer cells start forming a neoplastic lesion that develops within the tumor microenvironment of the epithelium (Kalluri, 2016). Even at this stage, that is known as carcinoma in situ, tumor has a reactive fibrotic microenvironment meaning that quiescent fibroblasts residing in the host tissue starts expanding due to the developing neoplasm (Ronnov-Jessen et al., 1996). In addition to residence fibroblasts that are found in the organ that cancer starts growing from, CAFs can be also recruited to the tumor side from a distant source such as bone marrow (Shiga et al., 2015) (Please see Chapter 1.3.). Trans-differentiation of pericytes, endothelial-to-mesenchymal and epithelial-to-mesenchymal transition programs have been also shown to generate CAF like cells (LeBleu & Kalluri, 2018). Growth factors such as TGFβ, PDGF and FGF2 secreted by cancer cells and infiltrating immune cells mediate recruitment of stromal fibroblasts to the tumor. TGFβ that stimulates local CAF proliferation and invasion is the major cytokine in the tumor microenvironment for the activation and recruitment of CAFs (Elenbaas & Weinberg, 2001). Although in theory, origin of CAFs can be determined by lineage tracing studies using tumor bearing mice, lack of specific markers for CAFs makes this difficult (LeBleu & Kalluri, 2018). There are certain markers whose expressions are associated with CAFs but most of these are also expressed by other cell types (Table 1). For example, the most

known activated fibroblast or myofibroblast marker is αSMA. However, its expression might be different in different CAF populations. It has been shown that CAFs that are isolated from breast cancer patients have bimodal distribution of ACTA2 expression that does not correlate with their activation status (Busch et al., 2017). This indicates that CAFs that are coming from the same tumor have different levels of ACTA2 expression that is either high or low.

It was speculated by LeBleu and Kalluri that fibroblasts are activated in the early stages of oncogenesis and become CAFs that in turn remodel the tumor microenvironment as a tissue repair response and exert antitumor functions. However, as tumor grows, cancer cells use CAF secreted factors to facilitate their own survival and proliferation, changing CAFs gradually from tumor suppressing to tumor promoting. The mechanisms underlying this transformation of fibroblasts from normal activated fibroblasts to CAFs remain largely unknown. In different tumor types, these changes can be different due to different background of residence fibroblasts. Even, in the same tumor type, CAFs can have distinct paracrine actions because of different sources of CAFs within the same tumor. All these factors contribute to create both inter and intra-tumor heterogeneity in CAF populations (LeBleu & Kalluri, 2018)

Table 1. Different CAF markers and their biological function (modified from LeBleu & Kalluri, 2018)

CAF markers	**Biological functions**
Collagen I, Collagen II, fibronectin, tenascin C, periostin	ECM components
LOX, LOXL1, MMPs, TIMPs	Remodeling enzymes
TGFβ, VEGFs, PDGFs, EGF, FGFs, PGE2, CTGF, SDF-1, WNTs	Growth factors and cytokines
PDGFRα/β, VCAM1, DDR2, TGFβRI/II, EGFR, FGFRs, BMPRI, BMPRII, podoplanin, FAP and decreased expression of CAV1	Receptors and other membrane-bound proteins
Desmin, vimentin, αSMA, FSP1	Cytoskeleton components and other cytoplasmic proteins

1.2.3.2. Roles of CAFs in tumor progression

There have been publications showing both tumor promoting and tumor restraining properties of CAFs that will be discussed in the following chapters.

Tumor promoting functions of CAFs

Early co-culture studies of CAFs and cancer cells have shown that CAFs contribute to tumorigenesis. For example, when SV40 transformed prostate epithelial cell were co-injected together with normal fibroblasts or CAFs into mice, only CAF co-injected cells formed tumors (Olumi et al., 1999). In another study, it has been shown that HSF1 upregulation in CAFs, support malignancy in breast cancer tumors (Scherz-Shouval et al., 2014).

ECM remodeling is one way of CAFs to promote tumor progression. For example, MMP3 that is produced by activated fibroblasts in breast tumors has been shown to promote invasiveness of cancer cells by stimulating epithelial to mesenchymal transition via cleaving E-cadherin (Lochter et al., 1997). In another study, it has been shown that ECM remodeling by periostin secreted by fibroblast is important step for preserving cancer stem cell niche (Malanchi et al., 2012). Also, CAFs that lack expression of Timp gene, has been shown to produce exosomes enriched in ECM proteins and the metalloproteinase ADAM10 that cause increase cancer cell motility, metabolic reprogramming and induction of cancer stem cell features (Shimoda et al., 2014).

CAFs also contribute to tumor angiogenesis. In a breast cancer model, xenograft breast tumors that were co-injected with CAFs showed increased tumor growth compared to the xenografts that were co-injected with normal mammary fibroblasts as a result of enhanced angiogenesis by CAF derived CXCL12 (Orimo et al., 2005). In another study that unraveled the CAF involvement in angiogenesis in lung carcinoma, it has been shown that PDGFA producing tumor cells recruit VEGF producing fibroblasts to the tumor microenvironment (Tejada et al., 2006).

CAFs are also important in secondary tumor growth at the metastatic site. CAFs, in the primary tumor, enhances metastasis by secreting potent growth factors and cytokines to promote growth and invasiveness of the cancer cells at a distant site (Kalluri, 2016). For example, PDGFC and VEGFA that are produced by stromal cells in breast cancer are key

for metastatic colonization to the lung (O'Connell et al., 2011). In colorectal cancer, stanniocalcin secreted by PDGF-stimulated CAFs, is an important factor for intravasation and metastasis (Peña et al., 2013).

Another important characteristic of CAFs in the tumor microenvironment is their immunomodulatory functions. The secretome of CAFs can dynamically evolve during tumor progression and affect tumor immunity differently at different stages of tumor development (Kalluri, 2016). According to the results of many studies investigating the role of CAFs in the regulation of immune system, CAFs have mainly immunosuppressive functions. However, it is worth to mention at this point, it has been also considered that these observations may be context dependent because most of the studies in this area are based largely on *in vitro* measurements or *in vivo* experiments using CAFs cultured *in vitro* (Kalluri, 2016). For example, CAFs that have active NF-kappaB signaling has been shown to mediate tumor enhancing inflammation by promoting macrophage recruitment, neovascularization and tumor growth that was (Erez et al., 2010). In another study in breast cancer, CAFs have been demonstrated to be an important factor involved immune polarization in cancer progression by promoting immune suppressive tumor microenvironment via recruitment of tumor-associated macrophages, myeloid derived suppressor cells and regulator T cells (Liao et al., 2009). In addition to secreting factors such as IL-6, IL-4, IL-8, IL-10, TNF, TGFβ, CCL2, CCL5, CXCL9, CXCL10, SDF1, PGE_2, NO, HGF and HLAG that are involved directly in immunosuppressive functions of CAFs, they can also affect immune response indirectly via acquisition of adhesion molecules such as ICAM1 that has been shown to provide a docking side for immune cells causing their activation, repression and polarization (Kalluri, 2016).

CAFs are also involved in metabolic regulation of the tumor microenvironment. Factors that cause activation of fibroblasts such as TGFβ, PDGF, HIFα and ROS mediated suppression of CAV1 also promote metabolic shifts in fibroblasts resulting in cells that rely on aerobic glycolysis similar to highly proliferating cancer cells (Guido et al., 2012) In addition to relying on aerobic glycolysis, CAFs also acquire increased catabolic activity and autophagy (Martinez-Outschoorn et al., 2014; Chaudhri et al., 2013). Moreover, it has been reported that CAFs produce increased levels of ketone bodies, fatty acids and

glutamine and supply anabolic cancer cells with fuel sources for mitochondrial respiration (Martinez-Outschoorn et al., 2014; Pavlides et al., 2009).

Another aspect of tumor progression that CAFs get involved in is drug resistance. It has been known for a long time now that organ specific microenvironments play an important role in therapy response and resistance (Fidler et al., 1994). In breast cancer, it has been shown that a stoma-related gene signature is associated with chemo-resistance and may be used to predict the response to chemotherapy in the neoadjuvant setting (Farmer et al., 2009). In another study, in Her2+ breast cancer, CAF produced HGF has been identified as an essential factor in resistance to lapatinib (Watson et al., 2018). Also in non-small cell lung cancer, HGF that is secreted by CAFs has been shown to contribute to resistance to EGFR tyrosine kinase inhibitors (Yano et al., 2011). In addition to direct effects of CAFs on drug response, they can also have indirect effects. CAFs have been shown to increase intra-tumoral interstitial fluid pressure thereby contributing to drug resistance by inhibiting drug uptake (Heldin et al., 2004).

Tumor restraining functions of CAFs

While tumor promoting effects of CAFs are generally based on secretion of pro-survival factors, their antitumorigenic roles are mostly associated with their functions as regulators of antitumor immunity (Kalluri, 2016). The studies, that try to target CAFs in the tumor microenvironment because of abundant literature about their tumor promoting functions, have been demonstrated the heterogeneity of CAFs and also showed possible antitumorigenic functions (LeBleu & Kalluri, 2018). For instance, in an *in vivo* setup of pancreatic cancer, the depletion of αSMA expressing CAFs, using genetically engineered mouse models, has resulted in more invasive tumors with increased proportions of regulatory T cells (Ozdemir et al., 2014). In another study in pancreatic cancer, the deletion of SHH gene from cancer cells has caused reduced stromal content but increased tumor aggressiveness (Rhim et al., 2014). Moreover, the secretome of CAFs might also have antitumor functions. For example, IL-10, TGFβs, IFNγ and IL-6 are involved in the recruitment and polarization of macrophages, NK cells and T cells that contributes to an immune control of cancer cells. All in all, it might be speculated that the net effect of CAFs on tumor progression might be bimodal mainly due to their heterogeneity (LeBleu &

Kalluri, 2018). Therefore, it is necessary to understand functions of different populations of CAFs that have differential gene expression and their different effects on tumor progression.

1.3. Mesenchymal Stromal/Stem Cells

1.3.1. Discovery, origin and plasticity

Adult stem cells which can be isolated from several tissues in the human body have a long-term self-renewal ability and they can give rise to more differentiated downstream cell lineages (Hsu & Fuchs, 2012). Mesenchymal stem cells (MSCs) were discovered in 1968 and initially described as bone-marrow-derived stromal cells that can form adherent fibroblast-like populations and differentiate into the mesenchymal cell lineages including adipocytes, chondrocytes and osteoblasts (Friedenstein et al., 1968). Although, their stem cell properties have still not been fully demonstrated *in vivo* (Ankrum et al., 2014), they were named as MSCs because of their *in vitro* multilineage differentiation potential and self-renewal capacity (A I Caplan, 1991). However, with in *vivo* heterotopic bone formation assays, single cell clones derived from only a minor subset of this population has been shown to have the potential to generate a miniature bone organ with bone tissue itself, cartilage, adipocytes, fibroblasts and hematopoiesis supporting stroma (Friedenstein et al., 1968) and have the ability to self-renew (Sacchetti et al., 2007). Therefore, it is now accepted that initially described mesenchymal ´stem´ cell population, which includes all the nonhematopoietic stromal cells in bone marrow, is a heterogeneous population and consists of stromal cells and stem cells that could generate skeletal lineages (bone, cartilage, adipocyte, hematopoiesis stroma). Not only stemness properties of these cells but also the extent of their multi-lineage potential has been a controversial topic. The reasoning behind naming these cells as mesenchymal stem cell was based on the hypothesis that in addition to their differentiation ability to the skeletal lineages, postnatal MSCs could also generate other mesenchymal tissues including skeletal muscle, myocardium, smooth muscle and tendon (Caplan, 2005). However, the differentiation ability to the latter is highly controversial and still not proven by proper *in vivo* experiments (Bianco et al., 2008). Therefore, some scientists referred them as skeletal stem cells to emphasize their *in vivo* proven differentiation potential into skeletal lineages (Bianco et al., 2013)**.** There have

been different reports showing that the subpopulation of bone marrow stromal cells with real stem cell properties is a very small fraction of all nucleated cells in bone marrow and different studies came up with different markers to identify these cells (Table 2).

Table 2. Human and mouse bone marrow mesenchymal stem cell markers tested *in vivo* (modified from Frenette et al., 2013)

	Cell phenotype
Mouse	$Nestin^+$ $CD45^-$ $CD31^-$ (later identified as $CD51^+$ $PDGFRa^+$ $CD45^-$ $Ter119^-$ $CD31^-$) $CD51^+$ $CD105^+$ $CD90^-$ $CD45^-$ $Ter119^-$ $PDGFRa^+$ $Sca\text{-}1^+$ $CD45^-$ $Ter119^-$
Human	$CD146^+$ $CD45^-$ $STRO\text{-}1^{bright}$ $CD146^+$ $STRO\text{-}1^{bright}$ $CD106^+$ $SSEA4^+$ $CD146^{+/-}$ $CD271^+$ Lin^-CD45^-

After their discovery in the bone marrow, heterogeneous MSC populations have been also identified in other tissue types such as adipose tissue, liver, placenta, umbilical cord blood and amniotic fluid. Because of their heterogeneity, a set of standards was defined to designate a cell as a MSC. They must be plastic adherent, express CD90, CD105 and CD73 on their cell surface, lack the expression of CD45, CD34, CD14, CD79 and HLA-DR and have tri-lineage (adipocytes, chondrocytes and osteoblasts) differentiation potential *in vitro* (Dominici et al., 2006). However, it was observed that MSCs express different levels of these markers and are variable in their differentiation potential which might be dependent on the donor, tissue of origin, isolation technique, culture conditions and passage number (Ankrum et al., 2014).

The origin of MSCs is not well defined. However, there are several reports demonstrating that they might be derived from neural crest or mesoderm during development (Morikawa et al., 2009; Dennis & Charbord, 2002). Mesoderm originated MSCs have a common progenitor with endothelial cells and it has been shown that vascular endothelial cells can transform into multipotent stem like-cells via a mutant *ALK2* receptor dependent mechanism (Medici et al., 2010) indicating that endothelial cells might be a source of MSCs after embryonic development. Interestingly perivascular cells, namely pericytes,

have also been reported to express MSC markers and have the tri-lineage differentiation potential which might designate the progenitor cells derived from blood vessel walls as the origin of MSCs (Crisan et al., 2008). However, these studies still require further investigation due to the lack of standardization in the handling of MSCs and the lack of rigorous *in vivo* data.

1.3.2. Physiological roles of MSCs

Although, the information about the stemness, origin and plasticity of MSCs is not complete yet, the physiological functions of these cells and especially of bone marrow derived MSCs (BM-MSCs) have been extensively studied. MSCs exert their functions either by differentiating into downstream lineages or by interacting with other cells directly or through paracrine mechanisms. In the bone-marrow, for example, BM-MSCs contribute to the homeostasis of the hematopoiesis via the osteoblasts which play a crucial role in the bone-marrow microenvironment by creating a niche for hematopoietic stem cells (HSCs) (Wilson & Trumpp, 2006). Also, they have been reported to directly interact with HSCs and be involved in the organization of their niches (Méndez-Ferrer et al., 2010).

Apart from the tri-lineage differentiation and direct interactions with the other cells, the major action mechanism of MSCs is the production and secretion of a wide range of cytokines and growth factors. These factors are produced under different conditions in response to different stimuli and have the potential to modulate several cellular processes including but not limited to cell proliferation, survival and migration (Table 3).

Table 3 The major paracrine factors secreted by MSCs and their roles in the cellular processes

Paracrine factors	**Biological functions**
VEGF, HGF, STC-1, SDF-1, TGFβ, IGF1, bFGF, TB-4	Cell Survival
VEGF, bFGF, IL-1, TNF-α, PDGF-BB, Ang-1, Ang-2, FGF-2, TGFβ, SDF-1, IGF-1, PIGF, MCP, HGF	Angiogenesis
VEGF, FGF-2, HGF, IGF-4, TB4	Anti-apoptosis
VEGF, HGF, IGF-1, TNF-α, TGFβ, G-CSF, SCF, LIF, M-CSF, IL-6, IL-11, Activin A	Cell differentiation
BDNF, NGF, neuregulin.1, BNP, IL-6, FGF-2, GDNF, VEGF, HGF, FGF-20	Neuroprotection and regeneration

IL-1, IL-10, TB-4, MMP-2, MMP-9, MCP-1, TSP-1, TGFβ, TIMP-1, TIMP-2, TIMP-9, HGF. NGF, ErbB-2	Tissue remodeling
VEGF, bFGF, FGF-2, HGF, TB-4, IGFBP-7	Cell contractility
IL-6, IL-10, IL-1β, TGFβ, INF-γ, GM-CSF, PGE-2, IDO, HGF, TNF-α, Activin A	Immunomodulatory effects
VEGF, endothelin, Smad-4, Smad-5, glypican-3, FGF-16	Cell proliferation and migration

One of the well-known outcomes of this wide secretome of MSCs is their impact on the immune system. MSCs have immune-suppressive effects on both adaptive and innate immunity. They have been shown to suppress T-cell proliferation and activation *in vitro* by secreting IL-10, TGFβ and HGF (Di Nicola et al., 2002; Tse et al., 2003). MSCs also inhibit the maturation of dendritic cells, the proliferation and activation of B-cells and natural killer cells via paracrine mechanisms (Beyth et al., 2005; Deng et al., 2005; Krampera et al., 2006). Moreover, in line with their immune-suppressive properties, MSCs can promote the generation of regulatory T-cells and M2 macrophages (Di Ianni et al., 2008; François et al., 2012). While inhibiting the effector cells of the immune system, MSCs themselves were for long believed to be immune privileged. They do not express major histocompatibility complex-II (MHC-II) or co-stimulatory molecules such as CD80, CD86 or CD40 and they express low levels of MHC-I. Thus they are poorly recognized by T-cells (Ryan, Barry, Murphy, & Mahon, 2005). One of the possible clinical applications of this feature is to use them as immune suppressors in organ transplantation or in autoimmune diseases (Tyndall, 2012). However, it should be mentioned that there are recent reports showing that allogeneic MSCs can cause an immune response and it has been stated that MSCs should be defined as immune evasive but not as immune privileged anymore (Ankrum et al., 2014). Another important function of MSCs is their tropism to the damaged tissues and participation in the wound healing process. There are several studies showing the homing ability of MSCs to the injury site after their systemic or local delivery in different hosts (Chen et al., 2001; Devin et al., 2003). Inflammatory chemokines released from the injured tissue facilitate the migration of MSCs since they express various chemokine receptors on their surface (Ponte et al., 2007). Although the exact molecular mechanisms of homing of MSCs is still under investigation, it has been shown that the extravasation process is similar to the well-known leukocyte homing (Rüster et al., 2006).

Once MSCs are in the injury site, they can be involved in each phase of wound healing by secreting factors such as VEGF, PDGF, bFGF, EGF, and TGFβ and thus directly facilitate tissue repair (Maxson et al., 2012).

1.3.3. Roles of MSCs in the tumor microenvironment

The tropism of MSCs towards the inflammatory signals also makes them migrate to the tumor site because tumors continuously produce inflammatory molecules that maintain the tumor microenvironment as mentioned above. BM-MSCs expanded *ex vivo* and injected into tumor bearing mice have been shown to accumulate at the sites of solid tumors including breast (Dwyer et al., 2007), lung (Suzuki et al., 2011), pancreatic (Beckermann et al., 2008), colon (Shinagawa et al., 2010) and ovarian carcinomas (Komarova et al., 2010) as well as other tumor types such as melanoma (Studeny et al., 2002), glioma (Nakamizo et al., 2005), and osteosarcoma (Xu et al., 2009). MSCs that are injected into a tumor bearing mice have been shown to migrate to the tumor site though different mechanisms. Adhesion of MSCs to the vascular endothelial cells and the following extravasation are among the first steps in the homing of circulating MSCs to the tumor site. TNFα, which is present in the tumor microenvironment at high concentrations, induces the expression of VCAM-I on the surface of MSCs and facilitate the adhesion of these cells to the vascular endothelium (Uchibori et al., 2013). The main source of TNFα in the tumor microenvironment of invasive breast carcinomas is the tumor associated macrophages (Leek et al., 1998) but also breast cancer cells secrete this factor (Meng et al., 2001). It has been reported to be highly expressed in the stroma of breast carcinomas and its expression has been correlated with the tumor grade (Miles et al., 1994). It has been shown that not only inflammatory cytokines and chemokines such as IL-6, IL-8, SDF-1, MCP-1, CXCL-1, CXCL-2 and TGFβ secreted by tumor tissue but also tumor derived growth factors including EGF, HGF, bFGF and PDGF can induce mobilization and migration of MSCs (Barcellos-de-Souza et al., 2013). Moreover, hypoxia has been reported to be an important factor in MSC homing. HIF-1 secreted by the tumor causes the expression of CXCR3 and

CXCR5 on the surface of the cancer cells thus facilitating the recruitment of MSCs which produce high levels of CXCL10 and CXCL5 (Chaturvedi et al., 2013).

The role of MSCs in cancer progression is highly controversial because there are reports showing both tumor promoting and suppressing effects of MSCs (Table 2). One of the important studies demonstrating tumor promoting roles of MSCs has been performed in a breast cancer model. Co-injection of BM-MSCs together with different breast cancer cell lines into immune-compromised mice enhanced the metastatic potential of all the cancer cell lines by CCL5 secretion that activates Akt signaling in the cancer cells (Karnoub et al., 2007).

Table 4 Tumor promoting and suppressing effects of BM-MSCs BM-MSCs can promote tumor progression by regulating several processes in the tumor microenvironment via secreting several cytokines and growth factors. However, there are also several reports showing their anti-tumorigenic functions (modified from Klopp et al., 2011).

Tumor promoting effects	Isolation	Tumor model	Findings	Proposed mechanism
Karnoub et al.	Human BM-MSC	Breast	Increased size and increased metastasis	CCL5 secretion
Zhu et al.,	Fetal and adults BM-MSCs	Colon	Increased incidence	Enhanced proliferation and angiogenesis
Djouad et al.	Mouse BM-MSC	Melanoma	Increased incidence	Immune system related
Muehlberg et al.,	Human and mouse ASCs	Breast	Increased size	SDF-1 secretion
Yu et al.	Human ASC	Lung or glioma	Increased size	Reduced apoptosis
Galie et al.	Mouse ASC	Breast	Increased incidence and size	Vasculogenic
Lin et al.	Human ASC	Prostate	Increased incidence and size	Vasculogenic and modulation of CXCR4
Kucerova et al.	Human ASC	Melanoma and glioblastoma multiforme	Decreased latency and increased size of melanoma xenografts	VEGF and SDF-1a/CXCR4
Shinagawa et al.	Human MSC	Colon	Increased size and metastasis	Increased angiogenesis and reduced apoptosis

Tumor suppressing effects	Isolation	Tumor model	Findings	Proposed mechanism
Ohlsson et al.	MPC1cE MSC	Colon	Tumor size smaller	Increased inflammatory infiltrate
Khakoo et al.	Human BM-MSC	Kaposi sarcoma	Tumor size smaller	AKT signaling
Qiao et al.	Human fetal skin	Hepatoma	Tumor size smaller	Wnt signaling
Qiao et al.	Human fetal skin	Breast	Increased latency, reduced tumor size and metastasis	Wnt signaling
Otsu et al.	Rat BM-MSC	Melanoma	Tumor size smaller	Inhibition of angiogenesis
Maestroni et. al	BM-MSC	Lung and melanoma	Tumor size smaller and decreased metastasis	Increased by treatment of MSC with GM-CSF

In an osteosarcoma model, BM-MSCs have been reported to increase proliferation by inducing STAT3 signaling in cancer cells via the secretion of IL-6 (Tu et al., 2012). IL-6 secretion by MSCs has been also shown to increase the number of tumor initiating cells in colorectal cancer (Tsai et al., 2011). Further, MSCs can contribute to EMT by secreting TGFβ. Breast cancer cells have been reported to express EMT specific markers upon co-culture with BM- MSCs (Q. Xu et al., 2012).

On the other side, there are several studies showing tumor growth inhibitory effects of MSCs. It has been shown that, for example, BM-MSCs inhibit *in vivo* tumor growth and *in vitro* proliferation, migration and invasion of lung adenocarcinoma cells by secreting oncostatin-M which is a cytokine that promotes differentiation (Wang et al., 2012). Their heterogeneity is the most likely explanation for different effects of MSCs on the tumor growth. It has thus been speculated that the origin of MSCs, passage number and culture conditions might create this difference. Another important factor might be the timing at which MSCs are introduced into the tumor microenvironment because when MSCs were introduced into the already established tumors, they had growth inhibitory effects.

However, in reports demonstrating tumor promoting roles of MSCs, they were always co-injected together with the tumor cells (Klopp et al., 2011).

It should be noted that most of these studies have been done either in vitro by co-culturing MSCs together with cancer cells or in vivo injecting in vitro cultured MSCs into tumor bearing mice. Therefore, they do not answer the question how bone marrow derived MSCs behave intrinsically when there is a solid tumor in the body. There have been two studies addressing this issue that are discussed in the following chapter.

1.3.4. MSCs as a source of CAFs

Cultured MSCs not only have morphological features similar to fibroblasts but also can be activated upon proper stimuli, produce ECM proteins and secrete various cytokines and chemokines (Kalluri, 2016). It was not clear until recently whether also MSCs contributed to CAF population in the tumor microenvironment. Initial studies have been reported that MSCs could gain activated phenotype in defined conditions and resemble CAFs. First of all, MSCs co-cultured with human colon cancer cells in the presence of TGFβ have been reported to express αSMA (Emura et al., 2000). Furthermore, when MSCs were exposed to tumor conditioned medium, they gain a CAF-like myofibroblastic phenotype such as expression myofibroblast markers and promoting tumor growth (Mishra et al., 2008). More recent *in vivo* studies investigating presence of endogenous bone marrow MSCs in the tumor microenvironment have finally proven the fact that MSCs indeed can migrate from bone marrow to the tumor microenvironment and contribute to CAF population. The first study carried out in an inflammation induced gastric cancer mouse model has demonstrated that 20% of CAFs in gastric cancer microenvironment have bone marrow origin (Quante et al., 2011). Similarly, also in a breast cancer mouse model, bone marrow derived MSCs have been shown as a substantial source of CAFs in the breast cancer microenvironment (Yaz et al., 2018).

1.4. Aims of the study

Part 1

The tumor microenvironment (TME) is now recognized as an important factor in breast cancer progression and significantly determines the response to anti-cancer therapies. Fibroblasts are activated in the early stages of oncogenesis and remodel the TME as an early tissue repair response and later these fibroblasts get transformed into cancer associated fibroblasts (CAFs) that have been reported to contribute breast cancer progression by different means. The mechanisms underlying this transformation of fibroblasts from normal activated fibroblasts to CAFs remain largely unknown. Although TGFβ and Wnt signaling pathways have both been associated with fibroblast activation, their role in this further transformation into CAFs is not clear. Gaining further insights into the activation of fibroblasts could offer a deeper understanding of their origin, plasticity and function within the tumor microenvironment. Furthermore, it creates opportunities to reverse this activation as a therapeutic option without depleting certain subpopulations of CAFs that might result in undesired effects on tumor progression.

Therefore, the aim of the first part of this PhD project was to understand how the changes in CAFs during the activation process are regulated at molecular level via

1. Treating bone marrow derived MSCs with conditioned media of MDA-MB-231 cells to induce a CAF-like activated fibroblast phenotype.
2. Performing gene expression and secretome analysis to find out differentially expressed genes and differentially secreted factors upon activation.
3. Identifying target genes that might have a role in the activation of fibroblasts.
4. Validating their effect on already activated patient derived CAFs.
5. Elucidating molecular mechanism underlying their effect on the activation of fibroblasts by profiling their impact on signaling pathways.

