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Differentiation of mesenchymal stem cells from the adipose tissue into carcinoma-associated myofibroblasts in interaction with human breast cancer cells

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1. Introduction

1.1 Breast cancer

Breast cancer is the most common female cancer in the world and the leading cause of cancer death among females both in economically developed and developing countries, resulting in nearly 1.4 million new cases (23% of the total new cancer cases) and approximately 460,000 cancer deaths (14% of all cancer deaths) in 2008. Although breast cancer death rates have been decreasing in the last two decades mainly because of major advances in understanding, diagnosing and treating this disease, several unresolved problems remain (Jemal, 2011). Even in highly developed countries such as the United States, breast cancer is the second most common malignancy among females as well as the second leading cause of cancer death in females with about 230,500 new cases and 39,500 breast cancer deaths expected for 2011 (DeSantis et al., 2011).

Female gender, age, family history and genetic mutations in the breast cancer 1 (BRCA1) or BRCA2 genes are the strongest risk factors for breast cancer. Further risk factors include early menarche or late menopause, nulliparous, oral contraceptives and hormone replacement therapy, increased breast density, lack of physical activity as well as alcohol consumption (Hulka and Moorman, 2008).

Unfortunately there are no early symptoms in breast cancer. Breast lumps as localized, palpable indurations or swellings of the breast are usually the first noticeable sign of an already advanced carcinoma. In economically developed countries most breast carcinomas are diagnosed as a result of an abnormal mammogram without any clinical symptoms at that time. Nevertheless, in patients with locally advanced breast cancer and inflammatory

breast cancer characteristic clinical signs can be observed (Giordano, 2003; Singletary and Cristofanilli, 2008).

Today most breast carcinomas are diagnosed as a result of abnormal mammography. When a suspect correlate is found in the mammogram further breast imaging characterization such as spot compression views and ultrasonography is performed to decide the need for breast biopsy. All primary breast tumors have to be assayed for estrogen (ER) and progesterone (PR) hormone receptors since hormone receptor-positive tumors have been shown to benefit from endocrine treatments with ER-antagonists such as tamoxifen or aromatase inhibitors. Furthermore it is being suggested that all primary breast cancers be assayed for the expression of human epidermal growth factor receptor 2 (HER2). Around 20% of breast cancer patients show HER2 overexpression and can benefit from treatment with trastuzumab, a monoclonal antibody that interferes with HER2 (Hayes, 2011).

In general the treatment of breast cancer is highly complex. With the goal being a tailored approach, the selection of a therapeutic strategy depends on clinical factors, TNM classification, morphological diagnosis, immunohistochemical assessment and recently also on molecular typing. In early stage breast cancer, treatment of the locoregional disease includes breast conserving surgical therapy (BCT) together with radiation therapy. Additionally, an adjuvant or neoadjuvant systemic therapy needs to be performed. Depending on the precise breast cancer classification, therapy is performed with endocrine drugs, one or a combination of chemotherapeutic agents and/or biological drugs (Hayes, 2011).

While major advances have been achieved in the treatment of early breast cancer, the management of metastatic breast cancer remains extremely challenging even with the availability of new and more efficient drugs, which have to some extent improved the patients' median survival (Gennari et al., 2005; Chia et al., 2007). Still, the majority of women with metastatic breast cancer will die of the disease.

In the last years major contributions have been made towards a better understanding of cancer biology. Nonetheless, the exact origin and etiology of breast cancer remains to be elucidated. It is now known that breast cancer is a heterogeneous disease with different tumor subclasses defined by gene expression patterns. The use of DNA microarray analysis, apart from morphological and immunohistochemical assessment, revealed a heterogeneous gene expression profiling of breast cancer. Elaborated studies have shown that there are five molecular tumor subclasses with different clinical outcomes including normal breast-like, basel-like, luminal A, luminal B and HER2+/ER- breast cancer with the main subtypes being the luminal A and the basel-like type (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2006). The tumor subtype-specific events as well as the cell of origin of these subtypes are being intensely studied and remain controversial (Visvader, 2011).

Breast cancer progression from atypical hyperplasia via ductal carcinoma in situ and invasive ductal carcinoma to subsequent metastatic disease is tremendously complex and poorly understood. Some studies have addressed the question of cancer progression by analyzing differences in geno-phenotypic patterns of breast carcinomas at different stages and propose a branching molecular evolutionary model for the development and progression of breast cancer (Shackney and Silverman, 2003). Yet this hypothetical cancer model, which is focused mainly on genetic profiling of epithelial cancer cells and does not take into account the potential role of the surrounding tumor microenvironment, cannot sufficiently explain breast cancer progression.

Our understanding of breast cancer progression and metastasis lags far behind and only a perception of breast cancer as whole entity consisting of “the cancer cell” as well as its microenvironment will allow scientists to understand the cancer continuum from initiation to metastasis and to eventually develop more efficient strategies in the battle against this fatal disease.

1.2 The tumor-stroma microenvironment

In the late 19th century Stephen Paget was the first scientist to hypothesize that both the cancer cell and its microenvironment are important for the spreading of cancer. Paget studied over 700 autopsy records of fatal breast cancer and noted that metastases were not randomly distributed in the body. Instead they were found more often in certain organs, suggesting that those organs provided better conditions for secondary cancer growth. Hence he hypothesized that even if “The best work in the pathology of cancer is now done by those who are studying the nature of the seed […]” (i.e. the cancer cell), the “[…] observation of the properties of the soil" (i.e. the surrounding) "may also be useful.” (Paget, 1889).

Ever since, researchers are studying the so-called “seed and soil” theory of cancer, trying to understand the molecular interactions of a tumor with its stroma. Especially in the last years it has become apparent that while tumor epithelial cells with their transforming genetic and epigenetic events are essential for the initiation of breast cancer and other cancers, a variety of cells of the surrounding microenvironment actively influence tumor progression and its hallmarks - proliferation, migration and invasion (Liotta and Kohn, 2001; Allinen et al., 2004).

In breast cancer and other cancers the stromal microenvironment, also referred to as “reactive stroma”, is defined as the extracellular matrix (ECM) together with the non-malignant cells surrounding the tumor. Although the cells that populate the stroma are not neoplastic, they seem to influence tumor cell behavior. These cells range from vascular cells (e.g. pericytes and endothelial cells), immune and inflammatory cells (e.g. lymphocytes, macrophages and monocytes) to reactive stromal fibroblasts (Liotta and Kohn, 2001). The neoplastic epithelial cancer cells and those of the microenvironment are in constant “conversations” and there is active recruitment of stromal cells into the tumor. The stroma cells produce additional tumor growth factors, cytokines, chemokines and matrix

metalloproteinases that essentially contribute to tumor progression. These stromal cells and the substances they produce do not only offer a promising target for new anti-cancer drugs, but could also serve as a source for novel biomarkers apart from those currently used, which are mostly expressed by the cancer cells themselves (Sund and Kalluri, 2009).

1.3 Carcinoma-associated fibroblasts within tumor stroma

From the variety of cells populating the stromal microenvironment the importance of reactive stromal fibroblasts (also called carcinoma-associated (myo)fibroblasts [CAFs]) in supporting tumor progression has been pointed out intensively in the past years (Kunz-Schughart and Knuechel, 2002; Kalluri and Zeisberg, 2006; Angeli et al., 2009). Although carcinoma-associated fibroblasts are a heterogeneous cell population, they consist mainly of myofibroblasts, which is why CAFs are often referred to as tumor-activated myofibroblasts. These cells differ from normal fibroblasts not only by their phenotype but also due to their production of various growth factors, chemokines, cytokines and proteins of the ECM, all known to possess tumor-promoting functions (Orimo and Weinberg, 2007; Ostman and Augsten, 2009).

Myofibroblasts have originally been described in skin wounds where they contract the stroma, bringing the epithelial borders closer together and thereby facilitate wound healing (Grinnell, 1994). However, apart from their importance in wound healing, myofibroblasts also play a crucial role in carcinomas, which Harold Dvorak referred to as “wounds that never heal” (Dvorak, 1986). Indeed wound healing and carcinomas seem to have quite remarkable molecular similarities since both, the process of wound healing as well as the

development and progression of cancer, rely on constant interactions between epithelial cells and the surrounding stroma (Schafer and Werner, 2008).