Part 2

Bone marrow MSCs have been identified as a substantial source of CAFs in breast cancer and reported to migrate from bone marrow to the breast TME. Bone marrow MSCs are a heterogeneous population consisting of stromal cells that support hematopoiesis and stem cells that could generate skeletal lineages. In the case of cancer, it is still not clear whether there is a preference for a specific subpopulation to be recruited to the tumor side and once there having been activated, to contribute to the CAF population. Moreover, the molecular mechanisms behind the activation of MSCs to become CAFs are a topic of ongoing research.

Therefore, the aim of the second part of this PhD project was to characterize MSCs migrating to the tumor microenvironment using a syngeneic mouse model via

1. Identifying the subpopulation(s) of bone marrow MSCs migrating to the breast cancer microenvironment by transplanting different stromal subsets into tibia of a breast tumor bearing mouse and monitoring their migration to the tumor microenvironment.
2. Profiling the changes in MSCs once they are in the tumor microenvironment by isolating MSCs from tibia or breast tumor to analyze the differences in their gene expression and DNA methylation levels.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Laboratory equipment

Aushon 2470 contact printer	Aushon Biosystems
Cell counter CASY	CASY, Roche
Cell culture incubator	Binder
Flow cytometry FACS Canto II	Becton Dickinson
Freezer (-20 °C)	Liebherr
Freezer (-80 °C)	Sanyo
Fridge	Liebherr
Laminar flow hood	HERA safe, Thermo Scientific
Light Microscope	Hund Wetzlar
Micro pipettes	Gilson
Microplate reader	Glomax Microplate Reader
Microvolume UV-Vis spectrophotometer	Nanodrop ND-1000, Thermo Scientific
Molecular Devices microscope IXM XLS	Molecular Devices
Near Infrared Scanner	Odyssey LI-COR Biosciences
PCR machine	Gene Amp PCR systems, Applied Biosciences
Pipetboy	Integra
Power supply	Consort
TaqMan reader	ABI Prism7900HT, Applied Biosystems
Table top centrifuge	Biofuge fresco, Heraeus
Vacuum aspirator	Laboport, KNF

Vortex mixer	Heidolph
Water bath	Julabo

2.1.2 Consumables

Adhesive Optically Clear Plate Seal	Thermo Fisher Scientific
Cell culture flasks (75 cm^2 and 175 cm^2)	Greiner Bio-one
Cell scraper	Corning
Cryo vials	Nunc, Thermo Fisher Scientific
Disposable Filtertips for micro pipettes	Starlab
Disposable tips for micro pipettes	Steinbrenner
Oncyte® Avid Nitrocellulose Film-Slide	Grace Bio-Labs
Optical 384ßwell plates for TaqMan	Applied Biosystems
PCR strips	Steinbrenner Laborsysteme GmbH
PVDF blotting membrane	Immobilon-P, millipore
RTCA CIM plates	Roche Diagnostics
Serological pipettes	BD Falcon
Trans-well system	Corning
1.5 ml microcentrifuge tubes	Eppendorf AG
2 ml microcentrifuge tubes	Eppendorf AG
15 ml conical tube	Becton Dickinson
50 ml conical tube	Becton Dickinson
6-well cell culture plate	Greiner Bio-one
96-well cell culture plate	Greiner Bio-one

96-well plate (ELISA)	Greiner Bio-one

2.1.3. Chemicals and reagents

BSA	PAA
CasyTon	Roche
Chloroform	Sigma-Aldrich
Cholera toxin	Sigma-Aldrich
Complete Mini Protease Inhibitor Cocktail	Roche
CytoPerm-CytoFix	Becton Dickinson
DMSO	Sigma-Aldrich
DMEM-F12 medium	Gibco
Ethanol	Sigma-Aldrich
Fast Green FCF	Carl Roth
Fetal Bovine Serum	Gibco
Hoechst 33258	Sigma
Lipofectamine RNAi max	Invitrogen
DMEM medium	Gibco
Methanol	Sigma-Aldrich
M-PER protein extraction reagent	Rockland
Nuclease-free water	Ambion
Opti-MEM	Gibco
PBS	Gibco
Pen/Strep	Gibco

Perm/Wash	Becton Dickinson
Proteinase K	Sigma-Aldrich
Rockland Blocking Buffer	Rockland Immunochemical Inc.
SDS	Carl Roth
Sodium fluoride (NaF)	Sigma-Aldrich
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich
TaqMan Fast Universal PCR Master Mix (2x)	Applied Biosystems
Trypsin-EDTA	Sigma-Aldrich
Tween 20	Sigma-Aldrich
Universal Probe Library	Roche

2.1.4. Solutions

Blocking buffer (ELISA)	PBS with 1% BSA
Blocking buffer (RPPA)	1:1 Rockland blocking buffer: TBS 1 mM Na_3VO_4 10 mMNaF
FCF staining solution	0.005% Fast Green FCF 10% acetic acid 30% ethanol
FCF destaining solution	10% acetic acid, 30% ethanol

Luciferase Buffer	40 mM Tricine 2 mMATP 10 mM $MgSO_4$ 0.5 mM EDTA 10 mM DTT 1 mM CoA 1 mM Luciferin
Protein Lysis Buffer	M-PER lysis buffer 1x Complete Mini Protease Inhibitor Cocktail 1x PhosSTOP Phosphatase Inhibitor Cocktail 1 mM Na_3VO_4 10 mMNaF.
Printing Buffer (RPPA)	10% glycerol 4% SDS 10 mM DTT 125 mM Tris–HCl pH 6.8
Staining buffer (FACS)	PBS with 2% FBS
Washing buffer (ELISA)	PBS with 0.25% Tween 20

2.1.5. Commercial kits

BCA Protein Assay Kit	Pierce
DNasey Blood and Tissue Kit	Qiagen
Human Stanniocalcin 1 DuoSet ELISA	R&D Systems
Lineage Cell Depletion Kit	MACS, Miltenyi
RNeasy Mini Kit	Qiagen
ReverdAid H Minus First Strand cDNA Synthesis Kit	Thermo Scientific
TaqMan	Applied Biosystems

2.1.6. Cells and growth media

Cell lines	Source	Derived from	Growth media
HCC-1937	ATCC CRL-2336	Breast ductal carcinoma	DMEM-F12 with 10% FBS and 1% PenStrep
HS578T	ATCC HTB-126	Breast carcinoma	
MCF7	ATCC HTB-22	Breast adenocarcinoma	
MDA-MB-231	ATCC HTB-26	Breast adenocarcinoma	
T47D	ATCC HTB-133	Breast ductal carcinoma	
Primary cells			
Mesenchymal Stromal Cells	LONZA	Isolated from bone marrow	
CAF1 cells	Patient material	Breast tumor	
CAF2 cells	Patient material	Breast tumor	

2.1.7. siRNAs

siRNA pools composed of four different siRNA sequences against a specific target gene were picked from the human siRNA library of siOnTargetPlus (Dharmacon)

Gene name	Catalogue number	Annotations	Target sequences
STC1 pool	LU-006477-00-0002	siSTC1	AAACGCACAUCCCAUGAGA GGGAAAAGCAUUCGUCAAA GUACAGCGCUGCUAAAUUU CAACAGAUACUAUAACAGA
ON-TARGETplus non- targeting pool	D-001810-10-20	siCTRL	UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA

2.1.8. TaqMan qRT-PCR primers and probes

Gene	Forward Primer	Reverse Primer	Probe number
IL-6	gatgagtacaaaagtcctgatcca	ctgcagccactggttctgt	40
IL-8	agacagcagagcacacaagc	atggttccttccggtggt	72
MMP1	gctaacctttgatgctataactacga	tttgtgcgcatgtagaatctcg	7
MMP2	tacgaccgcgacaagaagta	agttcccaccaacagtggac	29
MCP1	agtctctgccgcccttct	gtgactggggcattgattg	40
STC1	aagccatcactgaggtcgtc	caggcttcggacaagtctgt	44

2.1.9. Antibodies

2.1.9.1. FACS antibodies

Protein	Company/Catalogue number	Dilution
FAP-PE	RnD, FAB3715P-025	1:20
PE-isotype	RnD, FAB3715P-025	1:20
αSMA -eflour660	eBiosciences, 50-9760-82	1:1000
eflour660-isotype	eBioscineces, 50-4724-80	1:1000
CD45-PerCP	Biolegend, 103129	1:200
PerCP-isotype	Biolegend, 400629	1:200
Ter119-PerCP	Biolegend, 116225	1:200
CD31-APC	Biolegend, 102409	1:80
APC-isotype	Biolegend, 400511	1:80
PDGFRa-PeCy7	Biolegend, 135911	1:150
PECy7-isotype	Biolegend, 400557	1:150
CD51-PE	Biolegend,104105	1:20
PE-isotype	Biolegend, 400407	1:20

2.1.9.1. MACS depletion antibodies

Protein	Company/Catalogue number	Dilution
CD45	RnD, FAB3715P-025	1:400
CD31	RnD, FAB3715P-025	1:200
Ter119	eBiosciences, 50-9760-82	1:100

2.1.9.2. RPPA antibodies

Protein name	catalog #	Protein name	catalog #	Protein name	catalog #
CSNK1E	CST_12448	CSNK1E	CST_12448	Cdk2	Sigma C5223
Phospho-AMPKalpha_Thr172_40H9	CST 2535	Phospho-AMPKalpha_Thr172_40H9	CST 2535	Phospho-c-Raf_Ser259	CST 9421
p53_DO-1	sc 126	p53_DO-1	sc 126	Smad4	Millipore MAB1132
pak1	CST 2602	pak1	CST 2602	GSK-3alpha	CST 9338
beta-Catenin	CST 9562	beta-Catenin	CST 9562	APC	CST 2504
ERK5	CST3372	ERK5	CST3372	c-Myc	ab32072
Phospho-elF4B _Ser406	CST 5399	Phospho-elF4B _Ser406	CST 5399	Phospho-PRAS40_Thr246_C77D7	CST 2997
Caspase-9_Human Specific	CST 9502	Caspase-9_Human Specific	CST 9502	Phospho-PTEN_Ser380_Thr382-383_44A7	CST 9549
IkappaB-alpha	CST 9242	IkappaB-alpha	CST 9242	ADAM9_D64B5	CST 4151
MEK1	BD 610122	MEK1	BD 610122	Phospho-PKCalpha_Ser657	Millipore 06-822
p27_Kip1	BD 610241	p27_Kip1	BD 610241	ERK 2_6G11	sc 81458
PIK3CB	ab32569	PIK3CB	ab32569	Phospho-c-Jun_Ser73	CST 9164
Phospho-beta-Catenin_Ser675	CST 9567	Phospho-beta Catenin_Ser675	CST 9567	Axam	Sigma HPA029247
Phospho-MAP Kinase Kinase 1-2_MEK1-2_phosphoserine 217-220	CST 9154	Phospho-MAP Kinase 1-2	CST 9154	mTOR	CST 2972
Bcl-xL_54H6	CST 2764	Bcl-xL_54H6	CST 2764	PP2A B Subunit	CST 4953
Phospho-MEK1_Ser298	CST 9128	Phospho-MEK1_Ser298	CST 9128	S6 Ribosomal Protein_5G10	CST 2217
Met_L41G3	CST 3148	Met_L41G3	CST 3148	PKAalpha cat_C-20	sc 903
Rap1Alpha	Abcam ab124963	Rap1Alpha	Abcam ab124963	BRAF	CST 9434
LRP5_D80F2	CST 5731	LRP5_D80F2	CST 5731	P44/42	CST9102
Phospho-p38 MAPK_Thr180-Tyr182_3D7	CST 9215	Phospho-p38 MAPK	CST 9215	Caveolin 1	ab32577
Akt2_D6G4	CST 3063	Akt2_D6G4	CST 3063	AMPKalpha	CST 2532
Phospho-NF-kappaB p65_Ser536_93H1	CST 3033	Phospho-NF-kappaB p65_Ser536_93H1	CST 3033	TACE_D22H4	CST 6978
c-Raf	CST 9422	c-Raf	CST 9422	S6K1_phospho T421_S424_E135	ab 32525
MLCP	sc-7482	MLCP	sc-7482	PKCdelta_D10E2	CST 9616
Dishevelled 3_EP1991Y	ab 76081	Dishevelled 3_EP1991Y	ab 76081	Akt	BD 610860
c-Jun_60A8	CST 9165	c-Jun_60A8	CST 9165	p38 MAPK	CST9212
PDK1	CST 3062	PDK1	CST 3062	c-FOS	sc-7202
PP2A A Subunit	CST 2039	PP2A A Subunit	CST 2039	CSNK2A1	CST_2656
GRB2	CST 3972	PI3 Kinase p85 alpha_ep380y	ab 40755	ROCK2	atlas antibodies HPA007459
DKK1_4D4	WH0022943 M1	p70 S6 Kinase_49D7	CST 2708	Phospho-PDK1_Ser241	CST 3061
Phospho-mTOR_Ser 2448	CST 2971	CDH1	CST 4065	JNK1_2C6	CST3708

Crk	CST 3492	pSMAD3	CST 9520	Tuberin_TSC2_D57 A9	CST 3990
Jagged1_28H8	CST 2620	RBPSUH	CST 5442	Notch2_D67C8_XP	CST 4530
HER2/ErbB2_29D8	CST 2165	CBP_D6C5	CST7389	Snail	CST 3895
ILK	CST 3862	Phospho-MSK1_Ser376	CST 9591	DIAPH1	CST 5486
p70 S6 Kinase Phospho_pT421-pS424	Epitomics 1135-1	EGF Receptor	CST 2646	Jagged2_C23D2	CST 2210
Nicastrin	CST 5665	Src_32G6	CST 2123	Snail	CST 3879
HER4_ErbB4 Phospho_pY1162	Epitomics 2295-1	TCF7_EPR2035	ab 134127	Axin1_C7B12	CST 3323
pSMAD2	CST 3108	Smad6	CST 9519	Lunatic Fringe_D6V2V	CST66472
CDK7	CST 2090	Phospho-EGF Receptor_Tyr1086	CST 2220	Phospho-HER3-ErbB3_Tyr1289_21 D3	CST 4791
PEN2_D6G8	CST 8598	Phospholipase C gamma 1_M156	ab 41433	pSMAD2/3	sc-11769-R
NLK_EPR2012_3	ab 154200	Wnt16	ab 109437	LKB1_27D10	CST 3050
MSK2_D41A4_XP	CST 3679	Stat5_3H7	CST 9358	PLC Gamma I Phospho_pY771	Epitomics 2350-1
Gab3	CST 3232	Phospho-EGF Receptor_Tyr845	CST 2231	ErbB4	ab76303
Phospho-p44/42 MAPK_ERK1-2_Thr202-Tyr204	CST 4370	MEK7_EP1455Y	ab 52618	Phospho-Src Family_Tyr416	CST 2101
eIF4B	CST 3592	NF-kappaB p65	CST 3034	CTNNBIP1_EPR6697_2	ab 129011
p21_C-19	sc 397	Phospho-Stat3_Tyr705	CST 9131	pEGFR_Tyr1148	CST 4404
Phospho-S6 Ribosomal Protein_Ser240-244	CST 2215	IKKa	CST 11930	c-Cbl	CST 2747
Stat3	BD 610189	cyclin D1_M-20	CST 2926	NLK_EPR2012_2	ab 154199
CDK4_DCS156	CST 2906	rhoa	CST 2117	MSK2_D41A4_XP	CST 3679
pak2	ab76293	LRP6	ab 134146	Gab2	CST 3232
FAK	CST 3285	ERK1	RnD AF1575	Phospho-Src Family_Tyr417	CST 2101
Phospho-MEK1_Thr286	CST 9127	PI3 Kinase p110 beta_Y384	ab 32569	CTNNBIP1_EPR6697_3	ab 129012
4E-BP1_53H11	CST 9644	Phospho-cdc2_Tyr15	CST 9111	pEGFR_Tyr1149	CST 1090
CREB _48H2	CST 9197	Rac1-2-3	CST 2465	c-Cbl	CST 567
MEK4	ab 33912	casein kinase IIbeta_6D5 (CSNK2B)	sc 12739	pSMAD2	566415 Calbiochem
Ras, clone RAS10	Millipore 05-516	PI3 Kinase p110alpha_C73F8	CST 4249	MSK2_D41A4_XP	CST 3679
IGF-IR_clone JBW902	Millipore 05-656	SOS	CST 12409	Gab1	CST 3232
Paxillin	CST 2542	Smad3_C67H9	CST 9523		

2.1.10. Software

BD FACS DIVA	BD Biosciences
Graph Pad Prism 7.0	GraphPad Software, Inc.

Image J	NIH
Inkscape	Software Freedom Conservancy, Inc.
Odyssey 2.1	LI-COR
Roche UPL Design Center	Roche Diagnostics
SDS 2.2	Applied Biosystems
xCelligence Real Time Cell Analyzer Software 1.2	Roche Diagnostics

2.1.11. Databases

TCGA mRNA data	TCGA_BRCA_exp_HiSeqV2-2015-02-24
GSE14548	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14548
GSE35019	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35019
GSE9014	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9014
GSE5847	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE5847

2.2. Methods

2.2.1. Cell culture

2.2.1.1. Cultivation of cells

All breast cancer cell lines were obtained from ATCC. Cell lines were regularly authenticated by multiplex cell line authentication (Multiplexion GmbH, Friedrichshafen, Germany) and tested for mycoplasma contamination. Breast cancer cell lines used in co-culture experiments were modified by Ana Maia to express H2B tagged with yellow fluorescent protein. MSCs belonging to different donors were purchased from Lonza. CAFs were isolated from patient samples by Mireia Berdiell. All cells were incubated in a humidified incubator at 37 °C and 5% CO_2. Primary cells that are MSCs and CAFs were split when they reached to 80% confluency and medium was changed in every 3 to 4 days. They were used up to passage 9. Breast cancer cell lines were passaged every three to four days. To detach the cells, they were first washed with PBS and incubated with 0.25 % trypsin-EDTA. After detachment, trypsin was inactivated by the addition of full growth medium. Cells were counted using the CASY cell counter by counting 50µl of cell suspension diluted in 10ml CASYton. Cell were frozen in 1.5ml cryovials in an isopropanol bath at -80 °C using full growth media supplemented with 10% DMSO and additional 10% FBS. Frozen vials of cells were recovered by thawing quickly in a 37 °C water bath. Table 5 shows the cell numbers that were used in culture conditions and experimental conditions according to different flask types.

Table 5 Cell numbers in culture and experimental conditions

	Culture conditions	Experimental conditions
MSCs	75 cm^2 flasks: 4 $x10^5$	6 well plates: 5-8$x10^4$ 10 cm dishes: 1.6-2.5 $x10^5$ 96 well plates: 500-1000
CAFs	10 cm dishes: 2.5 $x10^5$-4 $x10^5$	6 well plates: 5-8$x10^4$ 96 well plates: 500-1000
Breast cancer cell lines	10 cm dishes: 2.5 $x10^5$-4 $x10^5$	10 cm dishes 96 well plates: 300-500

2.2.1.2. Generation of conditioned media of MDA-MB-231 cells and treatment of MSCs with conditioned media

$1x10^6$ MDA-MB-231 cells were seeded in 10 cm dishes in 10 ml DMEM-F12 supplemented with 1% FBS. Supernatant from the dishes was collected after 72 h and centrifuged in 1800 rpm and filtered using 0.25 uM filters to remove death and floating cells. Conditioned media was kept in -80 °C and thawed only once before treating MSCs. $1.6x10^5$ MSCs were seeded in 10 cm dishes and treated either with DMEM-F12 supplemented with 1% FBS or conditioned media of MDA-MB-231 cells for 3, 7 or 14 days. Media or conditioned media was changed every 3-4 days.

2.2.1.3. In-direct co-culture of MSCs and MDA-MB-231 cells using trans-well system

$2.5x10^5$ MSCs were seeded in the lower chamber of 10 cm trans-well dishes in 9 ml DMEM-F12 supplemented with 1% FBS. $4x10^4$ MDA-MB-231 cells were seeded in the upper chamber in 7 ml DMEM-F12 supplemented with 1% FBS. Trans-well were incubated for 3 or 7 days with a medium change every 3-4 days.

2.2.1.4. Transfection

All transfections were performed using Lipofectamine RNAi max (Invitrogen) transfection reagent according to the manufacturer's instructions. It is a cationic lipid-based method in which the positive charge of the liposome mediates the interaction between the nucleic acid and the negatively charged cell membrane, resulting in the endocytosis of the transfection complex (liposome/nucleic acid) into the cells (Life Technologies, Lipid transfection). Table 3 summarizes the amount of the Lipofectamine RNAi max and siRNAs used for the different size cell culture plates.

Table 6 Amount of Lipofectamine RNAi max and siRNAs used for transfections

	Lipofectamine RNAi max	Final concentration of siRNA
6-well plate	5 µl	30 nM
10 cm dish	20 µl	30 nM

2.2.2. Gene expression analysis

2.2.2.1. mRNA isolation and reverse transcription

mRNA was isolated using RNeasy Mini Kit of Qiagen according to manufacturer's protocol. RNA concentration and purity were measured by the NanoDrop-ND 1000 (Thermo Scientific). To synthesize the cDNAs, 500 ng total RNA was used and reverse transcription was performed using RevertAid H Minus First Strand cDNA Sythesis Kit. RNA was mixed with 1 µl of oligo dT-Primer to a volume of 12 µl. This mix was incubated for 5 min at 70 °C and mixed with 4 µl 5x reaction buffer, 1 µl RiboLock Ribonuclease Inhibitor (20 u/µl), 2 µl 10 mM dNTP mix and 1 µl Reverd Aid H Minus-MuIV RT (200u/ µl) to a final volume of 20 µl. This mix was incubated for 5 min 37 °C, followed by 60 min at 42 °C and 10 min at 70 °C.

2.2.2.2. Taqman qRT-PCR

To quantify specific genes, primers and probes for TaqMan qRT-PCR were designed using the Roche UPL Design Center. cDNA was diluted to 2 ng/µl and 5 µl of this dilution was mixed with 5.5 µl Abgene 2x mix, 0.11 µl left and right primers, 0.11 µl probe to a final volume of 11 µl. This mixed was incubated for 2 min 50 °C, followed by 15 min at 95 °C and 45 cycles of 15 sec at 95 °C and 60 sec 60 °C. The amplification and expression analysis were carried out using the ABI prism 7900HT sequence detection system using ΔΔCt method.

2.2.2.3. RNA Sequencing data alignment, differential analysis and pathway enrichment analysis

RNA sequencing (RNA-seq) was performed of MSCs treated either with media or conditioned media of MDA-MB-231 using Hiseq 4000 Paired-end 100 base pair technology by Genomics and Proteomics Core Facility of DKFZ. RNA sequencing analysis was performed by Naveed Ishak (Heidelberg Center for Personalised Oncology (DKFZ-HIPO)). RNA-seq reads were mapped the human reference genome (build 37, version hs37d5) using STAR (version 2.5.2b) [1] using a 2-pass alignment. Duplicate reads were marked using sambamba (version 0.6.5) [2] using 8 threads, and were sorted by position using SAMtools (version 1.6) [3]. BAM file indexes were generated using

sambamba. Quality control analysis was performed using the samtools flagstat command, and the rnaseqc tool (version v1.1.8.1) [4] with the 1000 genomes assembly and gencode 19 gene models. Depth of Coverage analysis for rnaseqc was turned off. featureCounts (version 1.5.1) [5] was used to perform gene specific read counting over exon features based on the gencode V19 gene model. The quality threshold was set to 255 (which indicates that STAR found a unique alignment). Strand unspecific counting was used. For total library abundance calculations, during FPKM/TPM expression values estimations, all genes on chromosomes X, Y, MT and rRNA and tRNA were omitted as they possibly introduce library size estimation biases.

Differential expression and expression fold change analysis were performed using DESeq2 (version 1.14.1) [6] and heat maps were visualized by ComplexHeatmap package (version 1.99.5) [7]. Genes with FDR < 0.05 were considered for further analysis.

All differentially expressed genes (Benjamini-Hochberg correct p-value < 0.05) with log2 fold change greater than 2 were used for KEGG enrichment analysis via the DAVID webtool. Default parameters were used and both the gene list and background were set to homo sapiens for all tests. Benjamini-Hochberg corrected modified Fischer's exact test (EASE score) p-values were used to determine if KEGG pathways were significantly enriched (Huang et al., 2009)

2.2.3. DNA methylation analysis

DNA methylation analysis was performed of MSCs treated either with media or conditioned media of MDA-MB-231 using Infinium MethylationEPIC Kit by Genomics and Proteomics Core Facility of DKFZ. Data analysis to find differentially methylated CpGs were carried out by Dr. Yassen Assenov (Post-doctoral researcher in the Division of Epigenomics and Cancer Risk Factors, DKFZ) using RnBeads (Assenov et al., 2014).

2.2.4. Protein expression analysis

2.2.4.1. Analysis of CAF markers by flow cytometry

Flow cytometry analysis was performed to measure the expression of FAP and αSMA at protein level. To this end, cells kept in different conditions collected by trypsinization and a single cell suspension was generated. $5x10^5$ cells per condition were pipetted into

different wells of a deep well plate and washed with staining buffer (PBS supplemented with 2% FBS). For surface staining, FAP antibody tagged with PE fluorophore (RnD) was used in a 1:2.5 dilution in 100 µl of staining buffer and cells were incubated on ice for 45 min. Next, cells were washed with 1x PermWash Buffer and incubated with Cytofix Cytoperm solution for 10 min on ice to fix and permeabilize the cells before intra-cellular staining. For intracellular staining αSMA antibody tagged with eFlour660 fluorophore was used in a 1:1000 dilution 100 µl of 1x PermWash buffer and cells were incubated on ice for 30 min. Cells were again washed with 1x PermWash Buffer and resuspended in 150 µl of 1x PermWash buffer. Cells were acquired with BD CantoII cell analyzer and analysis was carried out with BD FACS DIVA software.

2.2.4.2. Secretome analysis and intracellular protein analysis by mass spectrometry

Secretome analysis was performed by Gertjan Krammer (Post-Doctoral Scientist, Division of Proteomics of Stem Cells and Cancer) using previously established method (Eichelbaum et al., 2012) that enables detection of differentially secreted proteins that are newly produced in MSCs upon treatment with media or conditioned media of MDA-MB-231 cells. This method combines click chemistry and pulsed stable isotope labeling with amino acids in cell culture to selectively enrich and quantify secreted proteins. For this, cells are labeled with AHA that is incorporated into newly synthesized proteins and replaces methionine. Newly synthesized proteins are then coupled to alkyne-functionalized agarose resin by 1,3-cycloaddition (click reaction). To quantify secreted proteins another labeling with stable isotope-labeled amino acids (SILAC) is carried out. Secreted proteins can be then quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) after on-bead digestion and (optionally) peptide fractionation. Labeling with AHA and SILAC was carried out in MSCs treated with media or conditioned media of MDA-MB-231 cells for 7 or 14 days. While, MSCs treated with media were labeled with intermediate isotype labeled arginine and lysine (intermediate channel), MSCs treated with conditioned media were labeled with heavy isotype labeled arginine and lysine (heavy channel) making possible to quantify differentially secreted proteins among these two conditions. For this, $1.6x10^5$ MSCs seeded in 10 cm dishes were treated with media or conditioned media of MDA-MB-231 cells. At the end of the treatment, MSCs were washed two times by PBS

and incubated with depletion media for 30 min. Next, labeling media was pipetted on the cells and cells were incubated for 6 hours. At the end of incubation time, supernatants were collected and centrifuged in 1800 RPMI for 5 min to remove cell debris and death cells. Labeling of the cells were done by me and following sample preparation, measurement and analysis were carried out by Gertjan Krammer. Together with supernatants, cell pellets were also collected and analysis of differentially expressed intracellular proteins was carried out using the same method.