Tumor-activated myofibroblasts/CAFs are characterized as large, spindle-shaped cells defined by stress fibers, well developed cell-matrix-interactions and the expression of different immunohistochemical markers, most importantly alpha smooth muscle actin (α-SMA), which is used as the main detecting marker in most studies on the subject of CAFs. α-SMA, initially described in cells of smooth muscle lineage, is one of six different actin isoforms in mammalians. It is a major constituent of actin filaments forming the cytoskeleton which enables cell-contraction and locomotion (RonnovJessen and Petersen, 1996). It is known that α-SMA expressing myofibroblasts are abundant in most invasive human breast cancers (Sappino et al., 1988) and other epithelial carcinomas such as that of the prostate, colon, lung and uterus where they form reactive stroma (Orimo et al., 2001). Moreover, translational studies of the tumor stroma in different carcinomas including breast and colorectal cancer have demonstrated that α-SMA positive CAFs can reflect disease outcome or recurrence (Tsujino et al., 2007; Yazhou et al., 2004).

Orimo and his group studied the functional contributions of these altered fibroblasts in breast carcinoma. They found that CAFs present in invasive human breast cancer stimulate tumor growth and recruit circulating endothelial progenitor cells (EPCs) into the tumor furthering neoangiogenesis through secretion of stromal cell-derived factor 1 (Orimo et al., 2005) that binds to the C-X-C chemokine receptor 4 (CXCR4) expressed on cancer cells (Muller et al., 2001). Physiologically, the homeostatic chemokine stromal cell-derived factor 1 (SDF-1) mainly regulates hematopoietic cell trafficking and lymphoid tissue architecture. However within multiple malignancies the SDF-1/CXCR4 axis can be deregulated, leading to the promotion of cancer cell migration and metastasis (Teicher and Fricker, 2010).

Moreover, CAFs affect the invasive potential of cancer cells by providing multiple proinvasive factors through direct cell-cell contacts as well as paracrine signaling (De et al., 2008).

One important invasive-growth promoting factor specifically present in tumor-activated myofibroblasts is the hexameric extracellular matrix glycoprotein tenascin-C, one of four members of the ECM family of tenascins, and a well established cell marker for CAFs apart from α-SMA. Tenascins are typically expressed at sites of tissue remodeling as seen during wound repair and neovascularization or in pathological states such as inflammation or tumorigenesis and modulate the cell adhesion and migration (Hsia and Schwarzbauer, 2005). Tenascin-C produced by myofibroblasts *in vitro* has been shown to facilitate tumor cell invasion in human colon cancer (De et al., 2004) and a recently performed mouse study by O´Connell et al. proved that tenascin-C plays a crucial role in the metastatic colonization of murine breast cancer cells *in vivo* (O'Connell et al., 2011). Additionally, high expression levels of tenascin-C in patients with breast cancer have been reported to correlate with poor disease outcome (Suwiwat et al., 2004).

A further important proinvasive factor provided by the tumor stroma is the chemokine C-C motif ligand 5 (CCL5). Current studies indicate that this inflammatory chemokine is being secreted by tumor-associated fibroblasts in reactive stroma of breast cancer as well as by bone marrow derived stem cells (BMSCs) when admixed with breast cancer cells and promotes tumor-enhancing activities (Karnoub et al., 2007; Mishra et al., 2011). It was shown that human adipose tissue derived stem cells interacting with breast cancer cells can be a source of CCL5 which enhances cancer cell invasion *in vitro* (Pinilla et al., 2009). Furthermore CCL5 together with CCL2 support the migration of blood-derived monocytes into the tumor which consequently differentiate into tumor-activated macrophages and release a large variety of promalignant factors, again enhancing tumor progression (Mishra et al., 2011).

1.4 The origin of carcinoma-associated myofibroblasts

The molecular origin of CAFs and the mechanism by which CAFs develop into tumor promoting cells is not conclusively established and remains a subject of controversy (Haviv et al., 2009). It is likely that the origin of myofibroblasts within the desmoplastic stroma is determined by the type of carcinoma and that various cellular lineages can contribute to the overall count of CAFs, again depending on the type of cancer.