2.2.4.3. Detection of STC1 by ELISA

Enzyme linked immunosorbent assay (ELISA) is an antigen-antibody interaction based technique used to detect and quantify a specific protein in a solution. In this study, it was performed to quantify the amount of STC1 in the supernatants of MSCs using Human Stanniocalcin 1 DuoSet ELISA kit from R&D Systems according to the manufacturer's protocol. Briefly, 96 well plate was coated with a capture antibody against STC1 and incubated over night at room temperature. The next day, blocking was done using PBS with 1% BSA for 2h at room temperature. The standards and supernatants diluted in the blocking buffer were applied to the wells and incubated for 2h at room temperature. For the detection, the wells were incubated first with a biotinylated detection antibody for 2h and then with Streptavidin-HRP for 20 min. Afterwards, the wells were incubated with a substrate solution of HRP and reaction was stopped by adding stop solution after 20 min. The optical density was detected immediately ay 450 nm using Glomax Microplate reader. Standard curve was generated using the absorbance value of standards and absolute concentration of STC1 in the supernatants of MSCs was calculated.

2.2.4.3. Cell lysis, protein extraction and quantification

To harvest the cells, the media was removed and the cells were washed with PBS. Protein lysis buffer was added and the cells were detached using a cell scraper. Cells were incubated in the lysis buffer for 20 min and proteins were separated from the cell debris by centrifuging the samples at 13000 rpm for 5 min. Protein concentration was determined using the BCA Protein Assay Kit according to the manufacturer's instructions.

2.2.4.4. RPPA

Reverse Phase Protein Array (RPPA) was performed by Simone Borgoni (Phd student, Molecular Genome Analysis, DKFZ) using protein lysates of CAF1 cells transfected either with control siRNA or siRNA targeting STC1 mRNA. RPPA is (Paweletz et al., 2001). To this end, cell lysates were adjusted to a total protein concentration of 2 µg/µl. Samples were mixed with 4 x RPPA printing buffer and denatured at 95°C for 5min. The lysates were pipetted into 348-well plates and centrifuged for 2 min at 200 x g. As internal controls a dilution series of All samples were printed as technical triplicates on Oncyte Avid Nitrocellulose Film-Slides using an Aushon 2470 contact printer with 185 µm solid pins. 1.6 nl sample per spot was printed with an average spot diameter 250 µm. After the printing, slides were kept at -20°C. Before staining, the slides were blocked for 2 h at room temperature with blocking buffer. After blocking, the slides were incubated with primary antibodies at 4°C overnight. After incubation the slides were washed for 4 times for 5min with TBST and subsequently incubated with Alexa Fluor® 680 F(ab')2 fragments of goat anti-mouse IgG or anti-rabbit IgG in 1:12000 dilution for 1h at RT in dark. After 4 more washing round with TBST, the slides were washed 2 more times for 5 min with ultra-pure water. Afterwards, the slides were dried and scanned with the Odyssey® Infrared Imaging System with an excitation wavelength of 685nm and a resolution of 21 µm. Total protein quantification done using Fast Green FCF protein dye was used for normalization.

Signal intensities of individual spots were quantified using GenePixPro 7.0 software. The TIFF image of each slide and gene pix array list file (generated by the printer to map the sample location on the slide) was matched into a gene pix result file. A visual inspection of each spot was performed and slides without uniform background signal were excluded from further analysis. RPPA raw data preprocessing and quality control were performed using the RPPanalyzer R-package (Mannsperger et al., 2010). The raw signal intensities of the control samples were plotted against the respective total protein concentration. Only data of antibodies showing a linear correlation between target signal intensity and protein concentration were used for further analysis. Afterwards, target signals were normalized to the total protein amount per spot via Fast Green FCF control. After median calculation of technical replicates, normalized target signal intensities were plotted against the signal intensities obtained by incubation of primary antibody controls (blank signal). Machine

running, raw data processing and data generation was done by Simone Borgoni and antibody incubation and imaging of slides were done by Corinna Becki (Research Technician, Division of Molecular Genome Analysis, DKFZ).

2.2.5. Functional assays

2.2.5.1. Collagen gel contraction assay

Collagen contractility, a characteristic of activated fibroblasts, can be evaluated by Collagen Gel Contraction Assay. Collagen gel contraction assay was performed according to a previously published protocol (Su & Chen, 2015). Briefly, cells kept in different conditions collected and the concentration was adjusted into 1.5×10^5 cells/mL in DMEM-F12 supplemented with 1% FBS. 400 µl of cell suspension was pipetted into 24 well plates and mixed with 200 µl of Collagen type I isolated from rat tail (Thermo scientific). 7 µl of 1 M NaOH was added in each well immediately and mixed well. The mix was incubated at room temperature for 20 min until solidified and 600 µl of DMEM-F12 supplemented with 1% FBS was added into each well and the collagen gel was dissociated by gently running the tip of a 200 µL pipet tip along gel edges. The plates were incubated in a humidified incubator at 37 °C and 5% CO_2 for 24 to 72h and images was taken using a scanner. Gel contraction that is proportional to the area of the gel was measured using the Image J software.

2.2.5.2. Cell proliferation

Analysis of proliferation of MSCs by Hoechst staining

Hoechst as a fluorescent dye can bind to DNA. Total nuclei count can be determined by fluorescence microscopy after staining the cells with Hoechst. In this study, different cell types were stained with Hoechst -33258 to analyze the changes in their proliferation rate upon different conditions. To this end, cells seeded on 96 well plates were stained with Hoechst-33258 in 1:5000 dilution in media and incubated at 37c for 30 min. Fluorescent images were taken using the Molecular Devices microscope IXM XSL and nuclei counting was performed using Molecular Devices Software. The number obtained was considered as cell number. MSCs treated with media or conditioned media of MDA-MB-231 cells for

3 or 7 days were seeded in 96 well plates (750 cells per well) and after 3 days incubation nuclei counting was performed.

Analysis of proliferation of breast cancer cells

Proliferation analysis was performed using breast cancer cell lines, that were either directly co-cultured with MSCs or treated with conditioned media of MSCs or directly co-cultured with CAF1 cells. For direct co-culture experiments, MSCs or CAF1 cells were seeded in 96 well plates (500 cells per well) and next day breast cancer cells that express H2B tagged with YFP were seeded on top in DMEM-F12 supplemented with 1% FBS (500 cells per well). Fluorescent images were taken using the Molecular Devices microscope IXM XSL every day for 5 days. For conditioned media treatment experiments, breast cancer cells were seeded in 96 well plates (1×10^3 cells per well) and next day conditioned media treatment was started. Fluorescent images were taken using the Molecular Devices microscope IXM XSL every day for 5 days. Having YFP tagged H2B expression only in breast cancer cells made it possible to follow the growth of only these cells.

2.2.5.3. RTCA based migration assays

Migration ability of MSCs treated with media or conditioned media was measured by using real time cell analyzer (RTCA) system. To this end $2x10^4$ MSCs were seeded in upper chamber of trans-well RTCA plates in 100 µl DMEM-F12 supplemented with 1% FBS. As chemoattractant 175 µl of DMEM-F12 supplemented with 10% FBS was used in the lower chamber. When cells migrate from the upper chamber of the trans-well towards to the lower chamber, they first attached to the bottom part of the trans-well which is covered by electrodes. RTCA machine measures the changes in impedance as a result of changing cell number and this value is proportional to the number of cells that are attached to the bottom part of the trans-well. Migration was monitored real time for 30 hours.

2.2.5.4. Trans-well migration and invasion assay

$4x10^4$ CAF1 cells transfected with control siRNA or siRNA against STC1 were seeded on the upper chamber of a 24 well plate format trans-wells in 200 µl DMEM-F12 supplemented with 1% FBS. As chemoattractant 500 µl DMEM-F12 supplemented with

10% FBS was used in the lower chamber. Migration was stopped by fixing cells using 4% PFA after 16 h and crystal violet staining was used to stain the cells fixed on the bottom of the trans-wells. Images acquired using a fluorescent microscope.

2.2.6. *In vivo* experiments

All in vivo experiments were done under the animal experiment project number of 133/2015. Isolation and characterization of bone marrow stromal cells by flow cytometry analysis were carried out by me. FACS analysis was carried out in Imaging and Cytometry Core Facility of DKFZ. Injection of bone marrow stromal cells into mice and in vivo imaging were carried out by the group of Dr. Karin Müller-Decker (Tumor Models, DKFZ).

2.2.6.1. Isolation of bone marrow stromal cells

Femur and tibia were collected from wild type 6-8 weeks old BALB/C mice that were sacrificed by cervical dislocation. Either wild type BALB/C mice or L2G85.BALB/c mice that harbor the CAG-luc-eGFP L2G85 transgene exhibiting widespread expression of firefly luciferase directed by the CAG promoter were used for isolating bone marrow stromal cells. After removing tissue around the bones, bones were crushed on ice in DMEM-F12 supplemented with 10% FBS. Total bone marrow that was released into the media was pelleted by centrifugation in 1600 rpm for 5 min and washed with PBS several times. Cell numbers were determined by using a cell counter. Total bone marrow either directly cultured in 10 cm cell culture dishes or flow cytometry analysis was performed for further characterization.

2.2.6.2. Characterization of bone marrow stromal cells and cell sorting

Surface staining was carried out with freshly isolated total bone marrow by using CD31-APC, CD45-PerCP, Ter119-PerCP, PDGFRa-PeCy7, CD51-PE antibodies as previously described (Chapter 2.2.4.1.). CD45, CD31, Ter199 positive cells were gated out to eliminate lymphocytes, endothelial cells and erythrocytes respectively. Remaining bone marrow stromal cells were further characterized according to their CD51 and PDFGa expression and double positive cells were studies as rare mesenchymal stem cell

population. Bone marrow stromal cell population cell ($CD45^-CD31^-Ter119^-$), rare mesenchymal stem cell population ($CD45^-CD31^-CD51^+PDGFRa^+$), or bone marrow stromal cell population without rare mesenchymal stem cell population ($CD45^-CD31^-CD51^-PDGFRa^-$) were sorted using FACSAria II in Imaging and Cytometry Core Facility of DKFZ.

2.2.6.3. Enrichment of bone marrow stromal cells by magnetic cell sorting

Magnetic cell sorting (MACS) was used to enrich stromal cells in whole bone marrow population. To deplete the blood cells in bone marrow, Lineage Cell Depletion Kit of MACS, Miltenyi was used according to manufacturer's protocol. Another method that depletes just lymphocytes was also carried out suing CD45 beads of MACS, Miltenyi according to manufacturer's protocol. However, the highest enrichment was obtained using a homemade antibody cocktail. To this end, total bone marrow cells were stained with biotinylated antibodies against CD45, CD31 and Ter119 diluted in PBS supplemented with 2% FBS for 30 min on ice. Afterwards, cells were washed and incubated with Streptavidin beads (MACS, Miltenyi) for 15 min on ice and magnetic sorting was carried out according to manufacturer's protocol.

2.2.6.4. Luciferase Assay

Luciferase activity was measured in bone marrow stromal cells isolated either from wild type BALB/C mice or L2G85.BALB/c mice. For this $3x10^3$ cells were seeded in white 96 well plates. MCF7 cells transfected with a luciferase expressing plasmid was used as control. Next day, cells were lysed and treated with the luciferase buffer that contains substrate luciferin. Luciferase activity was measured after 3 min incubation using an Infinite M200 microplate reader.

2.2.7. Graphical Illustrations

Unless otherwise mentioned, data are presented as mean ± SD and statistical analyses were performed by unpaired two-tailed Student's t-test using GraphPad Prism Version 7.0. and p-values <0.05 were considered statistically significant. p-values <0.05, <0.01 and <0.001 are indicated with one, two and three asterisks respectively.

2.2.8. Statistical analysis

All graphs were generated using the GraphPad Prism Software and illustrated via Inkscape v 0.91

3. RESULTS

3.1. Part 1

3.1.1 Activation of MSCs with conditioned media treatment or with indirect co-culture

To study the effect of breast cancer cells on the activation of MSCs, a triple negative breast cancer cell line, namely MDA-MB-231, was selected as the model system. MSCs were either co-cultured in an indirect trans-well (TW) setting with MDA-MB-231 cells or treated with the conditioned media of these cells for 3 or 7 days to find out the best condition in which MSCs become activated. To assess the activation, a collagen gel contraction assay was carried out. Figure 3a shows the contraction of MSCs embedded in the collagen matrix in different conditions that was quantified by measuring the gel area (Figure 3b). Only after conditioned media treatment for 7 days, MSCs were found to be contracting more than the control cells. The TW setting, in which MSCs were either co-cultured with MDA-MB-231 cells or media, did not induce any difference in the contraction ability of the cells, even after seven days.

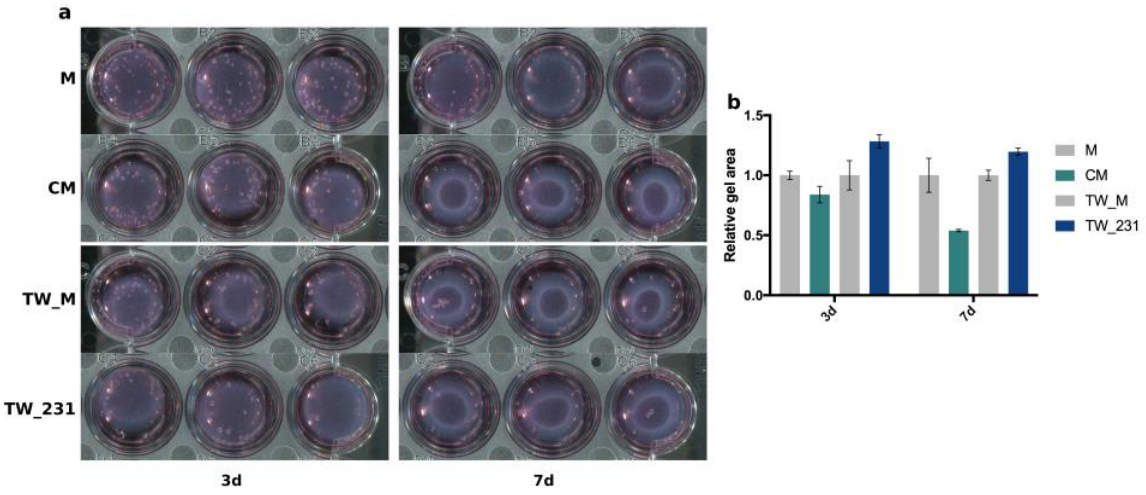

Figure 3 Effect of CM treatment or TW co-culture on the contractility of MSCs. a. Collagen gel contraction assay with MSCs either treated with media (M) or conditioned media (CM) for 3 or 7 days (d) or co-cultured with MDA-MB-231 cells in a trans-well (TW_231) format or cultured with media (TW_M) for 3 or 7 days **b.** Quantification of collagen gel area showing the fold changes relative to respective media samples and the values are presented as mean ± SD of 3 technical replicates.

Another way to check the activation of MSCs is to measure the changes in the expression levels of activation markers such as αSMA and FAP. Indeed, these markers were detected at the protein level upon treatment with either conditioned media or in the TW co-culture setting (Figure 4). While TW co-culture increased the expression of FAP after 7 days, αSMA levels went down both after 3 and 7 days (Figure 4a). In the conditioned media setting, however, FAP was already upregulated after 7 days of treatment and remained up after 14 days. αSMA expression was upregulated after 14 days of conditioned media treatment that might imply a gradual increase in the activation of MSC. In conclusion, the conditioned media setting activated the MSC more than the TW setting did. Therefore, I decided to use the conditioned media of MDA-MB-231 cells to activate MSCs. It should be noted at this point that even the media-treated MSC population had cells expressing FAP and αSMA demonstrating that these cells already had a baseline activation.

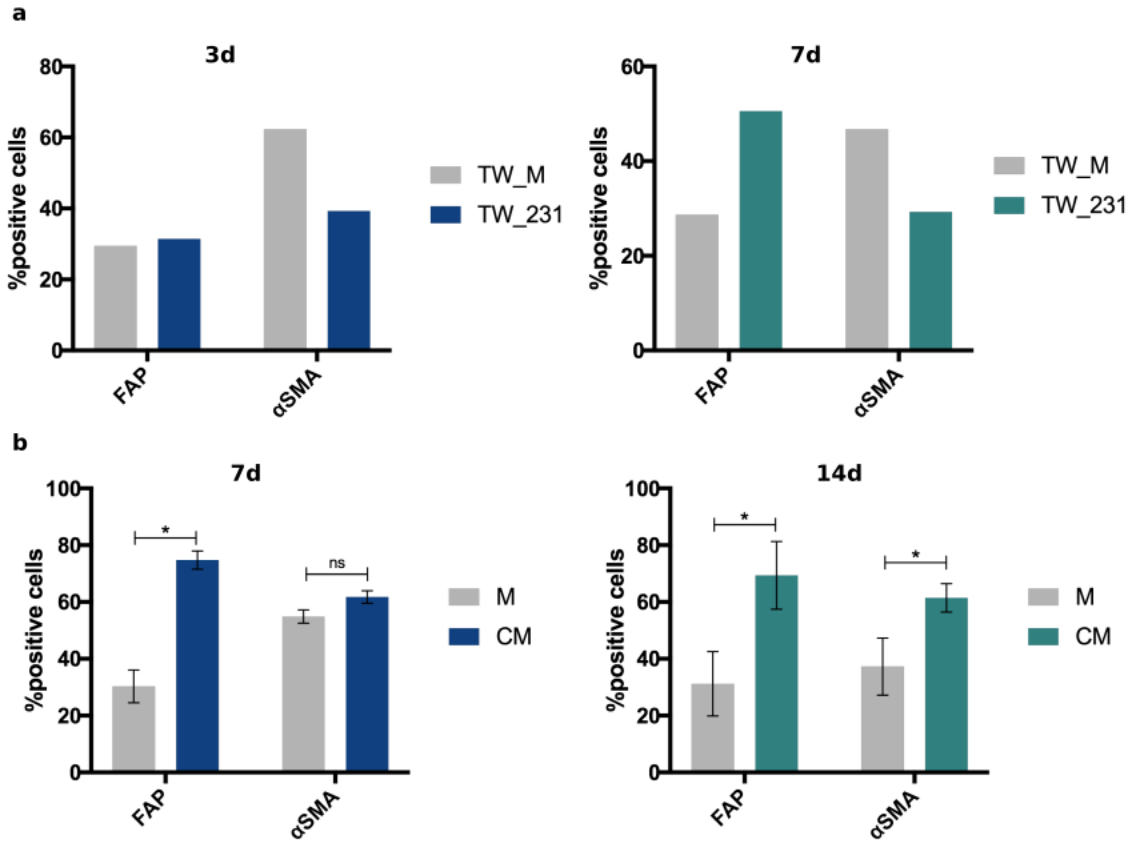

Figure 4 Effect of CM treatment or TW co-culture on the expression of αSMA and FAP Flow cytometry analysis of MSCs either **a.** co-cultured with MDA-MB-231 cells in a trans-well (TW_231) format or **b**. treated with media M or CM for 7 or 14 days. The graphs demonstrate the percentage of positive cells for FAP or αSMA expression (a-b) and the values are presented as mean ± SD of 3 biological replicates (b). * $p < 0.05$, ns: not significant

3.1.2. Activation of MSCs with prolonged CM treatment

To understand whether an extended treatment with conditioned media would have an additive effect on the activation of MSCs, conditioned media treatment was continued to 21 days. Then, a collagen gel contraction assay was performed to measure the activity of the cells. An increase in the contractility of MSCs was observed both at 7 and 14 days of treatment with conditioned media (Figure 5a-b). However, this difference was not significant after 21 days of conditioned media treatment. Hence, I chose to continue experiments using conditioned media treatment taking the 7 and 14 days time points.

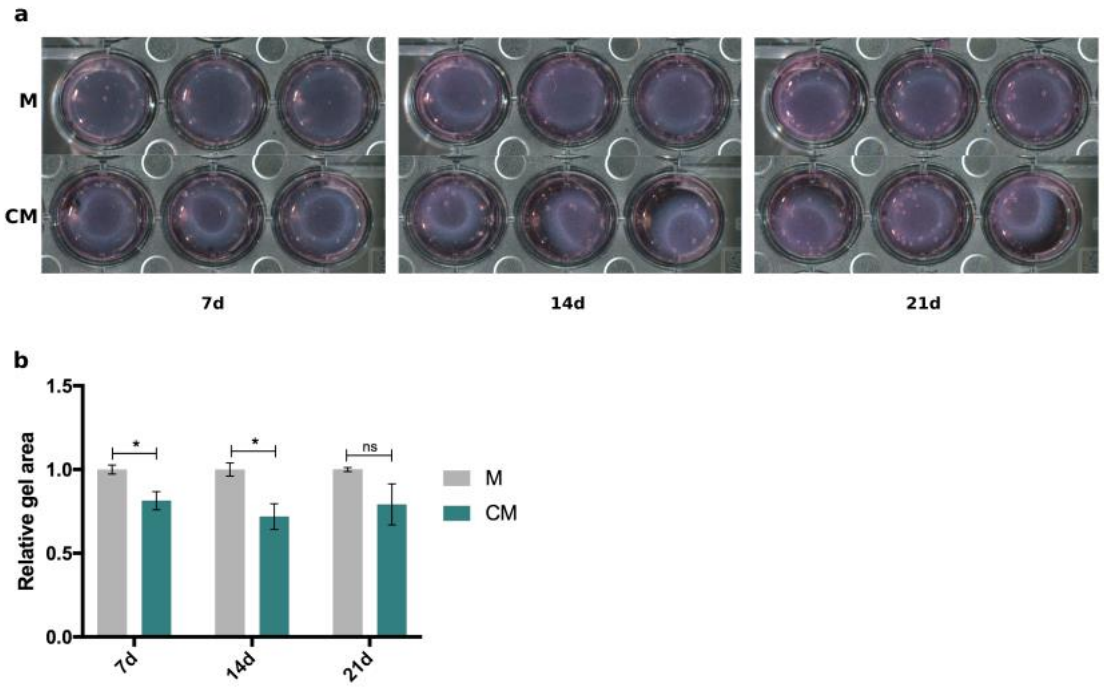

Figure 5 Effect of prolonged CM treatment on the contractility of MSCs Collagen gel contraction assay with MSCs either treated with M or CM for 3, 7 or 21 days **b.** Quantification of collagen gel area showing the fold changes relative to respective media samples and the values are presented as mean ± SD of 3 technical replicates. * $p < 0.05$, ns: not significant

3.1.3. Expression of cytokines upon CM treatment

Upon activation, fibroblasts start secreting various factors, including cytokines and chemokines. Hence, I next wanted to know which cytokines were regulated in my experimental system. To this end, MSCs were treated with conditioned media from MDA-MB-231 cells for 7 and 14 days, and the expression of selected cytokines and other relevant genes was tested. Indeed, the expression of *IL6*, *IL8*, *CCL2*, *CCL5* and *MCP1* expression

was upregulated at mRNA level both after 7 and 14 days. Out of two MMPs that were measured, while *MMP1* was upregulated, the expression of *MMP2* did not change (Figure 6).

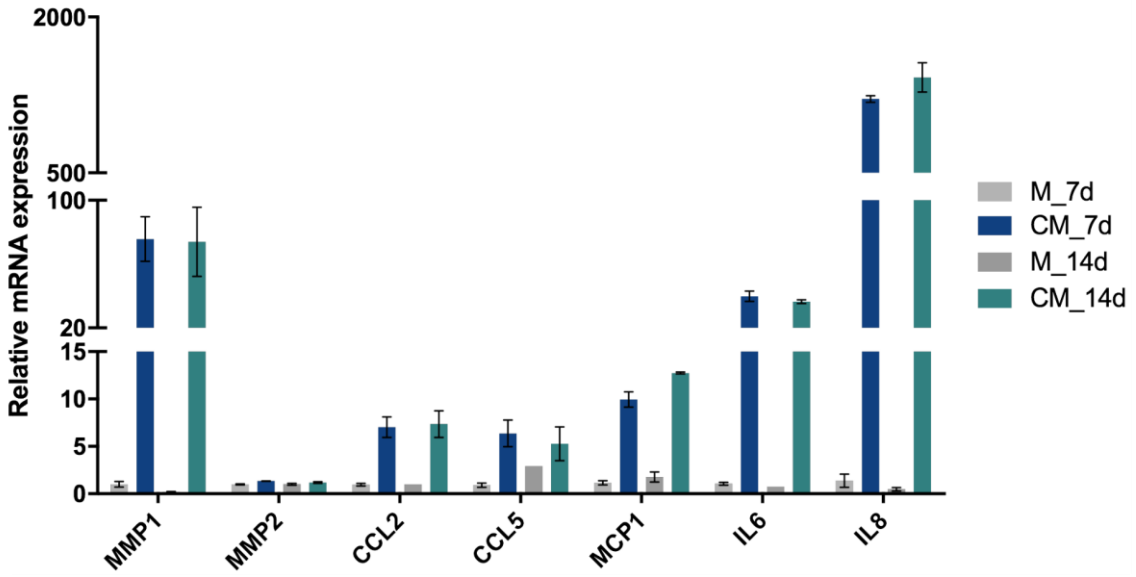

Figure 6 Effect of CM treatment on the expression of various cytokines Expression analysis of various cytokines in MSCs treated with media M or CM for 7 or 14 days using TaqMan qRT-PCR. The graph shows the fold changes relative to 7d M sample and the values are presented as mean ± SD of 3 biological replicates.

3.1.4. Phenotypic changes in MSCs upon activation with CM

Next, I investigated possible phenotypic changes that can be induced in MSCs with the CM treatment. Therefore, changes in the proliferation and migration ability of the cells were measured as increased proliferation and migration are associated with activated fibroblasts and CAFs. For this, a proliferation assay by nuclei count and a real time migration assay in trans-well format were performed. There was no change in the number of cells counted upon conditioned media treatment that might indicate the lack of effect of conditioned media on the doubling time of MSCs (Figure 7a). However, an increase was observed in the number of migrating cells only after 14 days of conditioned media treatment which is in line with the probable gradual activation of MSCs upon 7 and 14 days of treatment (Figure 7).

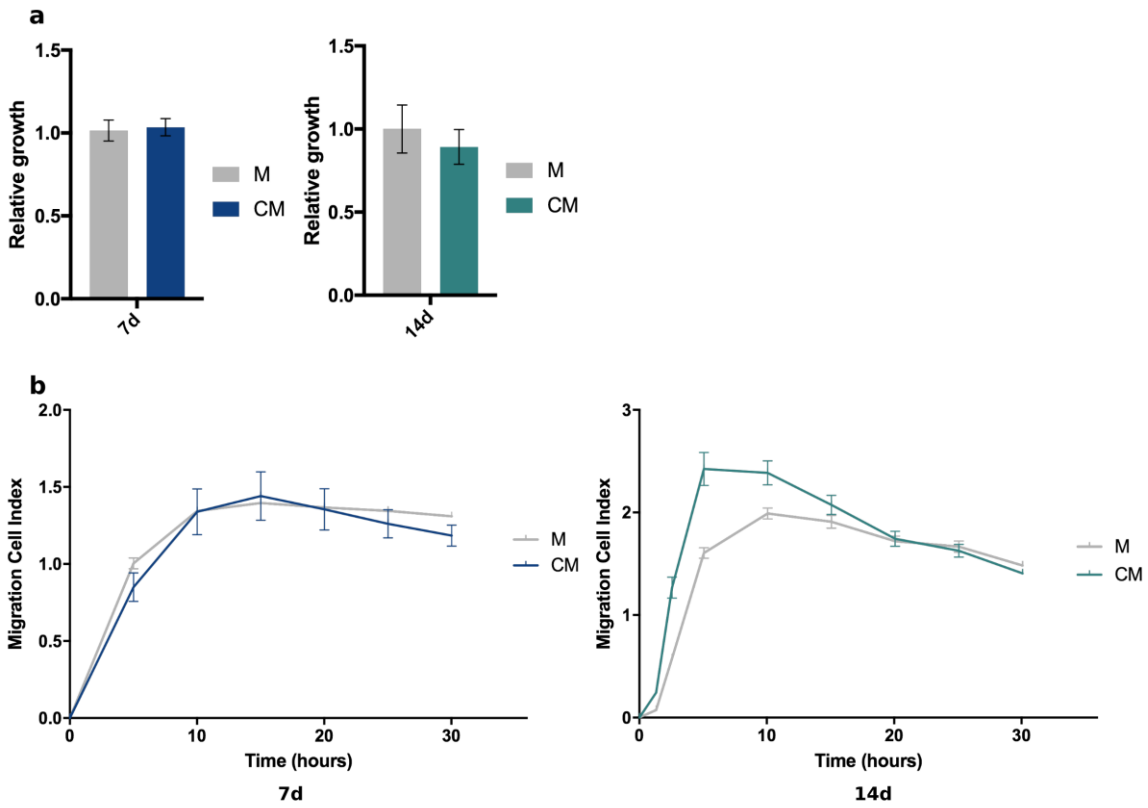

Figure 7 Effect of CM treatment on proliferation and migration a. MSCs were treated with media M or CM for 7 or 14 days and the changes in proliferation was measured by counting nuclei. The graphs demonstrate the fold changes in the growth of the cells relative to M control and the values are presented as mean ± SD of 6 technical replicates. **b.** TW migration assay using RTCA. Data for each time point is presented as mean ± SD of 4 technical replicates.

3.1.5. Impact of activated MSCs on cancer cell growth

The effect of CAFs on the tumor progression is well known (Kalluri, 2016). In order to investigate the influence of MSCs on the cancer cell growth, I directly co-cultured MSCs with various breast cancer cell lines and measured the changes in their growth. Figure 8 and 9 show the growth curves of the cancer cell lines that were co-cultured with media or conditioned media treated MSCs or mono-cultured for 7 (Figure 8) or 14 days (Figure 9). While growth of the two triple negative breast cancer cell lines, MDA-MB-231 and HCC1937 was not affected by the presence of MSCs, another triple negative cell line, namely HS578T, proliferated more in the co-culture setting with a higher proliferation with CM treated MSCs compared to media treated MSCs. The luminal cell lines, MCF7 and T47D also proliferated more in the co-culture with MSCs but there were no additive effects coming from the CM treated MSCs.

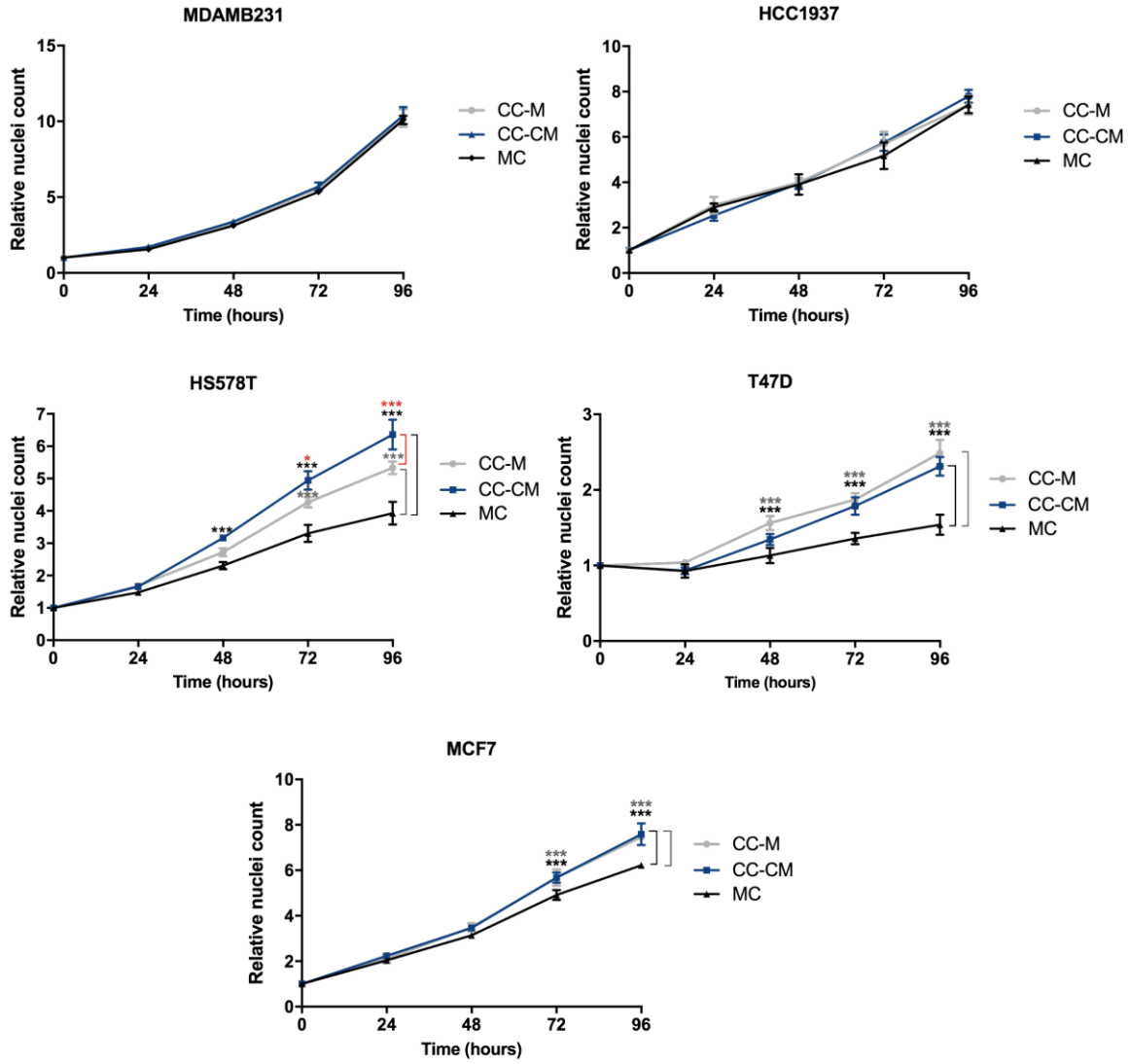

Figure 8 Effect of 7 days CM treated MSCs on the growth of breast cancer cell lines Breast cancer cell lines were co-cultured with 7 day M treated (CC-M) or CM treated (CC-CM) MSCs or mono-cultured (MC). The graphs demonstrate the growth of breast cancer cell lines as fold change relative to mono culture over time and the values are presented as mean ± SD of 2 biological replicates with 6 technical replicates each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

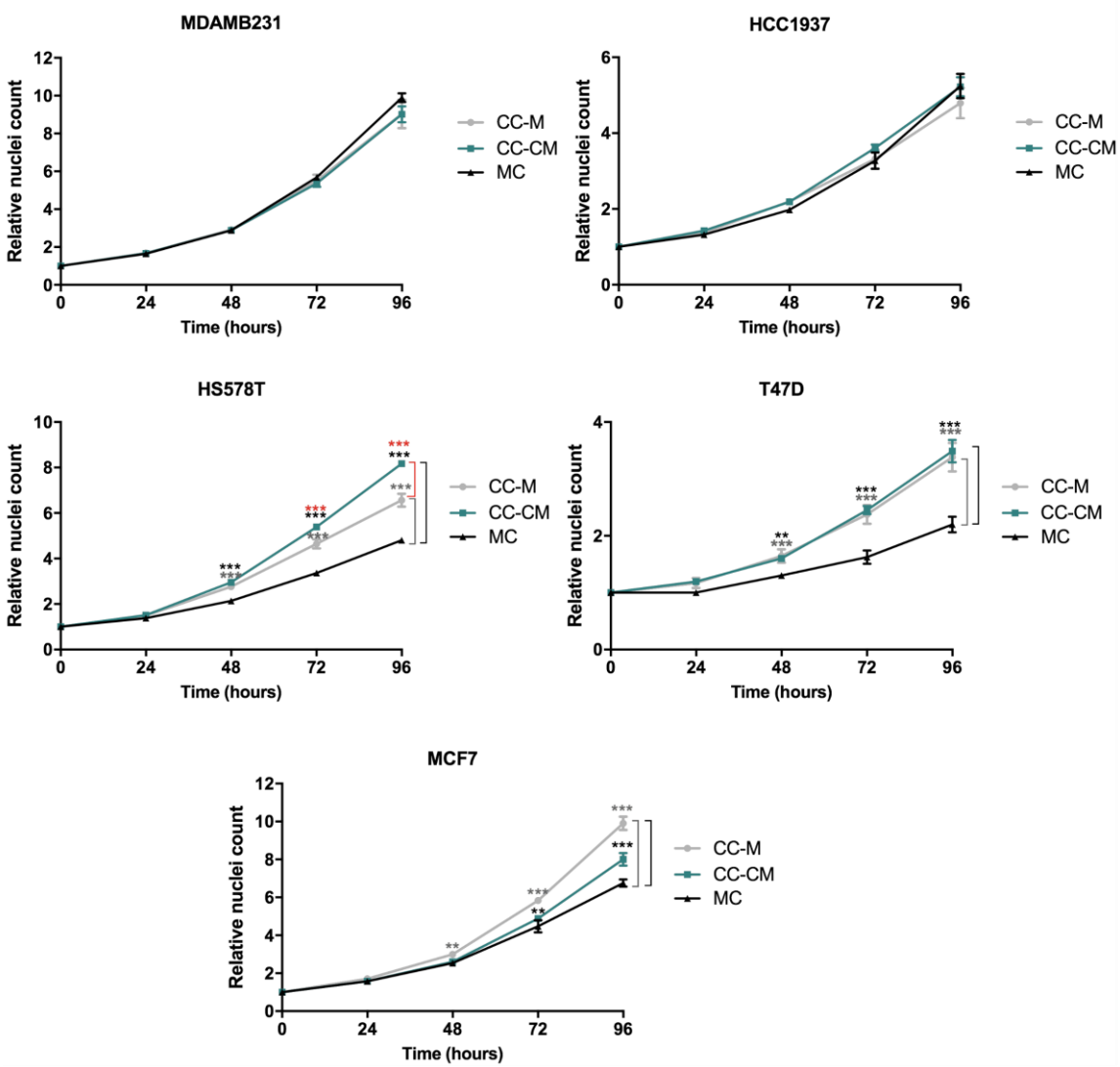

Figure 9 Effect of 14 days CM treated MSCs on the growth of breast cancer cell lines Breast cancer cell lines co-cultured with 14 day M treated (CC-M) or CM treated (CC-CM) MSCs or mono-cultured (MC). The graphs demonstrate the growth of breast cancer cell lines as fold change relative to mono culture over time and the values are presented as mean ± SD of 2 biological replicates with 6 technical replicates each. * p< 0.05, ** p< 0.01, *** p< 0.001

Next, in order to understand the mechanism behind this differential impact of MSCs on the growth of different breast cancer cell lines, the same experiment was repeated this time using the conditioned media of media or conditioned media treated MSCs to understand whether the observed effects were due to direct cell-cell contact or paracrine factors (Figure 10 and 11). Although HS578T cells grew more when they were exposed to the conditioned media of MSCs compared to the mono-culture control, there were no growth difference between the HS578T cells treated with the conditioned media of media and conditioned

media of conditioned media treated MSCs. Therefore, it might be concluded that direct cell-cell contact is required for the increased proliferation when HS578T cell were co-cultured with the conditioned media treated MSCs. Moreover, there was a small difference in the proliferation of MDA-MB-231 cells growing in mono-culture or treated with the conditioned media of MSCs that was not observed with the direct co-culture setup. This difference might be explained by the increased abundance and accumulation of secreted proteins in the conditioned media compared to more diluted co-culture condition.

All in all, although it was observed that MSCs, in general, have an impact on the growth of cancer cell lines, only HS578T cells grew better in the co-culture with the conditioned media treated and activated MSCs.

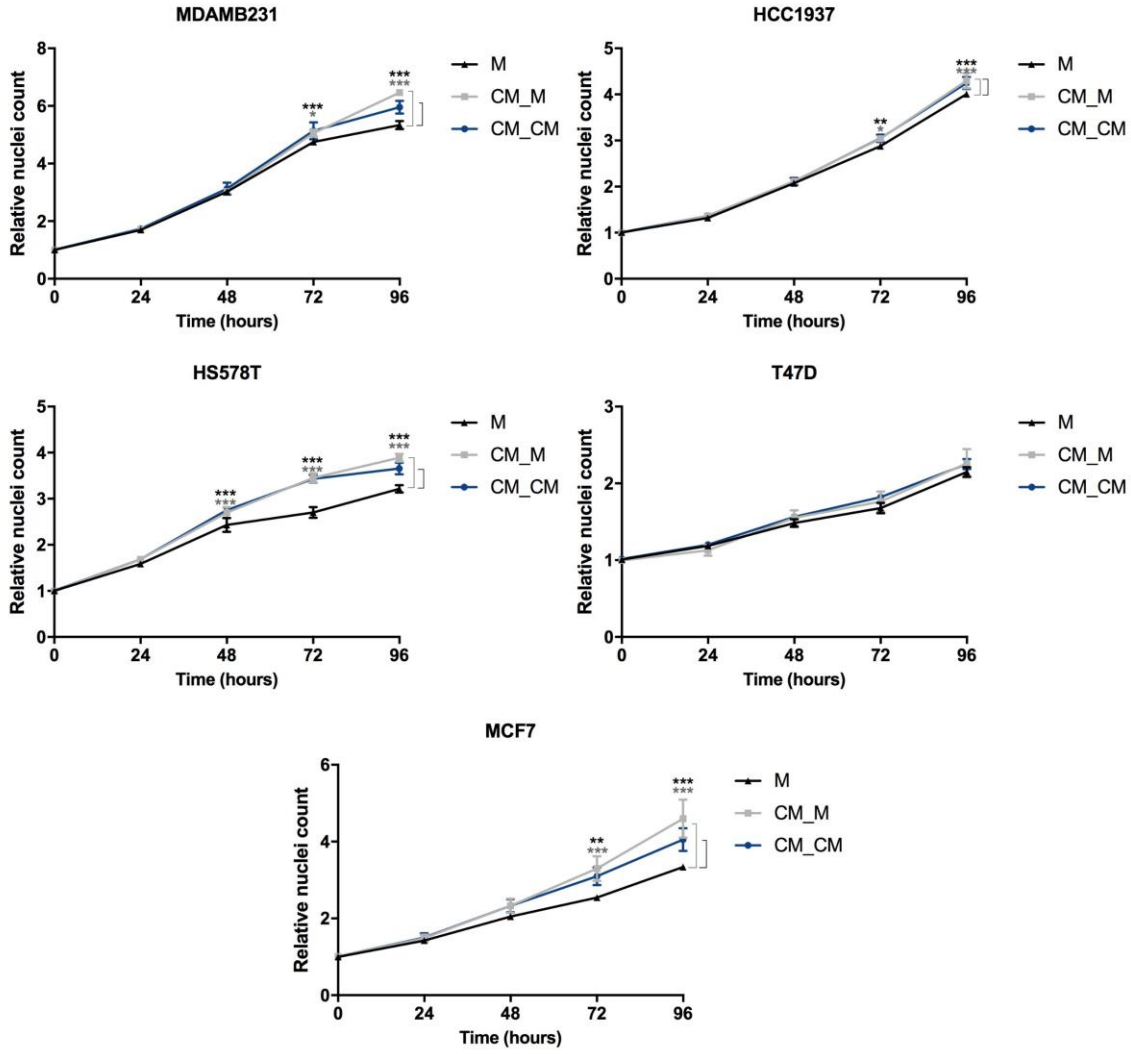

Figure 10 Effect of conditioned media of 7 days CM treated MSCs on the growth of breast cancer cell lines Breast cancer cell lines are treated with conditioned media of 7 day M treated (CM-M) or CM treated (CM-CM) MSCs or mono-cultured (MC). The graphs demonstrate the growth of breast cancer cell lines as fold change relative to mono culture over time and the values are presented as mean ± SD of 2 biological replicates with 6 technical replicates each. * p< 0.05, ** p< 0.01, *** p< 0.001

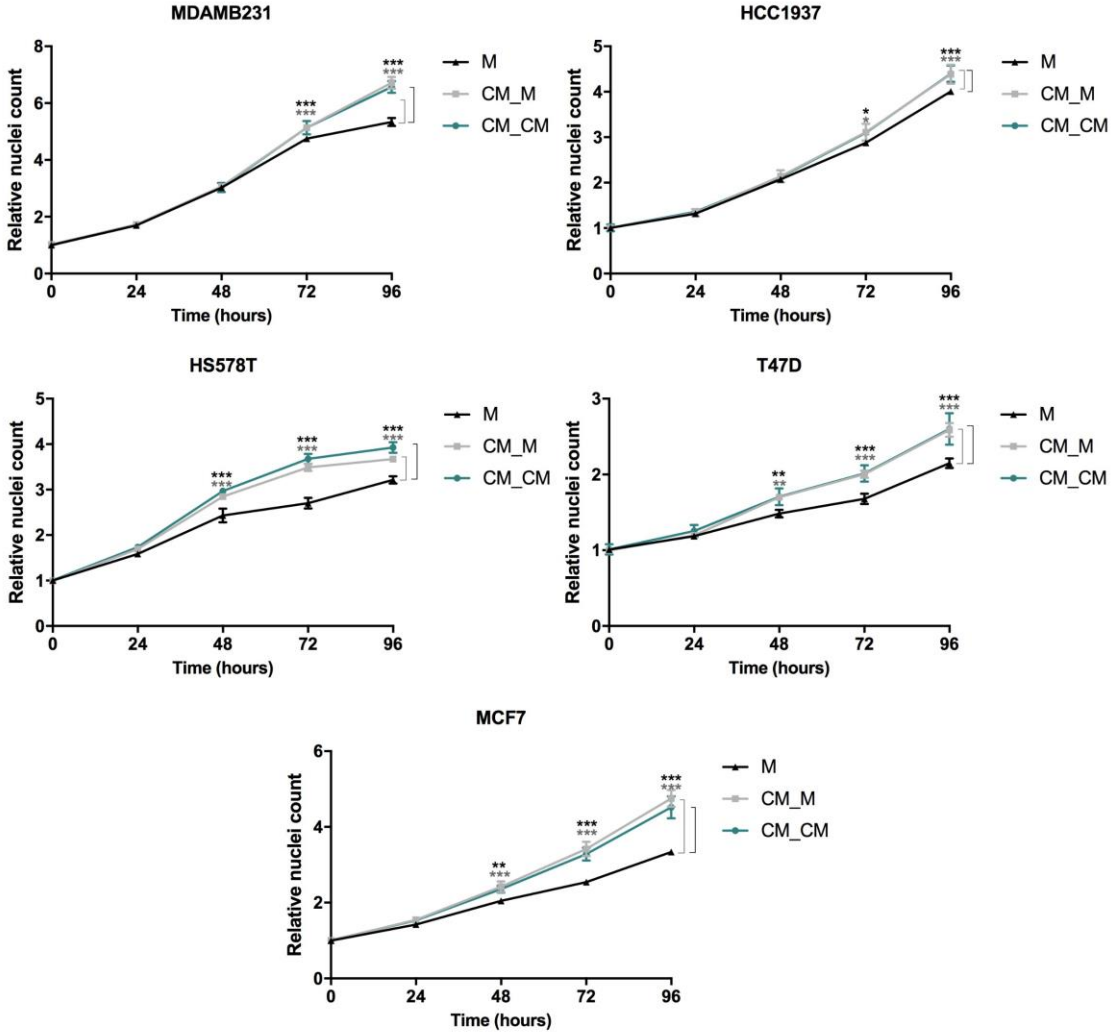

Figure 11 Effect of conditioned media of 14 days CM treated MSCs on the growth of breast cancer cell lines Breast cancer cell lines are treated with conditioned media of 14 day M treated (CM-M) or CM treated (CM-CM) MSCs or mono-cultured (MC). The graphs demonstrate the growth of breast cancer cell lines as fold change relative to mono culture over time and the values are presented as mean ± SD of 2 biological replicates with 6 technical replicates each. * p< 0.05, ** p< 0.01, *** p< 0.001

3.1.6. Differential gene expression and methylation analysis upon CM treatment

Next, I investigated whether epigenetic regulation has a role in the activation of fibroblasts. I hypothesized that changes in DNA methylation of MSCs might explain how cells get this activated fibroblast phenotype and might also contribute to the regulation of cytokines and other genes being upregulated during activation. Therefore, to determine the alterations in DNA methylation pattern upon conditioned media treatment, differentially methylated CpGs (probes) were determined using EPIC methylation array. RNA sequencing was also performed to assess whether an observed change in DNA methylation affects also the expression of a selected gene. Initial analysis of methylation data demonstrated that there were small differences (up to approximately 20%) in DNA methylation upon conditioned media treatment (Figure 12a). After 7 days of treatment with conditioned media, there were just very few CpGs differentially methylated while that number increased after 14 days of treatment (Figure 12b).

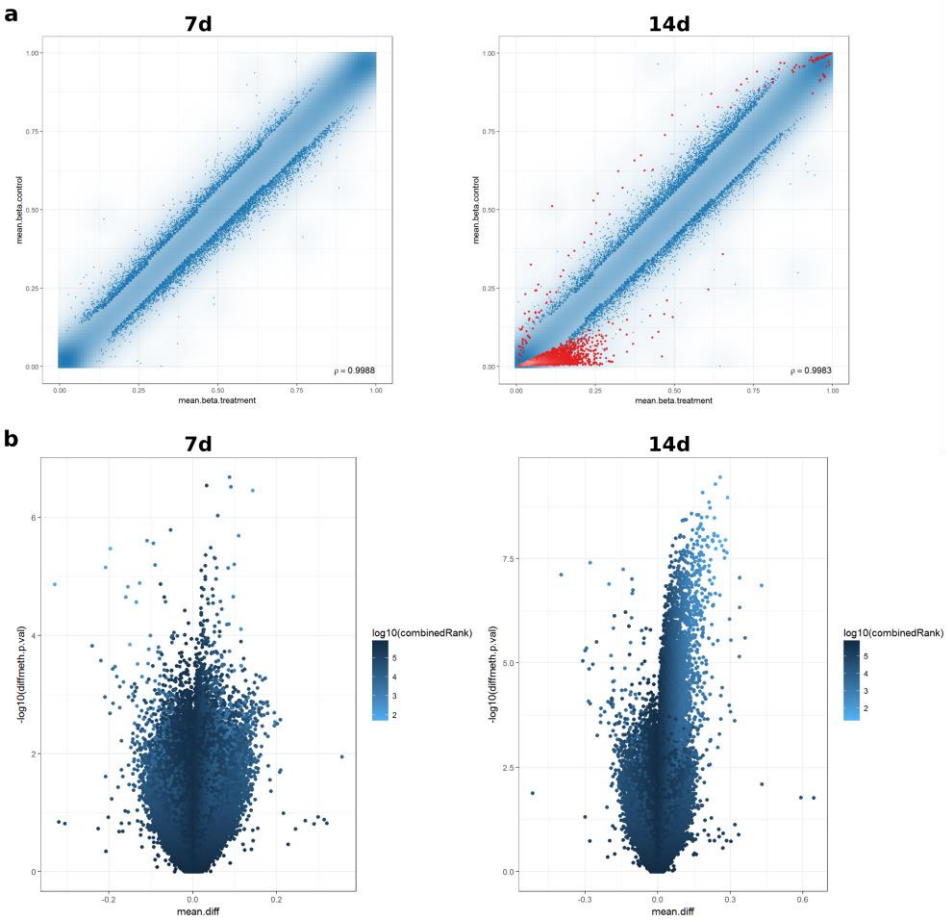

Figure 12 Differential methylation analysis of CM treated MSCs vs. control MSCs a. Comparison of methylation status of each probe in media and conditioned media treated MSCs. **b.** Volcano plots are depicted with the beta value of each probe relative to media treatment and the –log10p-value of the difference in methylation levels.

According to the differential gene expression analysis, the numbers of differentially expressed genes were similar for 7 day and 14 day of conditioned media treatment. Moreover, in most of the cases differential expression pattern for a gene was similar in 7 and 14 days samples meaning that if a gene was upregulated after 7 days of conditioned media treatment, it was still upregulated in the 14 day samples (Figure 13).

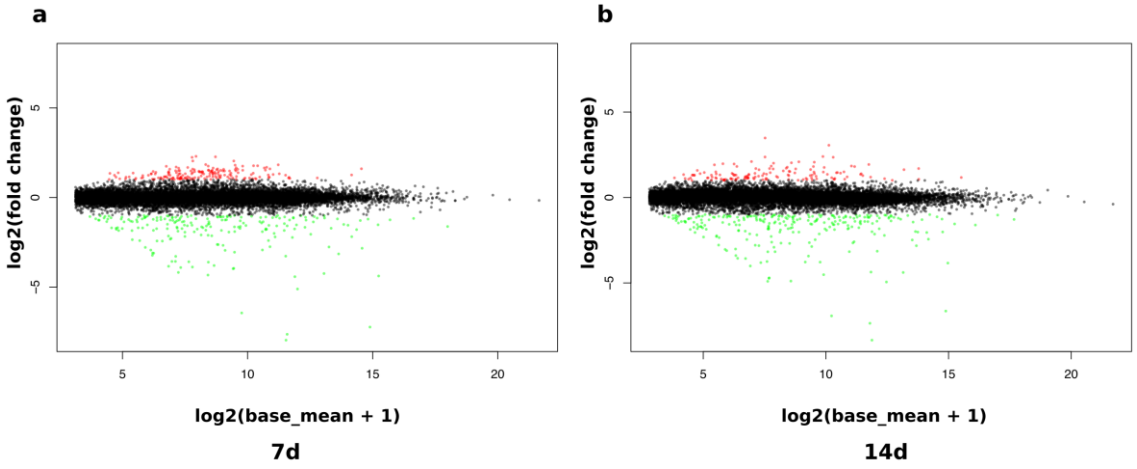

Figure 13 Differential gene expression analysis of CM treated MSCs. The graphs are depicted with log2-fold change and mean of normalized counts and they represent the distribution of magnitude of fold changes relative to mean expression for 7 **(a)** and 14 **(b)** day samples.

To find out common genes that are both differentially expressed and methylated an integrated analysis were carried out. Having at least one differentially regulated CpG in the promoter region (covering 1500 bp upstream of the transcription start site) or in the gene body was set as criterion to select a gene as differentially methylated. CpGs mapping to enhancer regions or to intergenic sequences were not regarded. This identified 273 genes that were both differentially methylated and differentially expressed after 7 days of treatment (Figure 14, 7d). For 14 days treatments, this number was 617 genes (Figure 14, 14d). Although several single CpGs were detected as differentially methylated, for most of the genes, there were no differentially methylated regions (two or more neighboring CpG differentially methylated at the same time) in which group of CpGs were differentially

methylated together. This observation would indicate that the methylation status of a single CpG located in the promoter region of a gene might not be playing a role in its expression. Therefore, I decided to focus on the RNA seq data to find out differentially expressed genes that might explain the activation phenotype of MSCs upon conditioned media treatment regardless of their methylation status.