It has often been suggested that CAFs could originate from host fibroblasts (Haviv et al., 2009). One recent study dealing with the origin of these cells found that CAFs present in liver metastases from colorectal cancer displayed a phenotype similar to that of the host portal-located liver fibroblasts (Mueller et al., 2007).

Other studies have suggested epithelial to mesenchymal transition (EMT) as well as endothelial to mesenchymal transition (EndMT) as a source of carcinoma-associated fibroblasts. As for EMT it has been argued that both epithelial cancer cells and normal epithelial cells adjacent to malignant cells could contribute to the pool of tumor-activated fibroblasts (Kalluri and Zeisberg, 2006; Radisky et al., 2007). Zeisberg et al. showed that TGFβ1 induced primary mouse endothelial cells to converse into fibroblast-like cells in vitro. This transition could also be observed in two different mouse models *in vivo* where EndMT significantly contributed to the total cell population of CAFs (Zeisberg et al., 2007).

Recent studies have shown that at least a subset of myofibroblasts in cancer originate from circulating bone marrow derived stem cells that infiltrate the tumor stroma (Direkze et al., 2004; Ishii et al., 2003; Mishra et al., 2008). Mishra et al. found that in breast cancer these BMSC-derived myofibroblasts did also exhibit functional properties of reactive stroma including the ability to stimulate tumor cell growth both *in vitro* and *in vivo* as demonstrated by a co-implantation model where cancer cells were admixed with BMSCs

(Mishra et al., 2008). In another *in vivo* study about inflammation-induced gastric cancer, approximately 20% of the carcinoma-associated myofibroblasts were derived from BMSCs (Quante et al., 2011). In a further study the human pancreatic cancer cell line Capan-1 was subcutaneously xenotransplanted into immunodeficient mice and the stromal formation was analyzed after 28 days revealing that approximately 40% of all CAFs originated from BMSCs (Ishii et al., 2003).

Jeon and his group showed that in ovarian cancer lysophosphatidic acid (LPA) present in patients´ ascites can stimulate the differentiation of adipose tissue derived mesenchymal stem cells towards carcinoma-associated myofibroblasts (Jeon et al., 2008). However, the involvement of tissue resident mesenchymal stem cells from the adipose tissue in breast cancer stroma formation has not been investigated so far. Recent studies from our and other groups indicate that adipose tissue contains multipotent stem cells. These cells express specific mesenchymal stem cell surface markers such as cluster of differentiation 29 (CD29), CD44, CD90, CD105 (Bai et al., 2007a; Bai et al., 2007b) and are capable of multi-lineage differentiation (Direkze et al., 2004; Gimble et al., 2007; Zuk et al., 2001). The multi-lineage differentiation potential of these cells into adipogenic, osteogenic, neurogenic and hepatogenic lineage has also been demonstrated in our laboratory (Bai et al., 2007b).

Since adipose-tissue derived stem cells (hASCs) are locally adjacent to the breast tissue and the female breast is composed of a vast quantity of adipose tissue, we hypothesized that a considerable amount of tumor-associated myofibroblasts in breast cancer could arise from hASCs. Hence we sought to elucidate whether α-SMA-positive CAF-like cells can indeed originate from hASCs within the breast cancer microenvironment and if so, which molecular mechanisms are involved in the process of differentiation.

Since it is known that TGFβ can promote the differentiation of mesenchymal precursors into fibroblasts and/or myofibroblasts (Derynck and Akhurst, 2007) we focused on the role of TGFβ within the potential differentiation of hASCs towards α-SMA positive CAFs in breast carcinoma.