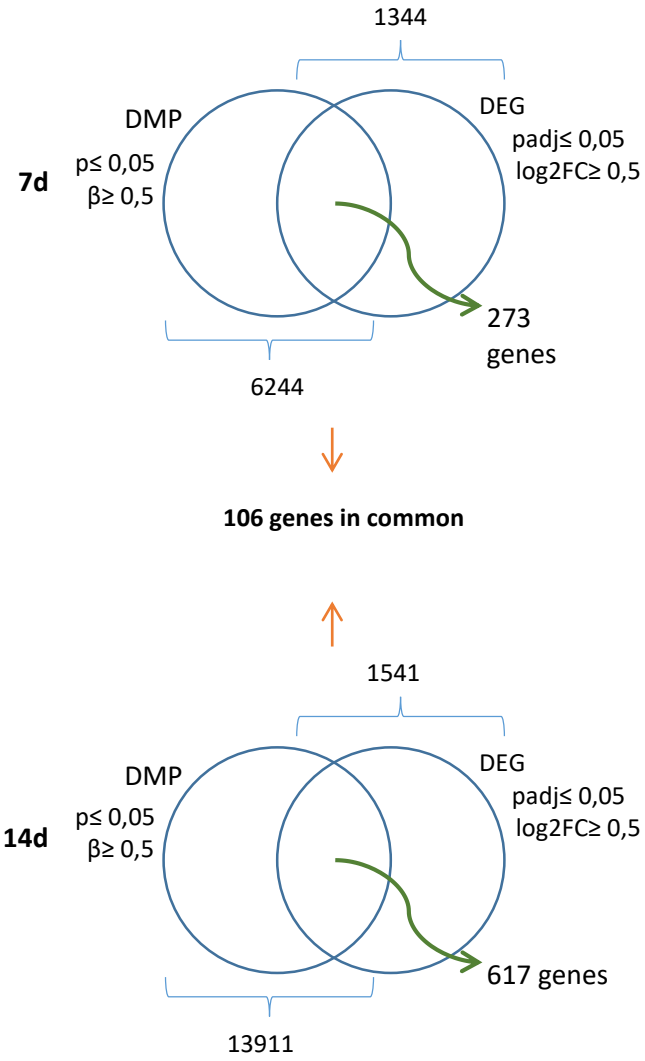

Figure 14 Correlation of DNA methylation and RNA sequencing. The VENN diagrams demonstrate the number of differentially methylated positions (DMP) detected by 850k EPIC methylation array and differentially expressed genes (DEG) detected by RNA sequencing upon CM treatment for 7 or 14 days. Common genes between DMPs and DEGs are depicted with green arrows.

Next, using RNA sequencing data, differentially regulated pathways upon conditioned media treatment were investigated. For this, KEGG enrichment analysis was performed using genes that are differentially expressed with a log2-fold change greater than 2. Most

of the identified pathways that are significantly regulated ($p<0.05$ after correction for multiple testing) were associated with inflammation (Figure 15). TNFα signaling and chemokine signaling pathways, cytokine-cytokine receptor interaction and the NF-κB signaling pathway were among the significantly regulated pathways both after 7 and 14 days of treatment. This observation might explain the upregulation of gene expression of various cytokines with the conditioned media treatment (Figure 6) and might indicate that conditioned media treatment induces the activation of inflammatory pathways that have a potential to also change the secretome profile of activated MSCs.

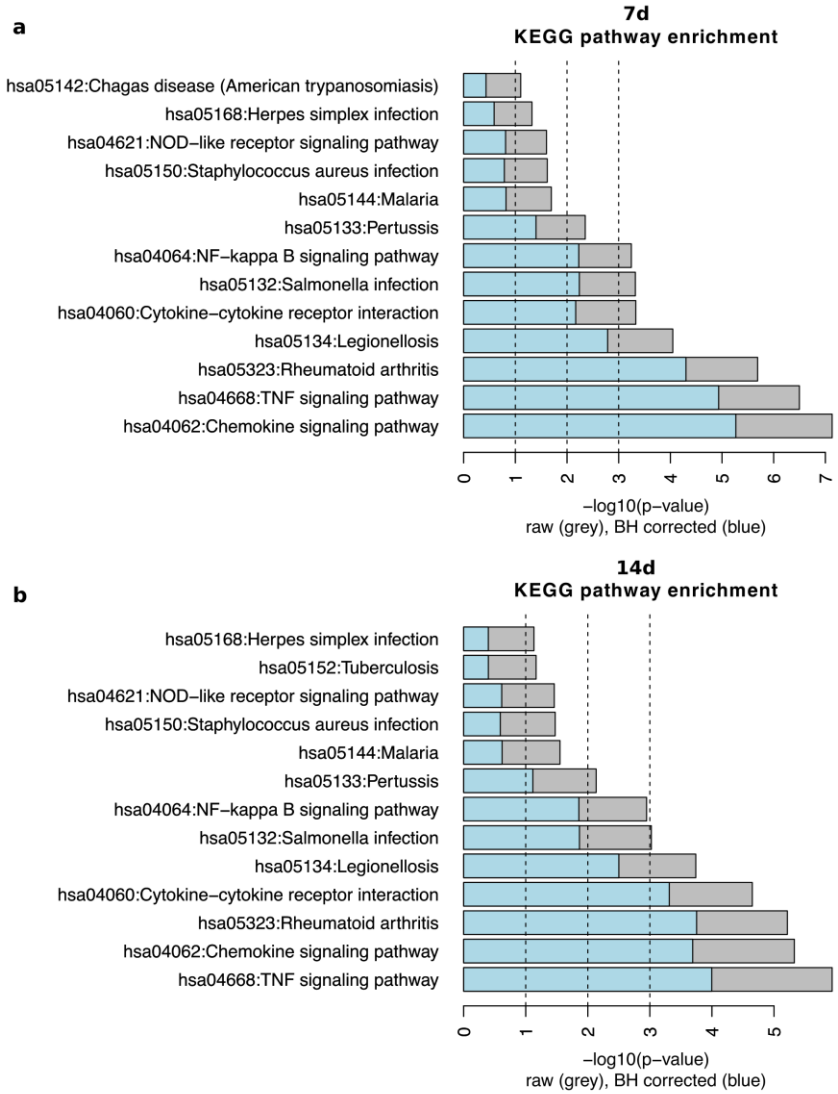

Figure 15 Pathway enrichment analysis. KEGG pathway enrichment analysis for differentially expressed genes upon 7 **(a)** or 14 **(b)** days of CM treatment. Raw and corrected p-values are shown on a -log10 scale.

3.1.7. Secretome analysis of activated MSCs

Detection of inflammatory pathways among the top differentially regulated pathways might suggest changes in the secretome of MSCs upon conditioned media treatment. To address this, proteomic analysis was performed to detect differentially secreted proteins in the activated MSCs in an unbiased approach. For this, after treatment with conditioned media for 7 or 14 days, cells were labelled with AHA and SILAC and subsequent analysis was carried out with LC tandem-MS to detect both differentially secreted and intracellular proteins. This method allows specific identification and quantification of newly synthesized proteins of labelled cells to distinguish these from proteins that had been present in the conditioned medium. Figure 16 shows the results of the proteomic analysis. Intracellular proteins were measured using cell pellets. For 7 days of conditioned media treatment, out of 882 different protein detected, 23 of them were differentially expressed.

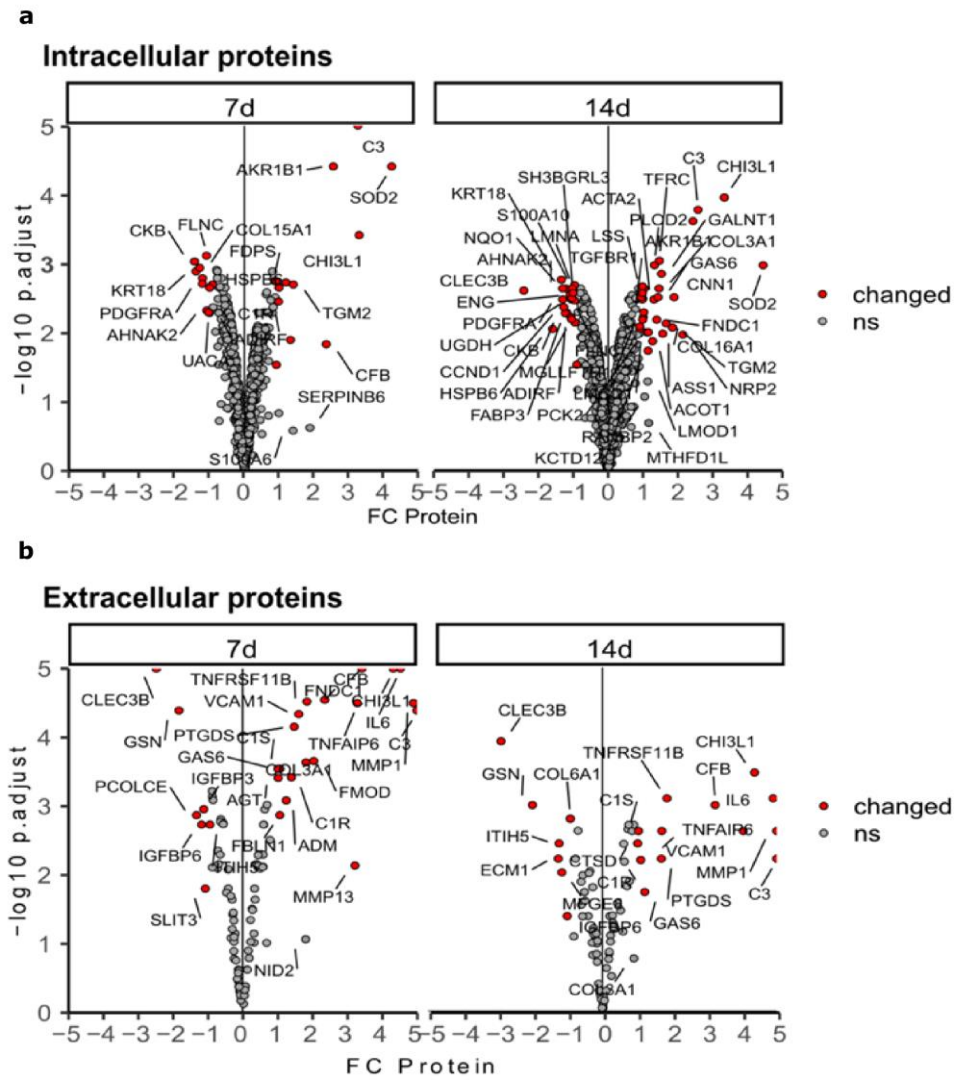

Figure 16 Mass spectrometry analysis of differentially regulated proteins upon 7 or 14 days of CM treatment. Volcano plots depict fold changes for each detected protein and the –log10 p value for intracellular **(a)** and extracellular proteins **(b)**. Changed: log2 fold change more than 0.8 with a p value less than 0.05. ns: no significant change

In 14 day samples, 1243 protein was detected and 52 of them were differentially expressed. Extracellular (secreted) proteins were measured using supernatants of MSCs upon labelling. For 7 days of conditioned media treatment, out of 82 distinct proteins detected, 20 were differentially expressed. In 14 day samples, 99 protein were detected and 52 of these were differentially expressed. In the intracellular and extracellular proteins which were found to be differentially expressed, there were several having been previously associated with activated fibroblasts or CAFs such as αSMA, GAS6, TGM2, IL6, VCAM1, MMP1 thus validating the approach. The analysis to detect differentially expressed proteins

requires at least two peptides to be detected in both SILAC channels in order to build a ratio (proteins shown in Figure 23). However, peptides from proteins close to the limit of detection or which are highly secreted in one of the two experimental conditions can sometimes fail to build such a ratio, for instance when peptides are predominantly detected on only one of two SILAC channels. To capture the dynamics of these proteins as well, a (pseudo)-ratio was built from the summed SILAC channels of all the detected peptides regardless whether both forms of a peptide were present giving an indication in which condition the protein is predominantly secreted (Table 7). If peptides were only detected in one of the two conditions then this condition is indicated.

Table 7 Analysis of extracellular proteins predominantly detected in one SILAC channel. Extracellular proteins altered upon conditioned media treatment for 7 or 14 days. CM: conditioned media channel M: Media channel nd: not detected

Extracellular proteins		**log2 fold change CM/M**		
	Gene	**Replicate 1**	**Replicate 2**	**Replicate 3**
7d	**CXCL1**	7,2	CM	7,7
	STC1	2,9	5,2	6,7
	MMP3	3,3	5,7	4,8
	POSTN	1,9	1,6	2,5
	KANK1	4,7	5,9	4,2
	SOD3	2,5	3,7	2,2
	STC2	nd	1,2	1,2
14d	**STC1**	CM	3,4	CM
	MMP3	5,6	2,1	4,9
	STC2	1,2	nd	1,1
	IGFBP1	M	-2,9	-1,4
	PLAU	-1,3	-1,5	-1,9

3.2.8. Target selection from RNA seq and secretome data

To select targets that might have a role in the activation of MSCs, I focused on the upregulated genes. Figure 17 demonstrates common genes that are found to be upregulated both at RNA and protein levels.

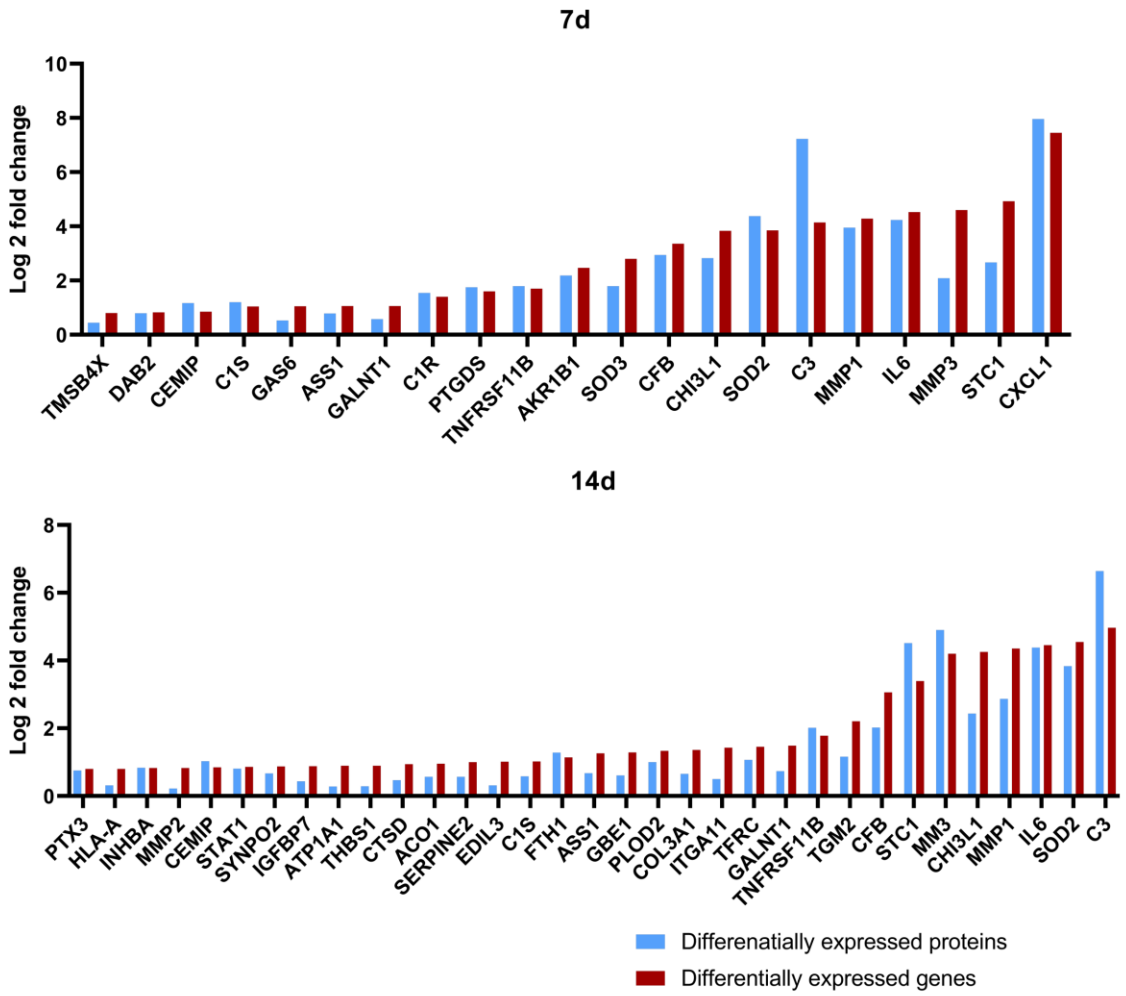

Figure 17 Upregulated genes and proteins upon CM treatment. The graphs represent log2-fold changes of upregulated genes detected both in RNA sequencing and respective proteins in mass spectrometry.

MMP1, MMP3, IL6 and CXCL1 are among the top 5 proteins that were upregulated after 7 days of conditioned media treatment and are all well-established CAF-secreted proteins (Kalluri, 2016). The other is STC1. This protein is not expressed in MSCs, however, its expression was stimulated by treatment with conditioned media from MDA-MB-231 tumor cells and could be detected also after 14 days of treatment with the conditioned media, both at mRNA and protein levels (Figure 17). Stanniocalcin 1 (STC1) is a secreted protein that has been shown to have inhibitory effects on calcium uptake in human cells (Xiang et al., 2016). Although the mechanism is not well established, STC1 has been also associated with CAFs and implicated as a CAF-secreted protein that is involved in metastasis of

colorectal (Peña et al., 2013) and therapy resistance in breast cancer (Roswall et al., 2018). STC1 secreted by MSCs has been also linked to fibrosis and shown to decrease collagen deposition (Ono et al., 2015). However, whether STC1 has an active role in the activation of fibroblasts or if its expression is a bystander effect in MSCs is still an open research question. Therefore, STC1 was chosen for further studies to better understand the mechanism of its upregulation and involvement in the activation of fibroblasts. Upregulation of STC1 in conditioned media-treated MSCs was validated both at mRNA and protein levels by real time PCR and ELISA respectively (Figure 18). Also, upregulation with conditioned media at mRNA level was confirmed in MSCs isolated from four other different donors (Figure 19).

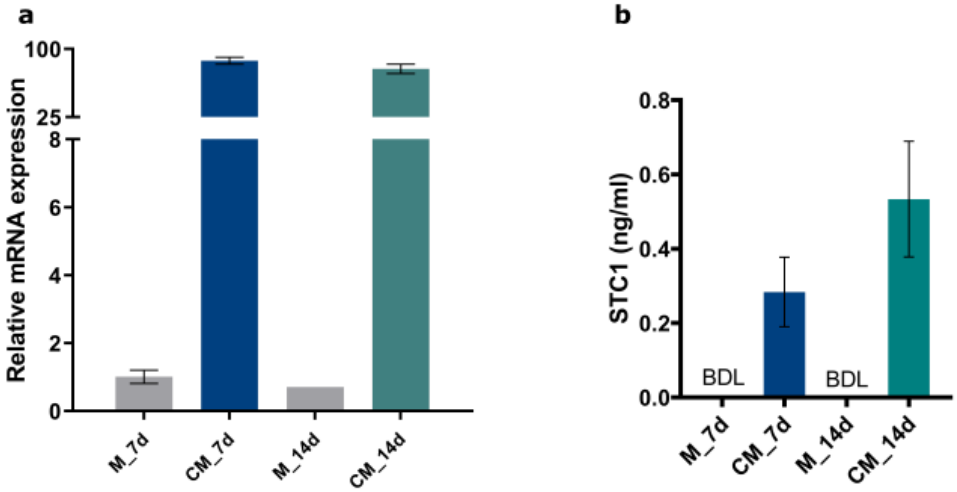

Figure 18 Validation of STC1 upregulation upon CM treatment. a. Expression analysis of *STC1* in MSCs treated with media M or CM for 7 or 14 days using TaqMan qRT-PCR. The graph shows the fold changes relative to 7d M sample and the values are presented as mean ± SD of 3 biological replicates **b.** ELISA analysis of STC1 using supernatants of MSCs treated with media M or CM for 7 or 14 days. The graph shows amount of STC1 in the supernatants and the values are presented as mean ± SD of 3 biological replicates. BDL: Below detection limit

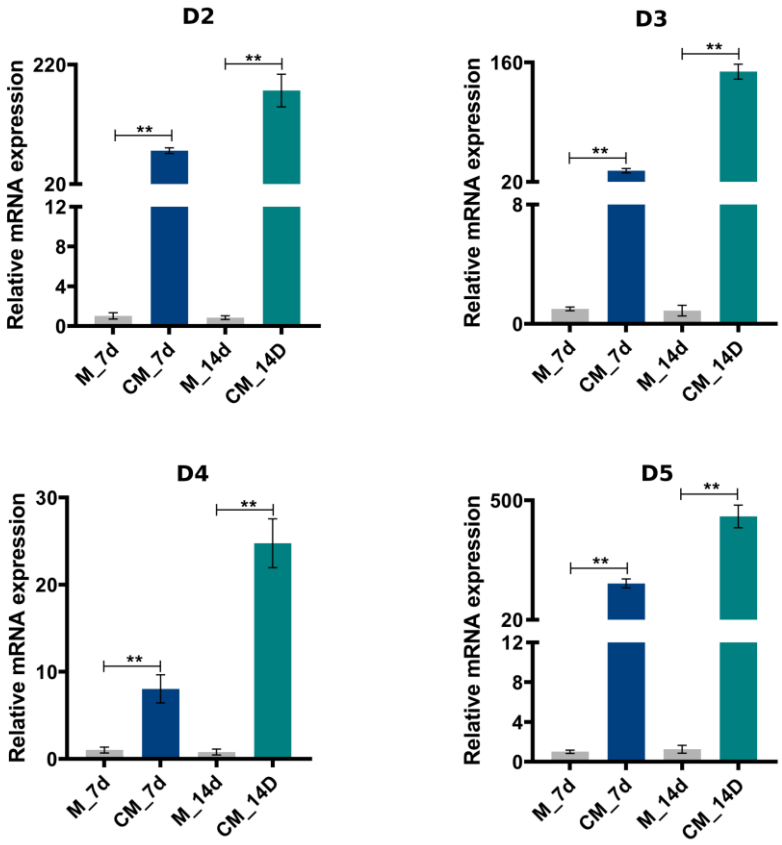

Figure 19 Analysis of *STC1* expression in MSCs isolated from different donors. Expression analysis of *STC1* in MSCs treated with media M or CM for 7 or 14 days using TaqMan qRT-PCR. The graph shows the fold changes relative to 7d M sample and the values are presented as mean ± SD of 3 biological replicates. D2-5: Donor 2-5. ** p< 0.01

3.2.9 Mechanism of STC1 upregulation upon conditioned media treatment in MSCs

Next, I wanted to investigate mechanisms behind the observed upregulation of STC1 and hypothesized that NF-κB signaling might play a role there. In pathway enrichment analysis (Figure 15) NF-κB pathway was among the most significantly differentially regulated pathways upon activation of MSCs with conditioned media treatment. Additionally, I focused on transcription factors that have predicted binding sites in the promoter region of the *STC1* gene. To understand the possible involvement of different transcription factors in the upregulation of *STC1*, MSCs were transfected with siRNAs targeting these transcription factors to down-regulate their expression. Afterwards, MSCs were treated with conditioned media of MDA-MB-231 cells for 24 hours and expression of *STC1* was determined at the mRNA level (Figure 20). In control conditions, even 24 hours treatment with conditioned media was sufficient to upregulate *STC1* expression. Moreover, out of

five transcription factors tested, downregulation of only *RELA* and *RELB* abrogated the upregulation of *STC1* induced by conditioned media treatment thus indicating the role of NF-κB signaling in the regulation of transcription of the *STC1* gene.

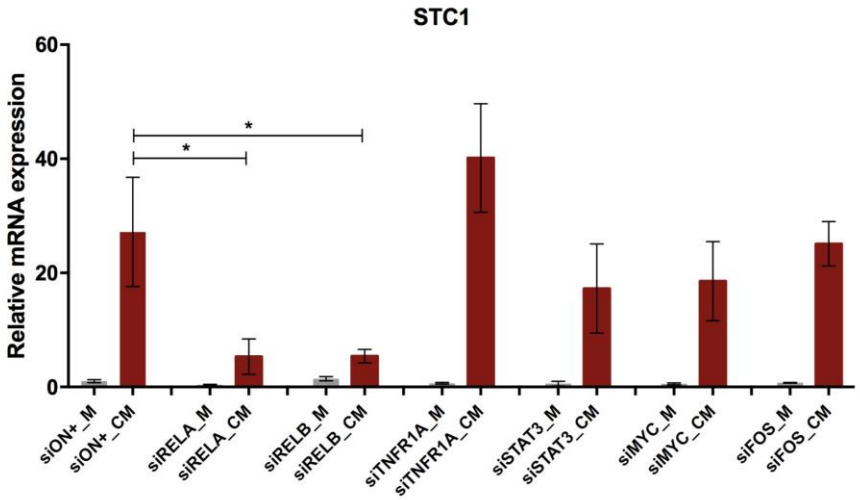

Figure 20 Analysis of STC1 expression in MSCs isolated from different donors Expression analysis of *STC1* in MSCs transfected with siRNAs targeting genes and treated with media M or CM for 24h using TaqMan qRT-PCR. The graph shows the fold changes relative to media treated control transfection (siCTRL_M) sample and the values are presented as mean ± SD of 3 biological replicates. * $p < 0.05$

3.2.10. Role of STC1 in the activation of conditioned media treated MSCs

Having shown the regulation of *STC1* expression at the mRNA level, *STC1* was down-regulated (RNAi) in MSCs treated with media or conditioned media to investigate the possible involvement of STC1 in the activation of MSCs. While downregulation of STC1 with a specific siRNA was efficient at the protein level, this was ~80% at the RNA level (Figure 21).

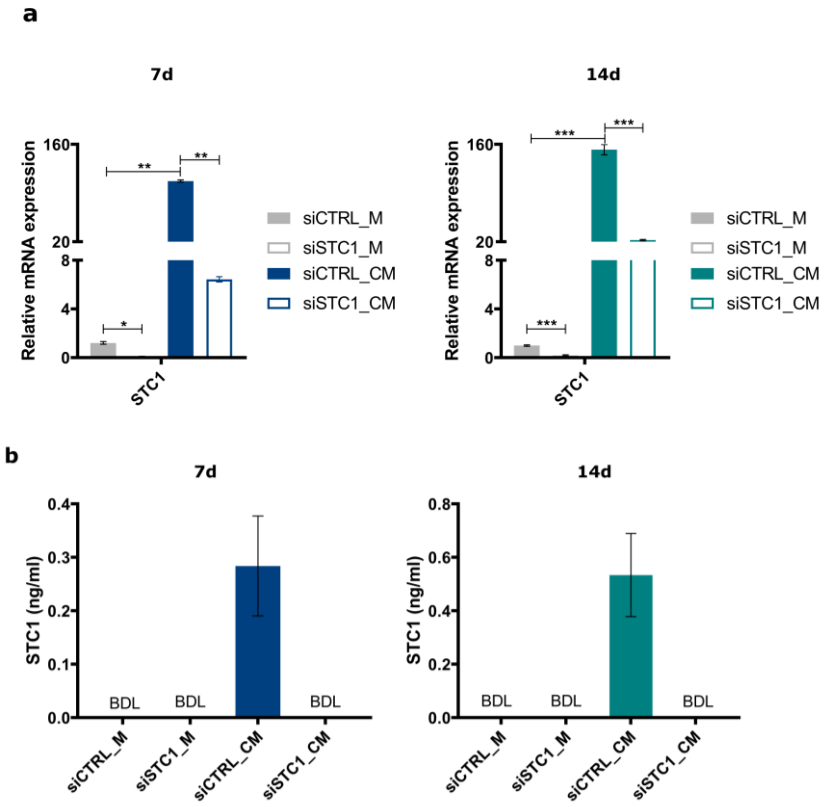

Figure 21 Analysis of STC1 expression upon siRNA knock-down. a. Expression analysis of STC1 in MSCs transfected with siCTRL or siSTC1 and treated with media M or CM for 7 or 14 days using TaqMan qRT-PCR. The graphs show the fold changes relative to media treated control transfection (siCTRL_M) and the values are presented as mean ± SD of 3 biological replicates **b.** ELISA analysis of STC1 using supernatants of MSCs transfected with siCTRL or siSTC1 and treated with media M or CM for 7 or 14 days. The graphs show amount of STC1 in the supernatants and the values are presented as mean ± SD of 3 biological replicates BDL: Below detection limit. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Having confirmed that RNAi of *STC1* indeed down-regulated the gene both at mRNA and protein levels, I wanted to check the effect of this down-regulation on the contraction ability of MSCs. To this end, STC1 was knocked down in MSCs, treated the cells with and without conditioned media for 7 (Figure 22a, b) and 14 (Figure 22c, d) days, and quantified the cells' contraction ability of MSCs.

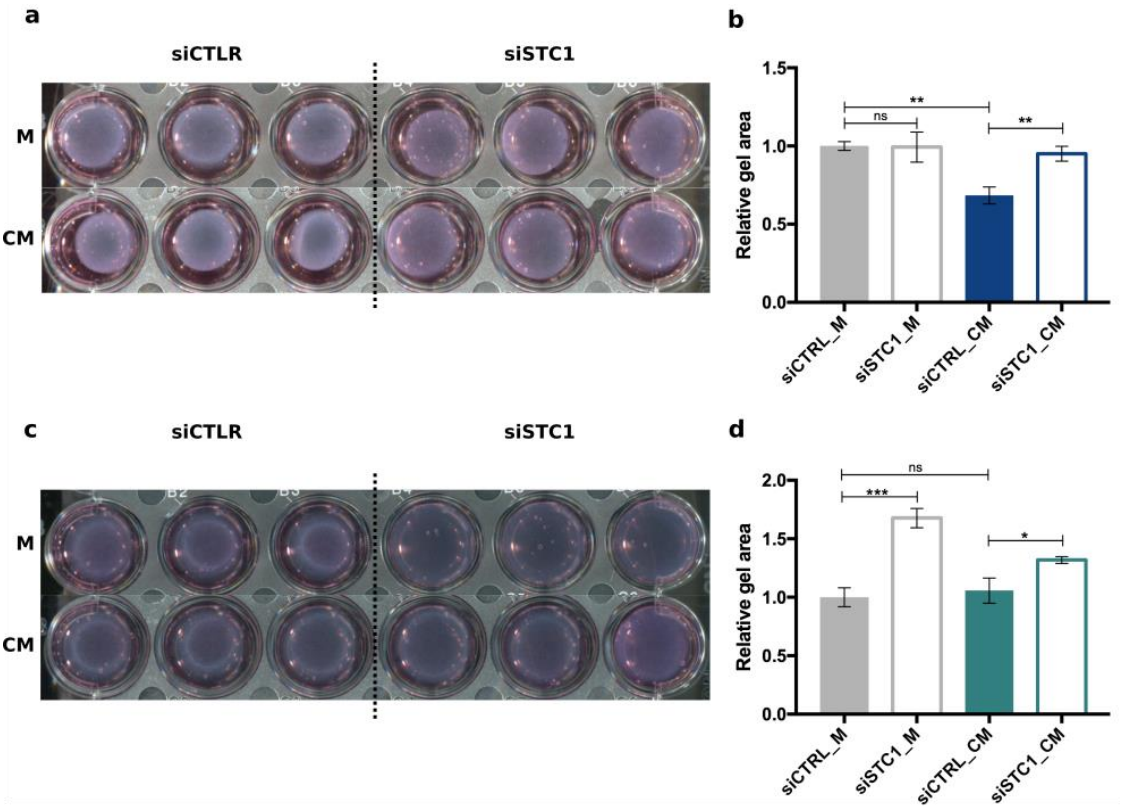

Figure 22 Effect of *STC1* knock-down on the contractility of activated MSCs. a-b Collagen gel contraction assay with MSCs transfected with siCTRL or siSTC1 and treated with media M or CM for 7 (a) or 14 (c) days**. b-d** The graphs represent fold changes in gel areas relative to siCTRL_M samples. Values are presented as mean ± SD of 3 biological replicates. * p< 0.05, ** p< 0.01, *** p< 0.001, ns: not significant

Next, effects of STC1 knock-down on the expression of CAF markers in MSCs treated with conditioned media were explored (Figure 23). Down-regulation of STC1 in cells treated with normal medium for 7 days caused down-regulation in FAP as well as αSMA expression. There, the increase in the levels of FAP was reduced in cells with downregulated STC1 treated with conditioned media, while the slight increase in αSMA expression observed in the control conditions was abrogated in knock-down cells. In contrast, at 14 days, for FAP expression, the initial strong difference between control and STC1 know-down cells treated with conditioned media were not seen anymore, while the reduced expression of αSMA expression upon *STC1* knock-down was still visible. Hence, the effect of STC1 seems to be temporal for FAP expression, however, sustained for αSMA.

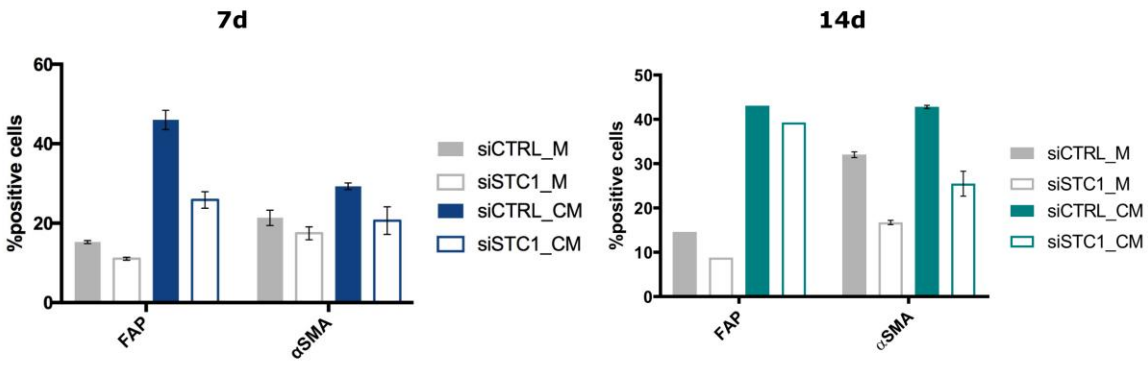

Figure 23 Effect of STC1 knock-down on the expression of αSMA and FAP in activated MSC
Flow cytometry analysis of MSCs transfected with siCTRL or siSTC1 and treated with media M or CM for 7 or 14 days. The graphs demonstrate the percentage of positive cells for FAP or αSMA expression and the values are presented as mean ± SD of 2 biological replicates (except for FAP 14d).

3.2.11. Role of STC1 in the activation of CAFs

After having shown that STC1 indeed has an effect on the activation of MSCs, next, I analyzed the impact pf STC1 knock-down on already activated CAFs. For this purpose, I determined the expression levels of STC1 in CAFs isolated from breast tumor samples of three different donors (Figure 24). CAF1 and CAF2 were chosen for further validation because they had the highest expression of STC1 among the CAFs that were tested. However, it is important to note that although the level STC1 expression in CAF1 and CAF2 cells was found higher than in MSCs after treatment with media, their expression did not reach the high levels that were measured in MSCs having been treated with conditioned media from tumor cells. Interestingly, STC1 was expressed at high levels also in most tumor cell lines, including MDA-MB-231.

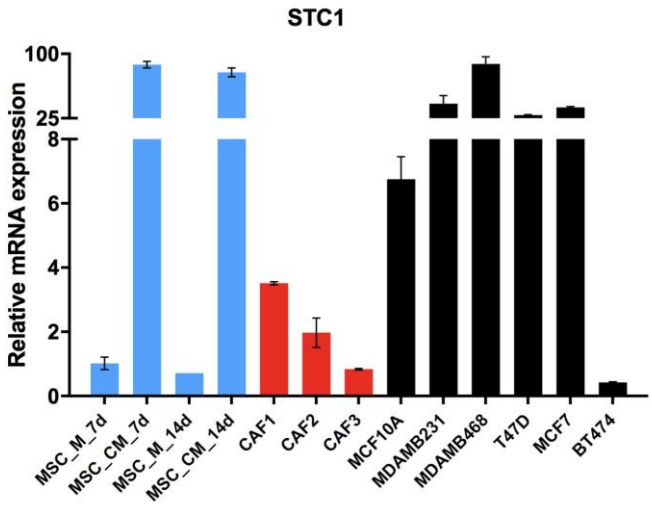

Figure 24 Expression of STC1 in MSCs and CAFs. Expression analysis of *STC1* in MSCs, in primary CAFs isolated from different donors and in several breast cancer cell lines using TaqMan qRT-PCR. The graph shows the fold changes relative to media treated control MSC sample and the values are presented as mean ± SD of 3 biological replicates.

In order to understand the effect of STC1 on the activation of selected CAFs a collagen gel contraction assay was performed upon siRNA knock-down of *STC1*. Interestingly, the two CAF cell lines showed contradictory results in this assay. While knock-down of *STC1* resulted in inhibition of contraction in CAF1 cells, this was not observed in CAF2 cells (Figure 25).

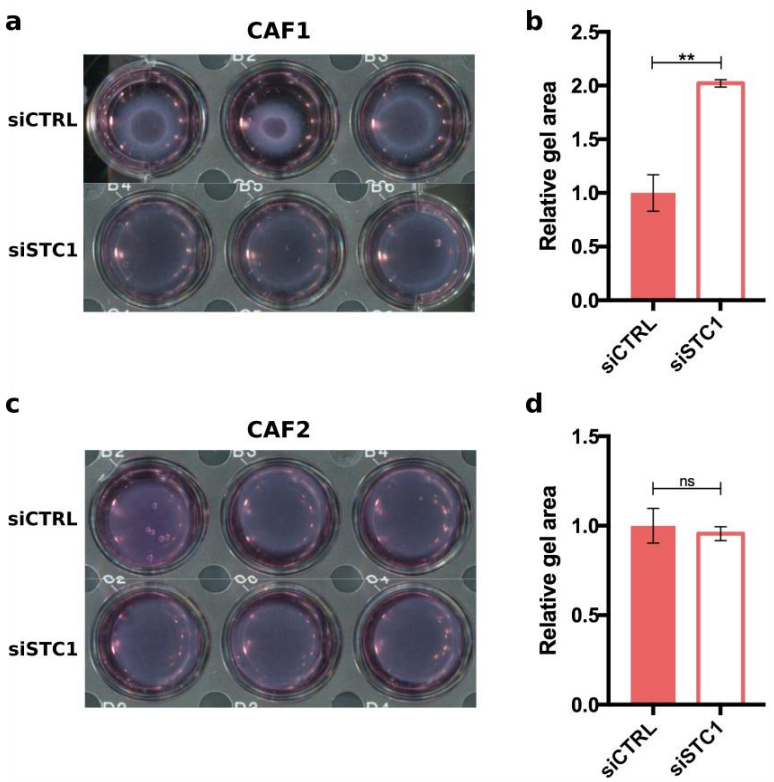

Figure 25 Effect of STC1 knock-down on the contractility of CAFs. a-b Collagen gel contraction assay with CAF1 (a) and CAF2 (b) transfected with siCTRL or siSTC1 **b-d** The graphs represent the fold changes in gel area relative to siCTRL samples and the values are presented as mean ± SD of 3 biological replicates ** p< 0.01, ns: not significant

To identify potential causes of the different phenotypes having been observed with CAF1 and CAF2 cells, I investigated whether knock-down of *STC1* would also have an impact on the expression of CAF markers in these cells (Figure 26). While the number of CAF1 cells expressing αSMA was reduced upon STC1 knock-down, this effect was not seen in CAF2 cells. However, the fraction of αSMA-positive cells was higher in the CAF1 than in the CAF2 cell populations at baseline conditions. Similar to αSMA, also FAP was expressed in a smaller percentage of CAF2 cells compared to CAF1. Hence, there seem to be differences in the level of activation between the two CAF entities. Therefore, CAF1 was chosen for further experiments due to its higher contractility and higher expression of FAP and αSMA.

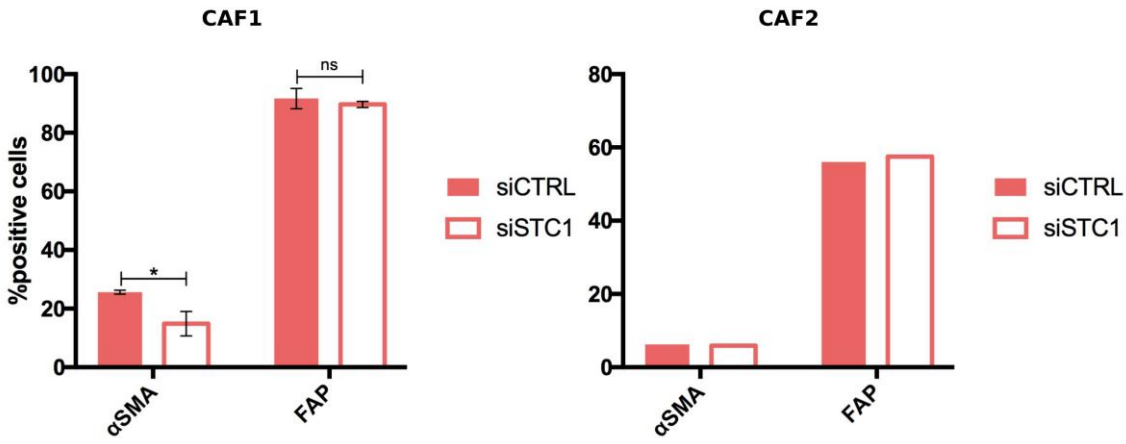

Figure 26 Effect of STC1 knock-down on the expression of αSMA and FAP in CAFs. Flow cytometry analysis of CAF1 and CAF2 cells transfected with siCTRL or siSTC1. The graphs demonstrate the percentage of positive cells for FAP or αSMA expression and the values are presented as mean ± SD of 3 biological replicates (CAF1).

Activated CAFs have increased migration and invasion abilities compared to naïve fibroblasts. Therefore, in order to determine if STC1 would also have an impact on these activation phenotypes, trans-well migration and invasion assays were carried out. Indeed knock-down of STC1 caused a decrease in the number of migrating or invading CAF1 cells (Figure 27).

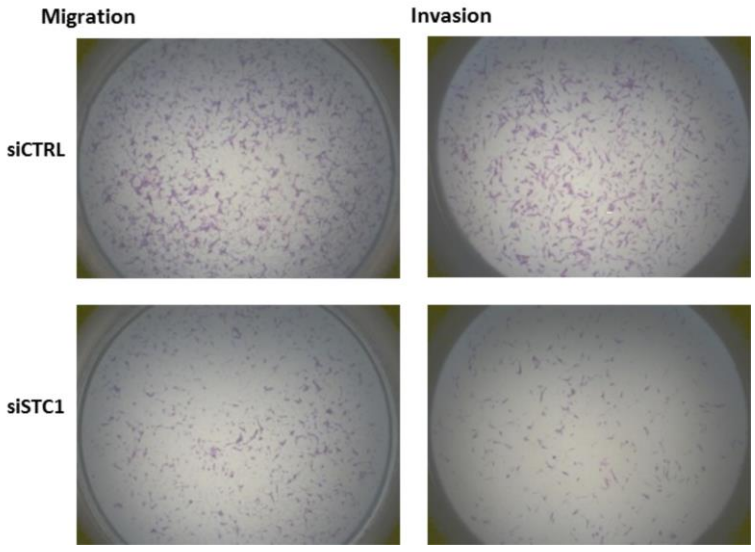

Figure 27 Effect of STC1 knock-down on migration and invasion of CAFs. Trans-well migration and invasion assays of CAF1 cells transfected with siCTRL or siSTC1.

3.2.12. Role of STC1 in the impact of CAFs on cancer cell growth

The effect of CAFs on tumor progression is well known (Kalluri, 2016). In order to investigate the influence of CAFs with down-regulated STC1 expression on the cancer cell growth, I directly co-cultured CAF1 cells transfected with control siRNA or siRNA against STC1 with various breast cancer cell lines and measured the changes in their growth. Figure 28 is showing the growth curves of cancer cell lines during 4 days of co-culture or mono-culture. While the presence of CAFs did not affect the growth of MDA-MB-231 cells, all the other cell lines grew better in the presence of CAF1 cells. However, no difference was observed when cancer cells were co-cultured with CAF1 cells transfected with control siRNA or siRNA targeting STC1 indicating that STC1 secreted by CAFs does not have any role on the growth of cancer cells.

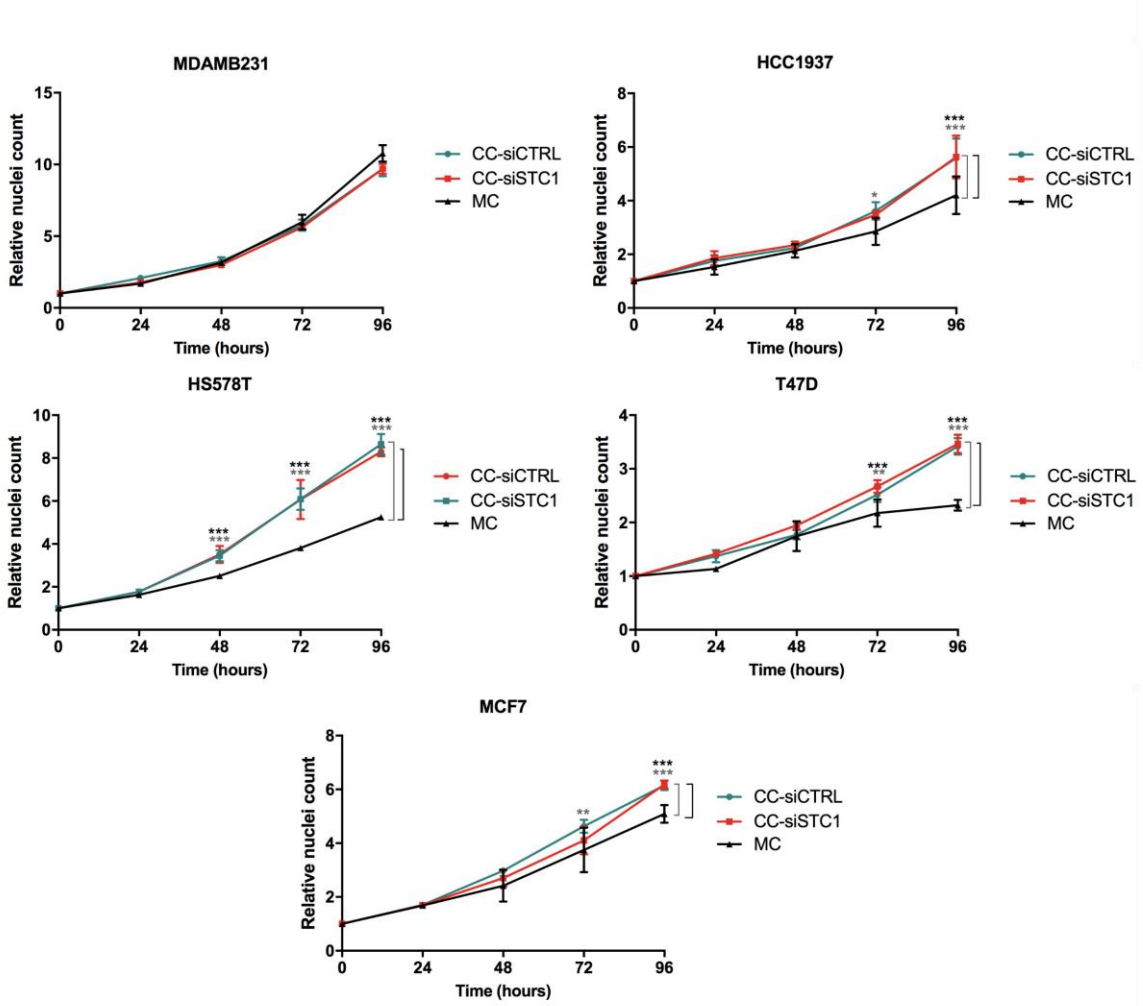

Figure 28 Effect of CAF1 cells on the growth of breast cancer cell lines Breast cancer cell lines were co-cultured with CAF1 cell transfected with siCTRL (CC-siCTRL) or transfected with siSTC1 (CC-siSTC1) or mono-cultured (MC). The graphs demonstrate the growth of breast cancer cell lines as fold change relative to mono culture over time and are presented as mean ± SD of of 2 biological replicates with 6 technical replicates each. * p< 0.05, ** p< 0.01, *** p< 0.001

3.2.13. Deconvolution of siSTC1 pool

Deconvolution of the 4 siRNAs of the STC1 pool demonstrated that all siRNAs decreased *STC1* expression at the mRNA level (Figure 29d). However, their effects on the contraction ability of CAF1 cells and ACTA2 expression were different (Figure 29a and d). While siRNA numbers 1, 2 and 3 had similar effects as the pool, siRNA number 4 did neither affect the contraction nor *ACTA2* expression. I also tested the effect of siRNAs on cell growth and demonstrated that siRNA number 3 slightly but insignificantly, reduced growth

of CAF1 cells (Figure 29c). Since siRNA number 1 both downregulated *ACTA2* and inhibited the contraction, this siRNA was chosen for use in further experiments.

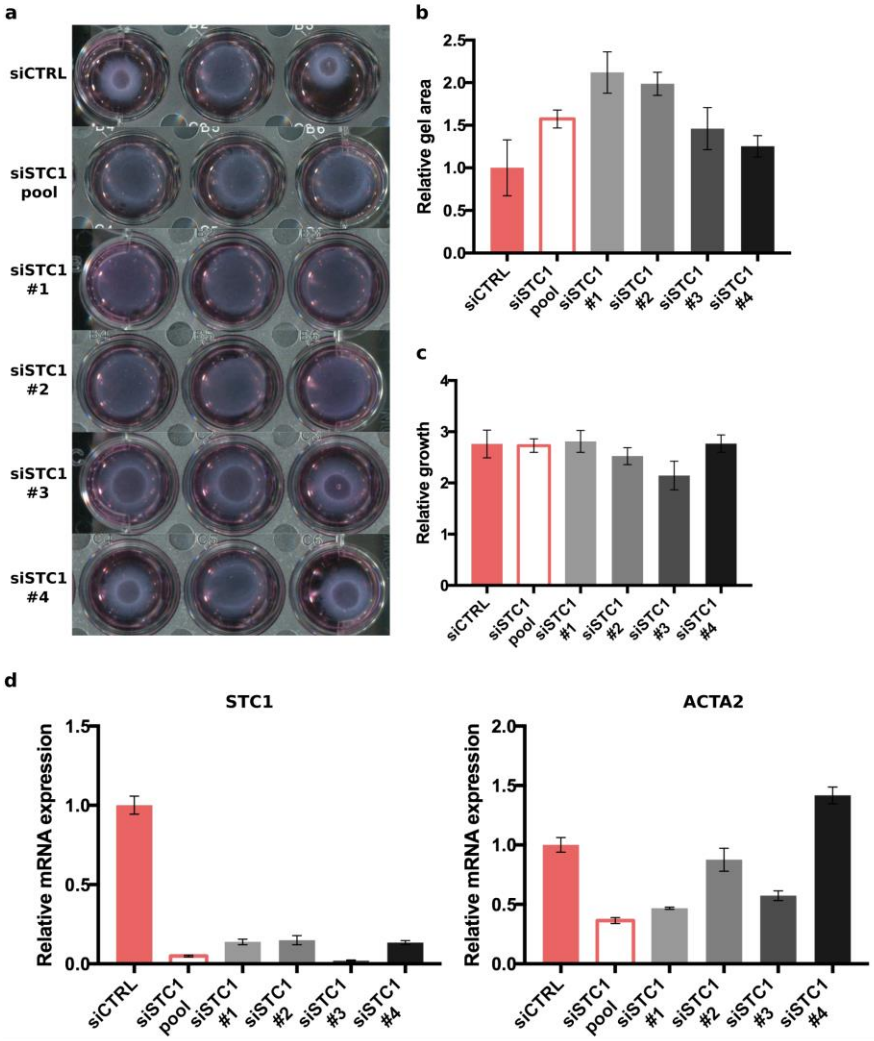

Figure 29 Deconvolution of siSTC1 pool a. Collagen gel contraction assay with CAF1 cells transfected with siCTRL or four different siRNAs for STC1 that are in siSTC1 pool. **b.** The graph represents the fold changes in gel area relative to siCTRL samples and the values are presented as mean ± SD of 3 technical replicates. **c.** Nuclei count of CAF1 cells. The graph represents the fold changes in growth relative to siCTRL samples and the values are presented as mean ± SD of 6 technical replicates. **d.** Expression analysis of STC1 and ACTA2 in CAFs. The graph shows the fold changes relative to media treated relative to siCTRL samples and the values are presented as mean ± SD of 3 biological replicates.

3.2.14 Pathway analysis upon STC1 knock-down in CAF1 cells

The results described above suggested that STC1 might have a role in the activation of fibroblasts. To validate this hypothesis, it was required to identify pathways that are regulated by knock-down of STC1 in CAF1 cells. This should help identifying mechanisms by which STC1 impacts on contraction, αSMA expression, migration and invasion. For this, proteomic analysis by reverse phase protein array (RPPA) was performed to identify pathways that are differentially activated upon STC1 knock-down. Out of several pathways that were analyzed (MAPK, Akt/mTOR, PI3K, NOTCH, Wnt), NOTCH signaling and Wnt signaling were the most changed ones (Figure 30). The figure shows heat maps for the changes in the expression of proteins belonging to these two signaling pathways. Red stars indicate statistically significant changes. For Notch signaling, two receptors of the pathway, namely, NOTCH2 and NOTCH3 were found to be downregulated upon knock-down of STC1. Moreover, for Wnt signaling, a down-regulation on β-catenin and phospho-β-catenin was observed.

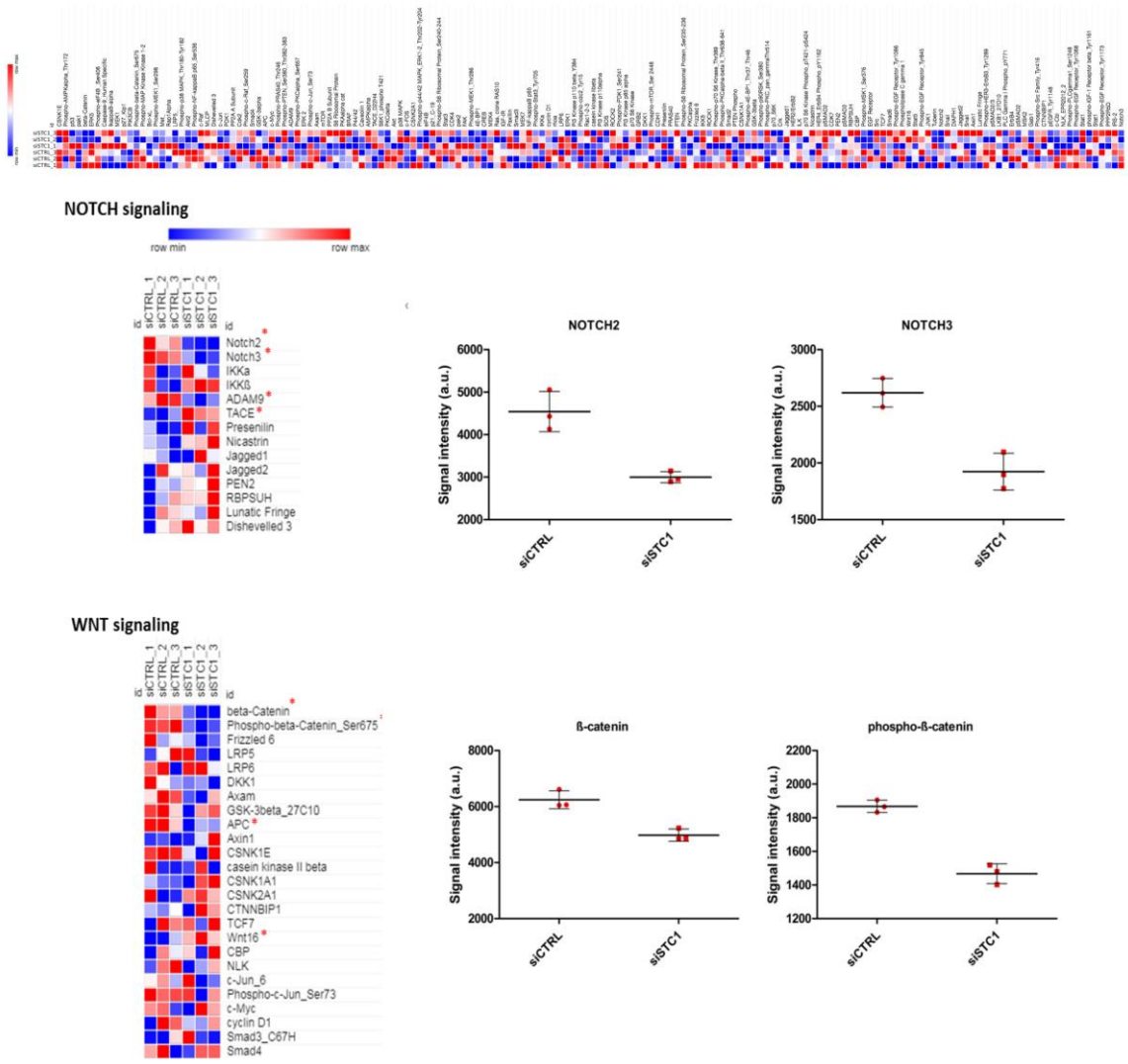

Figure 30 Effect of STC1 knock-down on different pathways Heat map of all analyzed proteins (Upper panel). RPPA results of NOTCH and Wnt signaling pathways. Heat maps shows the signal intensities of the proteins belonging to Wnt and NOTCH signaling in CAF1 cells transfected with siCTRL or siSTC1. Significantly changing proteins are labeled with red stars ($p<0.05$). The graphs show the signal intensities for specific proteins and the values are presented as mean ± SD of 3 biological replicates. * $p< 0.05$, ** $p< 0.01$

3.2.15. STC1 expression in patient

After having demonstrated the possible role of STC1 in the activation of fibroblasts *in vitro*, in order to investigate the relevance of this finding in breast cancer patients, publicly available datasets were analyzed for the expression of *STC1* in human tumors. First, I checked the expression of *STC1* in different molecular subtypes of breast cancer using the TCGA dataset. This contains RNA sequencing data of tumors from 1200 individual

patients. No difference in the expression of *STC1* was observed among the different subtypes (Figure 31). Since TCGA data set has information about the whole tumor without making a distinction between tumor and stromal cells, I decided to analyze microarray datasets that were obtained using RNA samples from stroma or epithelium of patient tumor samples obtained with laser capture microdissection of tissues. For this, four publicly available microarray datasets were analyzed (GSE14548, GSE35019, GSE9014, GSE5847). *STC1* expression was indeed found elevated in the stroma of ductal carcinoma in situ (DCIS) compared to the stroma of normal breast tissue and epithelium of DCIS in the GSE14548 dataset (Figure 31b). However, in the case of, invasive carcinoma there is no significant difference in the expression of *STC1* between the stroma and epithelium (Figure 31b). No significant differences in *STC1* expression between different parts of tumors were obvious in another data set covering of a similar type of samples (Figure 31c). I checked the expression *STC1* in another two data sets and compared its expression between normal breast tissue stroma and tumor stroma (GSE9014) or between tumor stroma and tumor epithelium (GSE5847). I found data tumor stroma has higher expression of *STC1* compared to the normal breast tissue stroma (Figure 31d). However, there was no difference in the expression of *STC1* between tumor stroma and tumor epithelium (Figure 31e). Hence, the results from patient data sets were inconclusive.

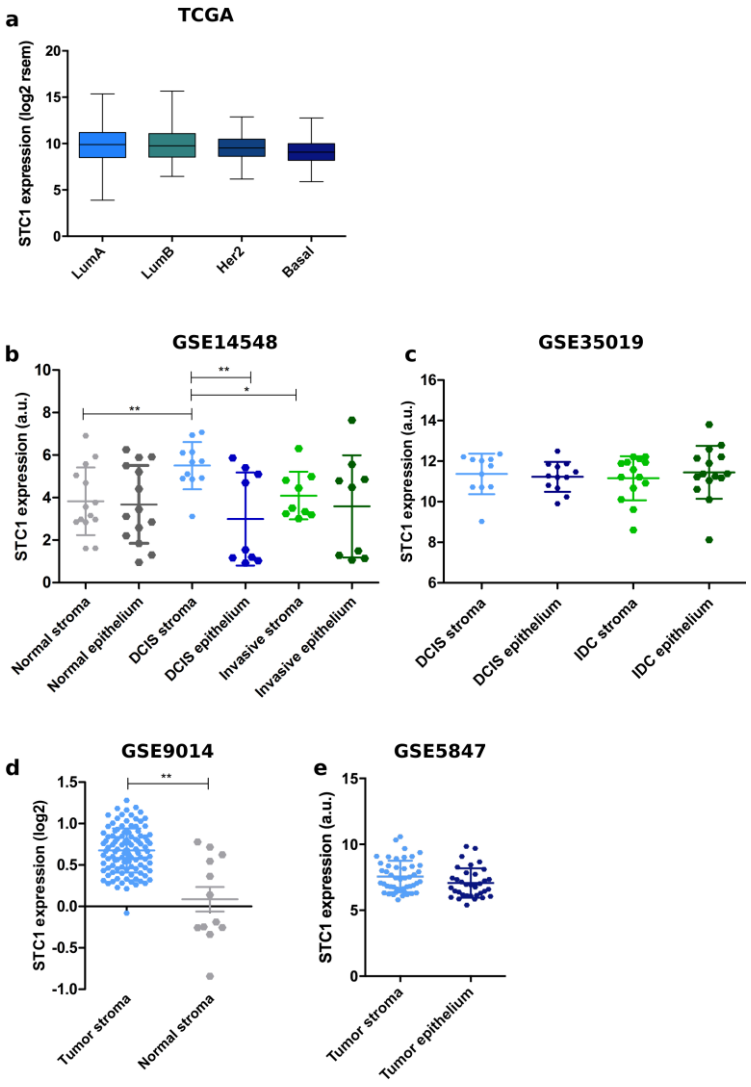

Figure 31 *STC1* expression in breast cancer patients a. *STC1* mRNA expression analysis of the TCGA dataset comparing the different molecular subtypes of breast cancer **b-c-d-e** STC1 mRNA expression analysis of different microarray datasets

Moreover, since it was observed that with the down-regulation of *STC1* expression, *ACTA2* expression was also going down, the correlation between the expression of these two genes in the stroma part of tumors was determined. In the two datasets that were analyzed, there was a significant correlation between *ACTA2* and *STC1* (Figure 32).

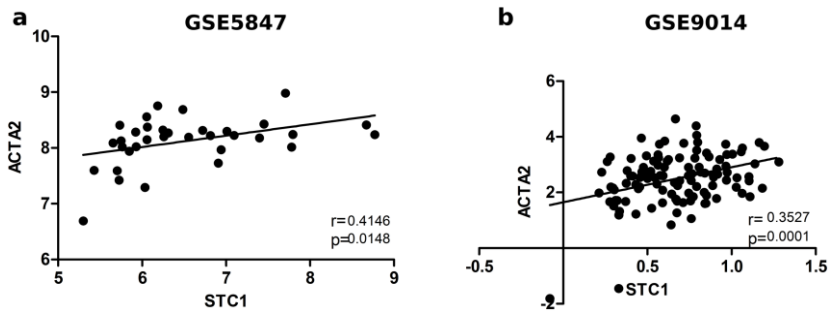

Figure 32 Correlation between *STC1* and *ACTA2* expression in patients Correlation analysis of stromal *STC1* with *ACTA2* gene expression of two different data sets. Correlation coefficient (r) was calculated using Pearson correlation.

3.2. Part 2

3.2.1. Characterization of bone marrow stromal cells

To study the recruitment of different stromal cell populations from the bone marrow to the tumor site, bone marrow cells were isolated from BALB/C mice and characterized either immediately after isolation or after keeping them in culture for 28 days. Figure 33a shows the bright field images of bone marrow cells immediately after isolation and after 7 or 28 days in culture. While there were still floating hematopoietic cells present after 7 days in culture, after 28 days in culture, the most abundant population was adherent fibroblast like stromal cells. To quantify the percentage of bone marrow stromal cells ($CD45^-CD31^-$), hematopoietic cells ($CD45^+$), endothelial cells ($CD31^+$) and rare mesenchymal stem cell ($CD45^-CD31^-CD51^+PDGFRa^+$) population, flow cytometry analysis was performed. Figure 33b demonstrates the dot plots of flow cytometry analysis of freshly isolated total bone marrow and bone marrow stromal cells cultured for 28 days. Directly after isolation, stromal cells constituted about 20% of bone marrow cells while the rare CD51 and PDGFRα double positive stem cell population was just 0.01% (Figure 33c). The percentage of mesenchymal stem cells stayed the same in the whole population of cultured cells, despite the stromal population was enriched over non-adherent hematopoietic cells that were efficiently removed after several medium changes. Therefore, I decided not to culture the cells in the bone marrow isolates but instead to either sort the whole bone marrow stromal population or only mesenchymal stem cells by fluorescence-activated cell sorting (FACS) directly after isolation of the bone marrow.

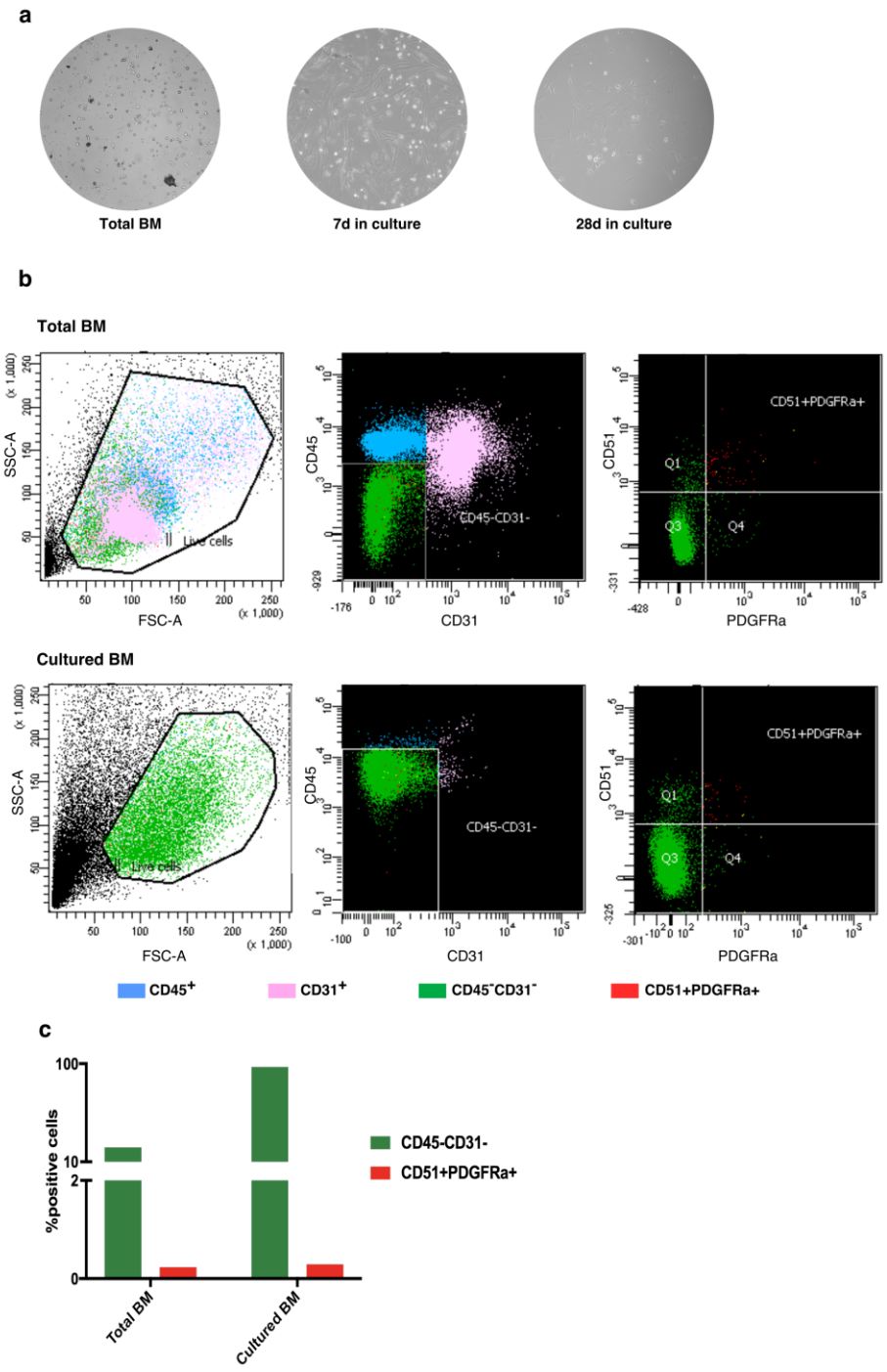

Figure 33 Characterization of bone marrow cells a. Bright field images of total bone marrow, 7 day cultured bone marrow and 28 day cultured bone marrow **b.** Flow cytometry analysis of total bone marrow and 28 days cultured bone marrow **c.** Graphical representation of flow cytometry data. BM: bone marrow

Since the rare mesenchymal stem cells represent a very small population, the non-stromal part of the bone marrow had to be depleted before cell sorting to reduce the time of sorting

and to increase efficiency. Therefore, different strategies were tested to deplete the non-stromal part of the bone marrow using magnetic cell sorting (MACS). Out of three different techniques (Figure 34a), depletion of $CD45^{+}Ter119^{+}CD31^{+}$ cells resulted in the highest enrichment of the stromal cell population with almost 60% of the cells being $CD45^{-}CD31^{-}Ter119^{-}$ (Figure 34b).

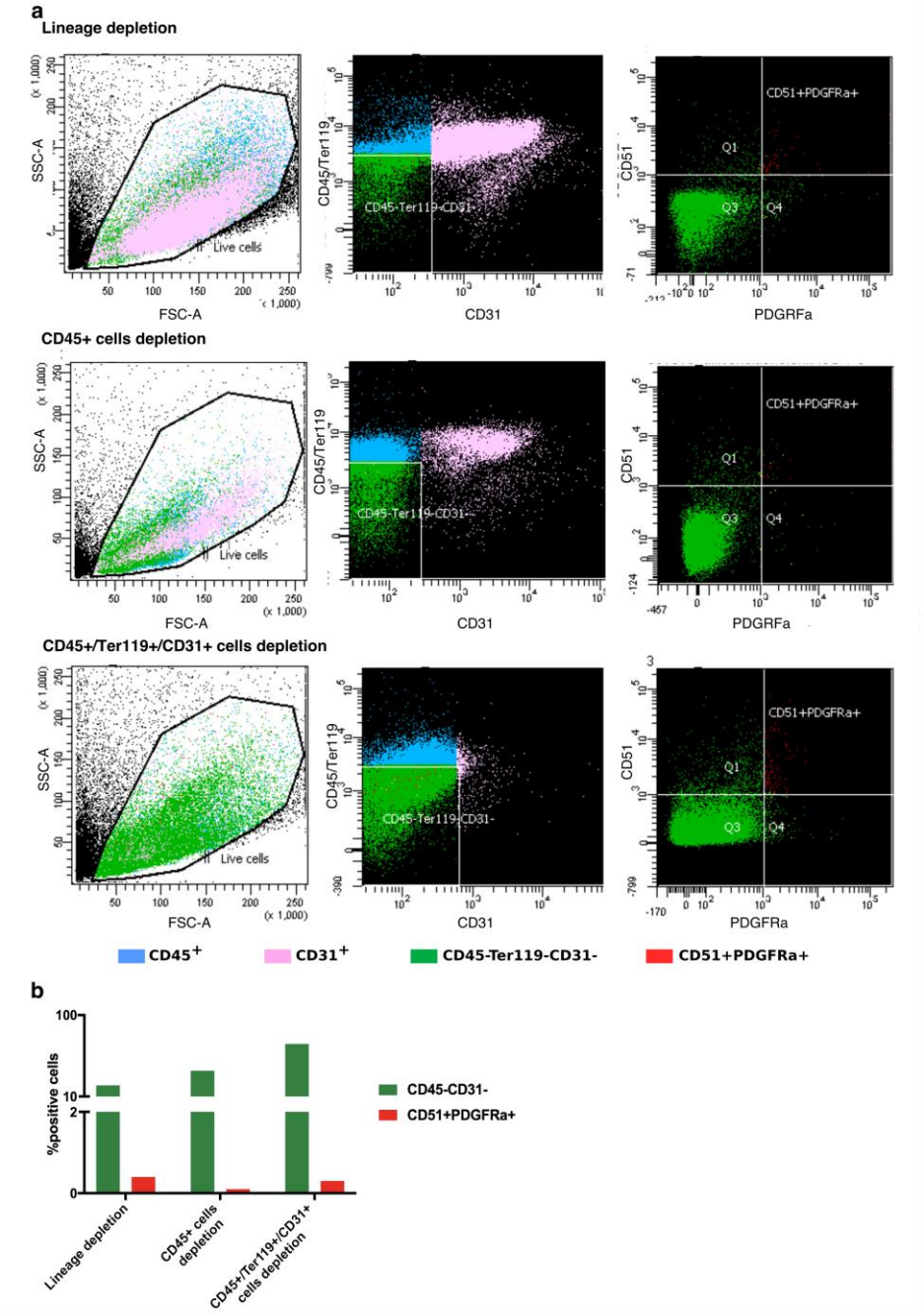

Figure 34 Enrichment of stromal cells by MACS a. Lineage depletion with lineage depletion kit of MACS Miltenyi, depletion of CD45 positive cells with CD45 beads from MACS Miltenyi, depletion of CD45+Ter119+CD31+ cells with homemade antibody cocktail **b.** Graphical representation of flow cytometry data.

3.2.2. Injection of different populations of bone marrow stromal cells into mice

After having isolated and characterized stromal cells from the bone marrow, the next step was to inject those cells into mice. In order to be able to trace the cells after injection, bone marrow stromal cells were isolated from mice that have luciferase expression in every tissue. To first test whether it was possible to monitor bone marrow stromal cells *in vivo*, cells from the whole bone marrow stromal population, mesenchymal stem cells, or bone marrow stromal cells having been depleted of mesenchymal stem cells were injected subcutaneously into wild type BALB/C mice. Figure 35 is showing images taken using IVIS, an *in vivo* imaging system, over three weeks after the injection. No signal above the threshold was detected

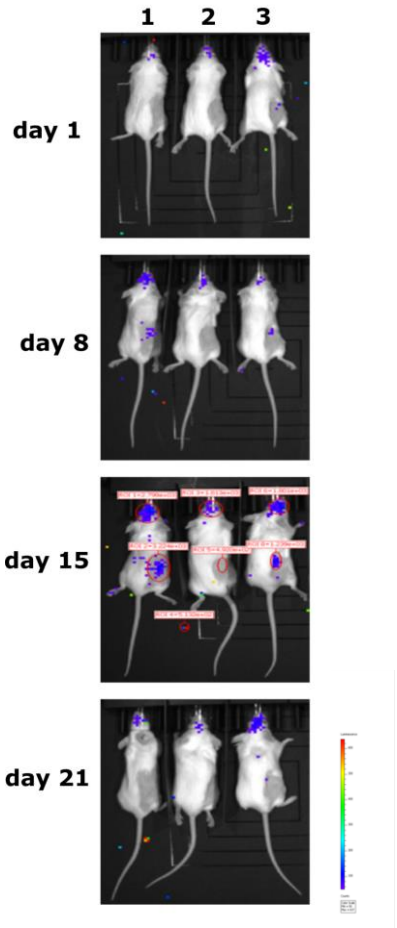

Figure 35 *In vivo* imaging of subcutaneous injection of bone marrow stromal cells Injection of different bone marrow stromal cell populations 1. $CD45^{-}Ter119^{-}CD31^{-}CD51^{+}PDGFRa^{+}$ **2.** $CD45^{-}Ter119^{-}CD31^{-}CD51^{-}PDGFRa^{-}$ **3.** $CD45^{-}Ter119^{-}CD31^{-}$.

To make sure that bone marrow stromal cells do not lose luciferase expression upon isolation, luciferase activity of freshly isolated bone marrow cells was determined using a luciferase assay. Figure 36 shows the luciferase activity of bone marrow stromal cells isolated from a wild type BALB/C (WT BALBc) mouse or three different luciferase reporter mice (L2G85 BALBc). While no luciferase activity was detected in bone marrow stromal cells of wild type mice, bone marrow stromal cells of the luciferase reporter mice had strong luciferase activity comparable with a breast cancer cell line in which the luciferase gene is stably overexpressed (MCF7_luc).

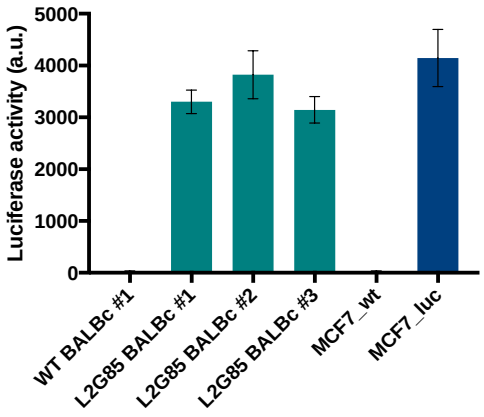

Figure 36 Detection of luciferase activity of bone marrow stromal cells In vitro luciferase assay of freshly isolated bone marrow stromal cells of different mice. The graph shows luciferase activity and the values are presented as mean ± SD of 3 technical replicates.

Having confirmed that the cells indeed expressed luciferase, I hypothesized that the subcutaneously injected cells might have disseminated under the skin. This might explain the lack of signals during in vivo monitoring. To test this hypothesis, intra-dermal and mammary fat pad injections with or without matrigel were carried out to prevent easy dissemination of the cells. However, again, *in vivo* imaging did not detect any signal above the background (Figure 37).

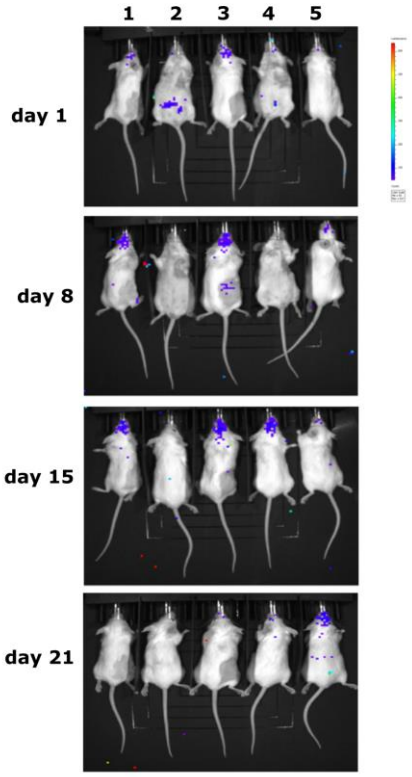

Figure 37 *In vivo* imaging of intra-dermal or mammary fat pad injection of bone marrow stromal cells Injection of $CD45^{-}Ter119^{-}CD31^{-}$ cells into different parts of the body **1.** Intradermal injection **2.** Intradermal injection with matrigel **3.** Mammary fat pad injection **4.** Mammary fat pad injection with matrigel **5.** No injection

After failing to detect a luciferase signal upon different injections, I next hypothesized that bone marrow stromal cells might survive better in their physiological microenvironment. Therefore, one more test round was performed with intra-tibial injection of cells. It was also speculated that presence of signals derived from cancer cells might support the maintenance of bone marrow stromal cells. Thus, mouse breast cancer cells, namely 4T1 cell line were injected into the mammary fat pat of mice and 7 days later, bone marrow stromal cells were injected either subcutaneously or into the tibia of the same mice. No signal above threshold was detected in any condition (Figure 38).

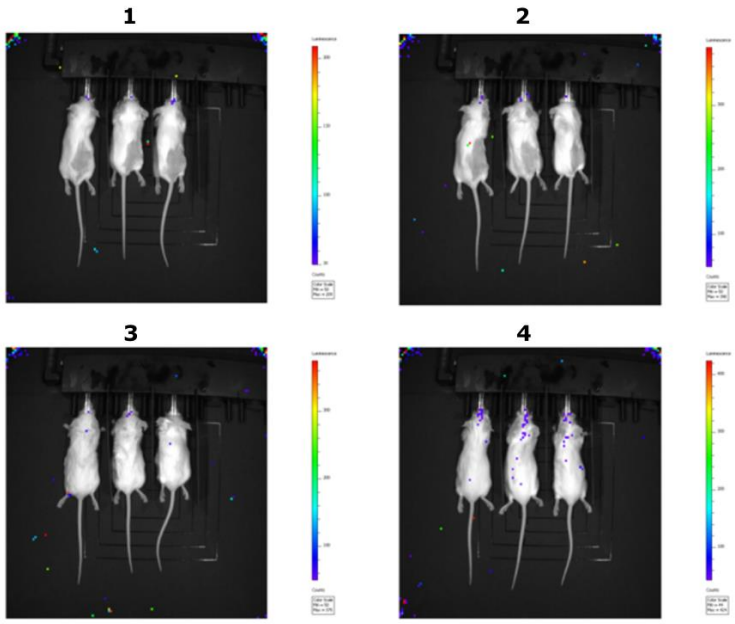

Figure 38 In vivo imaging of intra-tibia or subcutaneous injection of bone marrow stromal cells in tumor bearing mice Injection of $CD45^-Ter119^-CD31^-$ cells into mice that are bearing tumors formed by orthopedic injection of 4T1 cells **1.** 4T1 + $CD45^-Ter119^-CD31^-$ (subcutaneous) **2.** PBS + $CD45^-Ter119^-CD31^-$ (subcutaneous) **3.** 4T1 + $CD45^-Ter119^-CD31^-$ (intra-tibia) **4.** PBS + $CD45^-Ter119^-CD31^-$ (intra-tibia)

4. DISCUSSION

Breast cancer has remained one of the leading causes of cancer related death in women worldwide. It is a heterogeneous disease with several molecular subtypes. Identification and understanding the underlying biology of these molecular subtypes have resulted in the development of targeted therapies against specific molecules including tyrosine kinase inhibitors as well as antibodies targeting EGFR, HER2, c-MET and FGFR, inhibitors of intracellular signaling pathways targeting PI3K, mTOR and ERK, angiogenesis inhibitors and DNA repair interfering agents (Higgins & Baselga, 2011). However, resistance to these therapeutic agents and the disease relapse are still some of the major problems.

The tumor microenvironment has been recognized as a crucial part of tumor progression since tumor formation involves the co-evolution of neoplastic cells together with their microenvironment including extracellular matrix, tumor vasculature and immune cells (Junttila & de Sauvage, 2013). Therefore, understanding the role of the tumor microenvironment in cancer formation can reveal new targets for the development of new therapies. Since stromal cells in the tumor microenvironment are genetically more stable compared to the cancer cells, therapies targeting signaling molecules in the stromal part of tumor are likely to be less susceptible to the mechanism of therapeutic resistance (Quail & Joyce, 2013).

Cancer associated fibroblasts (CAF) are a dominant compartment of tumor stroma and they are involved in tumor progression by remodeling ECM and by metabolic and immune reprogramming of the tumor microenvironment (Kalluri, 2016). In the early tumor development, fibroblasts are recruited to the neoplastic legions as part of the host response to epithelial tissue injury that might reflect their role in the early antitumor response. This situation is similar to wound healing in which activated fibroblasts initiate the repair process and control and prevent further tissue damage. Moreover, in the case of wound healing, activated fibroblasts are also involved in inducing an intrinsic program that inhibits an excessive scarring response that could otherwise further injure the tissue. This program is failed to be initiated such as in the case of fibrosis and cancer, diseases associated with unabated fibroblast activation resulting in chronic inflammation (LeBleu & Kalluri, 2018). Although, the mechanisms behind this unabated activation of fibroblasts remain largely unknown, epigenetic reprogramming might be involved in this sustained activated state. It

has been shown that hypermethylation of the *RASAL1* promoter is associated with unabated fibroblast activation and fibrosis in the kidney (Bechtel et al., 2010). In addition it has been also demonstrated that CAFs have a global hypomethylation in their genome that might result in upregulation of genes associated with the CAF secretome (Jiang et al., 2008). In another study in dermal fibroblast it was reported that downregulation of the NOTCH protein effector CSL and p53 enables CAF activation and proliferation by overcoming the senescence (Procopio et al., 2015).

CAFs are a very heterogeneous population of cells that might be the outcome of the diverse origin of quiescent precursor fibroblasts. It was speculated that depending on their origin, activated fibroblasts could have diverse and unique functions in tumor progression (LeBleu and Kalluri, 2018). As mentioned in chapter 1.2.3.2., distinct subset of CAFs might have either cancer promoting or cancer retraining functions. Although, the early studies using CAFs isolated from solid tumors but expanded in vitro, showed mostly tumor promoting affects once re-introduced to mice together with cancer cells, it should be noted that tumor promoting CAFs, secreting pro-survival factors, might have a selective growth advantage over tumor inhibiting CAFs when cultured *in vitro* (Kalluri, 2016). Therefore, it is crucial to track the behavior of distinct CAF populations during different stages of tumor development *in vivo*. However, the lack of *in vivo* mouse models and imaging tools and the lack of specific markers to identify different CAF populations has remained a major challenge to study their diverse functions (LeBleu and Kalluri, 2018). Several studies targeting and depleting bulk CAF population in the tumor microenvironment for therapeutic purposes without having a precise information about subpopulations and their role in the tumor progression resulted in unexpected effects on tumor progression possibly due to the depletion of tumor restraining CAFs together with the tumor promoting ones (see chapter 1.2.3.2.). For this reason, a more effective therapeutic approach to target CAFs might be by perturbing the pathways that lead to the activation of fibroblasts instead of depleting the whole CAF population. For example, in pancreatic cancer, it has been demonstrated that vitamin D receptor ligand calcipotriol converted activated pancreatic stellate cells back to their quiescent state and reduced tumor volume when used in combination with a chemotherapeutic drug, gemcitabine, by reducing the tumor associated inflammation and fibrosis and increasing intra-tumoral gemcitabine (Sherman et al., 2014).

These findings emphasize that it is very important to explore the biology behind the activation of fibroblasts to unravel the molecular mechanisms behind it. Therefore, one of the aims of this PhD project was to understand how the changes in CAFs during the activation process are regulated at the molecular level and to identify signaling pathways that might be either directly leading this activation or might be involved in it by cross-talking with signaling pathways that have been already associated with the activation of fibroblasts.

4.1. Activation of MSCs upon CM treatment

In order to study the activation of fibroblasts *in vitro*, a triple negative breast cancer cell line MDA-MB-231 was used to activate bone marrow derived MSCs. MDA-MB-231 cell line was chosen due to the previous reports showing its effect on the transformation of MSCs to cancer associated fibroblast like cells (Mishra et al., 2008). MSCs were either exposed to conditioned media of MDA-MB-231 cells or indirectly co-cultured with them in a trans-well format. To assess the activation of MSCs, contractility of the cells was measured because increased fibroblast contractility is a major hallmark of CAFs and can be monitored by contraction of fibroblast-containing 3D collagen gels (Calvo et al., 2013). In addition to the contractility, changes in the expression of activated fibroblast markers namely, FAP and αSMA were determined. While the conditioned media treatment activated the MSCs, trans-well co-culture did not have any effect on the activation status of MSCs. This might be indicating that MDA-MB-231 secreted factors are potent enough to activate MSCs and MDA-MB-231 cells do not need any further stimulation by MSCs to secrete those required factors indicating that having no effect on the activation of MSCs upon indirect co-culture is due to technical reasons rather than the biological ones. These findings firstly demonstrate that factors secreted by MDA-MB-231 are sufficient to activate MSCs and that no feedback from MSCs to the tumor cells is necessary to stimulate secretion of those factors. Secondly, the experimental setup was likely responsible for the lack of MSC-induction in the trans-well assay, probably due to the different concentrations of factors. One of these might be having less concentrated conditioned media of MDA-MB-231 in the indirect co-culture settings due to having less starting cell number.

Conditioned media treatment was done for 7 or 14 days to be able to monitor possible gradual activation of MSCs. Indeed, flow cytometry analysis revealed that while FAP is upregulated at protein level even after 7 days of treatment, αSMA was upregulated only after 14 days. This might reflect that although some signaling pathways can be immediately activated by conditioned media treatment, activation of MSCs is a gradual process that requires continuous exposure to the stimuli.

Another very important point to discuss is the baseline activation of MSCs. Both collagen gel contraction assay and flow cytometry analysis of activated fibroblast markers revealed that MSCs are already activated to a certain extent in the normal culture conditions. This observation is in line with several studies that showed that artificial culturing conditions such as plastic cell culture dishes or presence of fetal bovine serum in culturing media is enough to activate the cells (Pal & Das, 2017). Hence, this baseline activation of MSCs reduced the dynamic range of the assay when measuring the effect of conditioned media treatment on the activation of MSCs. I could still identify major effects, validating my main focus on finding changes induced upon treatment with conditioned media. These included a upregulation of several cytokines, which have been shown to be secreted by CAFs, upon conditioned media treatment showing that although MSCs have already a baseline activation, conditioned media treatment still has an immense effect on MSCs.

4.2. Effect of activated MSCs on cancer cells

Since CAFs have been associated with almost all aspects of tumor progression in breast cancer (Qiao et al., 2016), another way to assess the activation of MSCs could be determining their effect on the phenotype of breast cancer cells. To this end, MSCs treated with media or conditioned media were directly co-cultured with different breast cancer cell lines and their proliferation was assessed. While, no change was observed in the growth of MDA-MB-231 and HCC1943 cells, the presence of MSCs had a positive effect on the growth of T47D, MCF7 and HS578T cell lines. However, the only difference between media or conditioned media treated MSCs was observed in HS578T cells. These differences might be explained by the fact that these cell lines are models of different breast cancer subtypes. HS578T cells are the only cells in this panel belonging to the mesenchymal sub-subtype of triple negative breast cancer. Therefore, this experiment

could be repeated using another mesenchymal cell line such as BT549 to test this hypothesis.

In addition to the breast cancer cell growth, other important phenotypes that can be affected by activated fibroblasts are migration and invasion ability of the breast cancer cell lines because one of the most important characteristics of CAFs is their supporting role in cancer metastasis (Gaggioli et al., 2007; Scherz-Shouval et al., 2014). When MDA-MB-231 cells were exposed to the conditioned media of activated MSCs, there was no difference in the invasion or migration ability compared to media-treated MDA-MB-231 cells (data not shown). This could be because of high baseline invasion and migration ability of this cells. To assess whether conditioned media-treated activated MSCs have a role in cancer cell motility, less motile luminal cell lines such as T47D and MCF7 could be also tested. It is important to note that CAFs in the tumor microenvironment affect tumor progression not only by interacting with cancer cells but also with immune cells and endothelial cells. Therefore, in addition to determining the impact of activated MSCs on cancer cell phenotype, effect of MSCs on immune cells could be also analyzed.

4.3. Methylation, gene expression and secretome analysis of activated MSC

Since it has been reported that epigenetic reprogramming might be involved in the activation of fibroblasts (Bechtel et al., 2010; Jiang et al., 2008), the changes in DNA methylation pattern of MSCs upon conditioned media treatment were analyzed at a global scale. Even with 14 days of CM treatment, out of $8.5x10^5$ tested probes, only up to 2% of them were differentially methylated. This might be due to short duration of conditioned media treatment. DNA methylation changes are introduced in the genome by successive cell divisions, however during the conditioned media treatment MSCs were passaged only once which might be a limiting factor for the detection of possibly induced DNA methylation changes.

In addition to the methylation analysis, expression analysis by RNA sequencing was performed. Pathway enrichment analysis was done using RNA sequencing data and it identified TNFα signaling, chemokine signaling pathways, cytokine-cytokine receptor interaction and NFκB signaling pathway as top regulated pathways both after 7 and 14 days of conditioned media treatment. This indicates an upregulated proinflammatory gene

signature that is in line with the upregulation of various cytokines. One of the hallmarks of cancer is the tumor promoting inflammation (Hanahan & Weinberg, 2011). Inflammation is associated with activation of the canonical NF-κB pathway (Barnes & Karin, 1997). Genes of many pro-inflammatory cytokines including but not limited to TNFα, IL-1, IL-6 and IL-8 have been shown to have NF-κB binding sites in their respective promoter regions and activation of NF-κB pathway has been reported to be crucial for their expression (Bonizzi & Karin, 2004). These cytokines can promote tumor progression by inducing angiogenesis, cancer cell proliferation and metastasis and they also maintain the inflammatory tumor microenvironment (Grivennikov & Karin, 2011). CAFs have been several times implicated as novel mediators of tumor promoting inflammation (Servais & Erez, 2013). Moreover, it is has been demonstrated that inflammatory features associated with CAFs are dependent on NF-κB signaling (Erez et al., 2010). Therefore, my observation of upregulation of inflammatory pathways upon conditioned media treatment is in line with the inflammatory characteristics of CAFs.

Due to the capability of CAFs to secrete vast number of soluble factors and extracellular matrix components, secretome analysis was done using mass spectrometry, in collaboration with Prof. J. Krijgsveld, to identify differentially secreted proteins upon conditioned media treatment. Several proteins identified as being secreted more upon activation of MSCs had previously been reported as CAF secreted factors such as IL6 (Kato et al., 2018), MMP1 (Cui et al., 2018), MMP13 (Folgueira et al., 2013) and GAS6 (Kanzaki et al., 2017). Since I wanted to study new factors that have a role in the activation of fibroblasts, I followed up several upregulated target genes. Several like CHI3L1, CHI3L2, GREM1 and TGM2, did not show any effect on the activation status of MSCs upon their knock-down by siRNAs (data not shown). The reason behind this could be their upregulation as a result of activation of MSCs without having a role as the driver of this activation. Moreover, they might be upregulated as a response to a factor in the conditioned media without being involved in the activation of MSCs. Another target, STC1, was selected because it was among the top differentially secreted proteins in mass spectrometry analysis and was also detected to be upregulated in expression analysis.

4.4. Stanniocalcin 1

Stanniocalcin (STC) was first discovered in bony fish as a glycosylated peptide produced by endocrine glands in the kidney (Wagner et al., 1986). The protein is expressed and secreted as a response to increased calcium concentration in water (Ellis & Wagner, 1995) and inhibits calcium uptake via inhibition of epithelial calcium channel mRNA expression (Tseng et al., 2009). In mammals, two members of the STC family have been identified (A. C. Chang et al., 1995; A. C. Chang & Reddel, 1998). *STC1* has 61% sequence identity with fish *STC* and *STC1* has 30% sequence identity with *STC2* (Yeung et al., 2012).

In the first in vivo study in mice that explored the role of STC1 in bone formation, when *SCT1* was expressed under the control of a muscle specific promoter, dwarf morphology with decreased bone formation was observed in mice (Filvaroff et al., 2002). Although similar to its fish homolog, recombinant human STC1 has inhibitory effects on calcium uptake in human cells (Zhang et al., 2000; Sheikh-Hamad et al., 2003), *STC1* knock-out mice (-/-) did not have any phenotype (A. C.-M. Chang et al., 2005) indicating that mammalian STC1 is not involved in calcium homeostasis and that the fish and mammalian STC proteins are not functional orthologs.

STC1 is expressed in wide variety of tissues including heart, lung, kidney, femur and ovary (A. C. Chang et al., 1995) and has been reported to be involved in diverse biological processes such as osteogenesis (Yoshiko et al., 2002) , inflammation (Kanellis et al., 2004) and cellular metabolism (McCudden et al., 2002). STC1 was demonstrated to be upregulated as a response to hypoxia (Zhang et al., 2000). Moreover, STC1 secreted by MSCs has been shown to have inhibitory effects on apoptosis that is induced by hypoxia (Block et al., 2009) or by reactive oxygen species (Ohkouchi et al., 2012). This observation might be explained by its localization in mitochondria upon internalization where it induces mitochondrial uncoupling proteins (UCPs) that in turn reduce the formation of reactive oxygen species (Y. Wang et al., 2009).

STC1 has been reported to be differentially expressed in paired human tumor and normal tissues with an upregulation in several types of cancer including colorectal cancers, hepatocellular carcinomas, non-small cell lung cancer, ovarian cancer, leukemia, and breast carcinoma (Chu et al., 2015) and to be associated with tumor progression in renal (Ma et al., 2015) breast (Welcsh et al., 2002) and ovarian cancers (Liu et al., 2010). It has been

shown to be involved in tumor growth, epithelial-mesenchymal transition and apoptosis (Chu et al., 2015).

There are also several studies that have reported STC1 as a CAF-secreted factor and have associated the protein with metastasis in colorectal carcinoma (Peña et al., 2013) and with therapy resistance in breast cancer (Roswall et al., 2018). However, the molecular mechanisms behind the roles of CAF secreted STC1 have not been elucidated.

4.5. Role of STC1 in the activation of MSCs and CAFs

In order to investigate the role of STC1 on activation of MSCs upon conditioned media treatment, *STC1* was down-regulated in MSCs using siRNAs before treatment with conditioned media. It was observed that *STC* knock-down not only decreased the contraction ability of MSCs but also reversed the upregulation of FAP in MSCs treated with the conditioned media for 7 days as well as the upregulation of αSMA in MSCs treated with the conditioned media for 7 or 14 days. Moreover, *STC1* knock-down also downregulated baseline αSMA expression. It has been reported that expression of αSMA is correlated with the contractility of fibroblasts (Shinde et al., 2017; B Hinz et al., 2001) however, there are also other factors involved in the contraction of these cells such as activation of transcriptional regulator YAP (Calvo et al., 2013). Therefore, STC1 could inhibit the contraction of activated MSCs, however, that this might not be a direct effect of αSMA downregulation.

To test the effect of *STC1* know-down on already activated patient-derived CAFs, baseline *STC1* expression was determined in different primary CAFs having been isolated from different patient tumors. Two CAF lines, namely CAF1 and CAF2 having highest expression of STC1, were chosen for further experiments. While *STC1* knock-down in these cells reduced the contractility of the CAF1 cell, it did not have any effect of CAF2 cells. In line with this observation, *STC1* down-regulation resulted in downregulation of αSMA in CAF1 cells but did not cause any change in CAF2 cells. This could be due to lower baseline levels of αSMA in and lower contractility of CAF2 compared to CAF1 cells. These results suggest that the activation level of CAFs is important to see an effect of *STC1* knock-down. Furthermore, it should be noticed that CAF1 cells express higher levels of STC1 compared to CAF2 cells which could further support a role of STC1 in the

activation of fibroblasts. However, to test this hypothesis, more CAFs from different tumors should be examined.

Using CAF1 cells, the effect of STC1 on another characteristic of activated fibroblasts was tested. *STC1* knock-down decreased the number of invading and migrating CAFs. In addition to their impact on cancer cell migration and invasion, migration and invasion are also important features for CAFs. It has been recently shown that CAFs exert a physical force via CAF-cancer cell adhesion and promote a cooperative collective invasion or migration of CAFs and cancer cells (Labernadie et al., 2017).

All in all, it could be summarized that due to its effect on contractility, αSMA expression, migration and invasion in CAFs, STC1 could have a role in the activation of fibroblasts. This presumed role of STC1 in the activation of fibroblasts does not fit with the results from a previous study where the inhibitory effects of recombinant STC1 in bleomycin-induced idiopathic pulmonary fibrosis by decreasing collagen deposition and intracellular ROS amounts was shown (Ono et al., 2015). In the same study, it was demonstrated that STC1 expression can be induced by TGFβ in MSCs and when MSCs injected in mice, they have the same effect on fibrosis as recombinant STC1 does. While the observed inhibitory effect on fibrosis might imply an inhibitory role of STC1 on the activation of fibroblasts, this study does not mention the effects of STC1 in the activated fibroblasts that are already in the fibrotic area. Therefore, the difference in the experimental setup and lack of evidence of direct effects of STC1 on the cells within fibrotic areas suggests that further studies need to be carried out to understand how STC1 is involved in the activation of fibroblasts.

4.6. Regulation of STC1 by NF-κB signaling

STC1 upregulation in MSCs treated with conditioned media was rescued almost 70% when two transcription factors of NF-κB signaling, RELA and RELB, were down-regulated using siRNA. This observation is in line with the study in which it has been shown that the *STC1* promoter has two putative binding sites for RELA and the transcriptional regulation of *STC1* by RELA was confirmed by chromatin immunoprecipitation (Law et al., 2008). Since STC1 upregulation by conditioned media treatment was indeed rescued with the downregulation of both RELA and RELB, it can be speculated that conditioned media treatment is inducing both canonical and non-canonical NF-κB signaling since RELA and

RELB take part in either one respectively (Shao-Cong Sun, 2018). These two pathways can be induced by the same or different ligands; therefore, it is hard to speculate about the factor(s) in the conditioned media inducing NF-κB signaling. Moreover, although it has been shown that RELA can directly bind to the promoter of STC1, upregulation via conditioned media might be also induced by NF-κB signaling indirectly as a result of the activation of the pathway. To address this question, binding of RELA or RELB to the *STC1* promoter with or without conditioned media treatment should could be determined by chromatin immunoprecipitation assay. If there is no direct binding, different transcription factors that might be activated by NF-κB signaling with a potential to bind to the STC1 promoter could be examined. Moreover, rescue of STC1 upregulation via RELA or RELB knock-down was not complete. This might be because of either that STC1 upregulation is controlled also by other means or knock-down level for RELA and RELB is not efficient enough to result in a full rescue. However, it can be still concluded that STC1 upregulation upon conditioned media treatment is an outcome of a proinflammatory programming that results in changes of inflammatory pathways of activated MSCs.

4.7. Pathways regulated by STC1 knock-down

In order to identify the molecular mechanism by which STC1 plays a role in the activation of fibroblasts, the pathways that are differentially activated upon STC1 knock-down were determined by RPPA. There were indeed changes in the expression of proteins of the Notch and Wnt signaling pathways. Two receptors of Notch signaling, NOTCH3 and NOTCH4 were found to be down-regulated. Notch signaling is involved in diverse biological process from embryonic development and stem cell regulation to heart morphogenesis and cancer. Upon ligand binding, receptors of the NOTCH family undergo proteolytic cleavage, releasing Notch intracellular domains of the receptors (NICD) which enter the nucleus and stimulate the transcription of target genes (Bray, 2016). Several studies have mentioning the involvement of NOTCH signaling in the activation of fibroblasts: it has been demonstrated that stimulation of dermal fibroblasts with recombinant Jag-1, a Notch ligand, results in gaining myofibroblast like phenotype and increased collagen release (Dees et al., 2011). In another study in oral squamous cell carcinoma, NOTCH3 expression in CAFs has been associated with poor prognosis (Kayamori et al., 2016). Therefore, it can

be speculated that STC1 knock-down by down-regulating Notch signaling can attenuate fibroblast activation. Interestingly, there is a publication demonstrating STC1 as a non-canonical NOTCH ligand and showing its interaction with extracellular domain of NOTCH1 thereby regulating stemness in glioblastoma (Li et al., 2018). Therefore, Notch signaling could be a possible target to explain the role of STC1 in fibroblast activation. In order to study this, Notch pathway inhibitors in the context of STC1 overexpression could be tested.

Another protein that was determined to be down-regulated by STC1 knock-down was β-catenin. β-catenin is the central protein in Wnt signaling pathway. Wnt signaling has been already associated with the activation of fibroblasts (see chapter 1.2.2.) mostly by cross-talk with the TGFβ pathway. STC1 knock-down also resulted in a decreased in the phosphorylated levels of SMAD3 protein. Therefore, it can be hypothesized that STC1 knock-down can cause inhibition of fibroblast activation by downregulating β-catenin thereby inhibiting TGFβ signaling. To test this hypothesis, effect of STC1 overexpression on the activity of Wnt signaling pathway could be analyzed. The putative involvement of STC1 in the Wnt pathway has recently been suggested as *STC1* seems to be a Wnt3a target gene in the context of osteogenesis (Sebastian et al., 2017).

4.8. STC1 expression in breast cancer patient datasets

Possible role of STC1 in the activation of fibroblasts opens up several research questions about localization of its expression in the tumor microenvironment. Although, STC1 has been several times shown to be a CAF secreted factor *in vivo*, most of the breast cancer cell lines also express it to some extent. Therefore, it is important to understand which compartment of the tumor expresses STC1. For this, breast cancer patient data sets which have separate information of stroma and epithelium of tumor samples were analyzed. Despite of having significantly more *STC1* expression in the tumor stroma of DCIS compared to the epithelium of DCIS in one data set (GSE14548), in another one this difference was not observed (GSE35019). Moreover, when *STC1* expression was compared between normal stroma and tumor stroma, although a significant difference was detected in one data set (GSE9014), it was not the case in another one (GSE5847). These

differences might be the result of small cohorts of these data sets and to have more concrete data, more patient data sets with bigger cohorts could be analyzed.

4.9. In vivo tracking of MSCs recruited from bone marrow to the tumor microenvironment

When this project started in 2015, there was no *in vivo* proof of recruitment of bone marrow stromal cells from bone marrow to the breast cancer microenvironment. Bone marrow stromal cells had been shown to be a heterogeneous group of cells. Therefore, I hypothesized that if bone marrow stromal cells are indeed migrating to the tumor microenvironment in breast cancer, different populations might have different behavior in this migration process. In order to address this question, bone marrow cells injected into tibia of mice were attempted to be monitored using their luciferase expression. However, the cells were never able to being detect either in tibia or in anywhere else in the mice. There could be several reasons for this. First of all, although it was shown that bone marrow stromal cells were expressing luciferase upon isolation, once they were injected into mice, they might have stopped expressing luciferase due to several reasons such as inactivation of the genomic locus of luciferase gene. Also, luciferase expressing cells might have generated an immune response and have been cleared up by immune cells. Although there are publications showing engraftment of stromal cells when injected directly in the bone marrow, due to several possible factors, engraftment might have failed. A better strategy for this could be performing a bone marrow transplantation instead of injecting bone marrow stromal cells alone that might facilitate their survival. Indeed, a recent study using bone marrow transplantation strategy has demonstrated bone marrow stromal cells to be a substantial source for CAFs in the breast cancer microenvironment (Yaz et al., 2018), thus validating my original concept.

5. CONCLUSIONS AND OUTLOOK

In conclusion, I have shown that bone marrow derived MSCs can be activated by treating them with conditioned media of MDA-MB-231 cells. Conditioned media treatment induced a transcriptional reprogramming in which inflammation related genes were upregulated. STC1 was identified as an upregulated gene upon activation of MSCs and this upregulation was controlled by two transcription factors of NF-κB signaling, RELA and RELB indicating that it might be also the result of the inflammatory response. The role of STC1 on the activation of MSCs as well as patient derived CAFs was demonstrated by down-regulating its expression. Furthermore, this down-regulation resulted in a decreased in the expression of several proteins such as NOTCH3, NOTCH4 and β-catenin suggesting possible mechanisms by which STC1 regulates the activation of fibroblasts.

Activation of MSCs upon conditioned media treatment was assessed with increased contractility and increased expression of two CAF markers, FAP and αSMA. To demonstrate that the activation of MSCs has an effect on tumor progression, the influence of activated MSCs on cancer cells and/or immune cells will be investigated *in vitro*.

There is evidence that STC1 upregulation in activated MSCs upon conditioned media treatment is regulated by RELA and RELB. To confirm NF-κB pathway is directly responsible for this upregulation, direct binding of RELA and RELB in the promoter region of STC1 will be explored.

While the regulatory role of STC1 in the activation of fibroblasts was demonstrated, the molecular mechanism behind this is not clear. To address this, the involvement of Notch and Wnt signaling in the activation of fibroblasts as well as their interaction with STC1 will be studied.

Gaining further insights into the activation of fibroblasts could offer a deeper understanding of their origin, plasticity and function in the tumor microenvironment and could provide new therapeutic targets. Investigating the role of STC1 in the unabated activation of CAFs will enable a deeper understanding of the tumor microenvironment and its role in the tumor progression and aid in the development of better therapeutic strategies.

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ABBREVIATIONS

AHA L-Azidohomoalanine

ATP Adenosine triphosphate

BM Bone marrow

BMPR Bone morphogenic protein receptor

BRCA Breast Cancer

CAF Cancer associated fibroblast

CBP CREB binding protein

CC Co-culture

CCL C-C Motif Chemokine Ligand

CCND1 Cyclin D1

CM Conditioned media

CSF1 Colony Stimulating Factor 1

CTGF Connective tissue growth factor

CXCL Chemokine (C–X–C motif) ligand

CO2 Carbon dioxide

cDNA Complementary deoxyribonucleic acid

D Donor

DNA Deoxyribonucleic acid

DEG Differentially expressed genes

DMP Differentially methylated probes

DMSO Dimethyl sulfoxide

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EGF Epidermal growth factor

EGFR Epidermal growth factor receptor

ELISA Enzymed-linked immunosorbent assay

EMT Epithelial-mesenchymal transition

ER Estrogen receptor

ERBB2 Erb-B2 Receptor Tyrosine Kinase 2

ESR1 Estrogen Receptor 1

FAP Fibroblast activation protein alpha

FBS Fetal Bovine Serum

FC Fold change

FSP1 Fibroblast specific protein 1

FGF Fibroblast growth factor

GTP Guanosine triphosphate

HER2 Human Epidermal Growth Factor Receptor 2

HSC Hematopoietic Stem Cells

HIF1α Hypoxia inducible factor 1α

IL Interleukin

IκB Inhibitor of kappa B

LOX Lysyl Oxidase

M Media

MAC-1 Macrophage 1 Antigen

MC Mono-culture

MPP Matrix metalloproteinases

MAPK Mitogen-Activated Protein Kinase

MPER Mammalian Protein Extraction Reagent

mRNA Messenger ribonucleic acid

MSC Mesenchymal stromal cells

mTOR Mechanistic Target of Rapamycin Kinase

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

NaF Sodium Fluoride

NEAA Non-essential amino acids

TP53 Tumor protein 53

PARP Poly (ADP-Ribose) Polymerase

PBS Phosphate buffered saline

PDGF Platelet derived growth factor

PDGFR Platelet derived growth factor receptor

PD-1 Programmed cell death protein

PD-L Programmed cell death protein ligand

PenStep Penicillin/Streptomycin

PGE Prostaglandin E synthase

PI3K Phosphatidylinositol-4,5-Bisphosphate 3-Kinase

PR Progesterone receptor

PTEN Phosphatase and Tensin Homolog

PUM1 Pumilio RNA Binding Family Member 1

qRT-PCR Quantitative reverse transcriptase polymerase chain reaction

RNA Ribonucleic acid

RNAi Ribonucleic acid interference

ROS Reactive oxygen species

RPPA Reverse phase protein array

RT Room temperature

RTCA Real time cell analyzer

SD Standard deviation

SILAC Stable isotope-labeled amino acids

siRNA Small interfering ribonucleic acid

TBST Tris buffered saline with Tween 20

SBE Smad binding elements

STC1 Stanniocalcin 1

TAM Tumor associated macrophage

TCGA The Cancer Genome Atlas

TGFβ Transforming growth factor beta

TGFβR Transforming growth factor beta receptor

TIL Tumor infiltrating lymphocytes

TIMP Tissue inhibitor of metalloproteinase

TNBC Triple negative breast cancer

TNFα Tumor Necrosis Factor

Tregs Regulatory T cells

TW Trans-well

VEGF Vascular endothelial growth factor

αSMA alpha smooth muscle actin

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