In cancer the cytokine TGFβ can be both suppressing and promoting cancer growth which is why it is also known as the molecular "Jekyll and Hyde" of cancer (Bierie and Moses, 2006). Human TGFβ belongs to a superfamily comprising more than 30 different members of which most exist in variant forms (e.g. TGFβ1, 2 and 3). The TGFβ receptor is a dimer composed of two pairs of receptors (serine/threonine kinases) known as the type I and type II receptors (TGFβ RI/II). The type II receptor phosphorylates the TGFβ I receptor upon binding TGFβ, which in turn transmits the signal by phosphorylating and activating Smad2 and Smad3 transcription factors. Within the cell nucleus Smad2 and Smad3 form a complex with Smad4 which binds to the DNA and can activate or suppress target genes (Shi and Massague, 2003; Massague, 2008).

The systemic and particular activities of TGFβ are highly complex and context specific. TGFβ can affect most physiological processes and specific actions on certain cells depend on the specific circumstances of these cells´ environment. In normal cells TGFβ modulates cell differentiation, cytostasis and apoptosis and can suppress inflammation and stroma-derived mitogens. In breast cancer however, malignant progression leads to loss of tumor-suppressive responses in cancer cells. The cancer cells can now exploit TGFβ to their advantage in order to initiate the production of tumor-supporting factors (e.g. cytokines, proteases), for evasion of immune surveillance, invasiveness and metastatic dissemination, and for the recruitment of other cells such as myofibroblasts into the tumor microenvironment (Massague, 2008). TGFβ is implicated in breast cancer progression and clinical studies in human primary breast cancer demonstrated that high protein levels of TGFβ1 were correlated with a shorter disease-free survival (Desruisseau et al., 2006; Sheen-Chen et al., 2001).

The origin of TGFβ in cancer depends on the cancer-type and can range from the cancer cells themselves to various cells of the microenvironment, with each source having its own functional effects (Massague, 2008).

1.5 Aim of the study

It has been widely recognized that carcinoma-associated myofibroblasts, frequently present in desmoplastic breast cancer stroma, play a crucial role in supporting tumor progression. Nonetheless, the cell type of origin and the molecular mechanisms by which these cells develop into tumor-promoting mediators has not been conclusively established and remains debated. Different types of cells have recently been proposed to be precursors of myofibroblasts in breast cancer. However the involvement of human adipose tissue derived stem cells (hASCs) in this context had not been investigated so far. The aim of this study was to investigate whether carcinoma-associated myofibroblasts can originate from hASCs, which are locally adjacent to epithelial breast cancer cells and might therefore represent early response cells within breast cancer.

The goal of this project was therefore to answers the following questions in the given order:

1. Can hASCs differentiate into myofibroblast-like cells when interacting with human breast cancer cells *in vitro*?
2. Is TGFβ1 involved in this differentiation?
3. Do myofibroblast-like cells generated from hASCs exhibit functional properties of carcinoma-associated myofibroblasts?

   In particular:

   a. Do these altered cells produce the tumor-promoting chemokines SDF-1α and CCL5?

   b. Do these altered cells influence the invasive potential of breast cancer cells *in vitro*?

2. Materials and methods

2.1 Materials

2.1.1 Apparatuses

Camera Cool SnapEZ	Photometrics
Centrifuge and micro-centrifuge	Beckmann
Centrifuge, Model 5682	Forma Scientific
Digital camera	Canon
FACSAria II cell sorter	BD Biosciences
FACSCalibur cell sorter	BD Biosciences
Heating block	Fisher Scientific
Hemacytometer	Hausser Scientific
Incubator, Steri-Cult 200	Forma Scientific
Laminar Flow Hood	NuAire
Microscope Axiovert 25	Carl Zeiss
Microscope Axiovert S100	Carl Zeiss
RotoShaker	Scientific Industries
Spectrophometer µQuant	BioTek Instruments

2.1.2 Cells

Human ASCs	Established from subcutaneous tissue of patients undergoing elective liposuction
MCF7 cancer cells	American Type Culture Collection
MDAMB231 cancer cells	American Type Culture Collection
GFP-labeled MDAMB231	American Type Culture Collection cells transfected with pLOX/EW-EGFP lentivirus

2.1.3 Tissue culture materials

Centrifuge tubes; 15ml, 50ml	Greiner Bio One
Fetal bovine serum	Atlanta Biologicals
HBSS	Cellegro
L-glutamine	Cellegro
Liberase Blendzyme 3	F. Hoffman-La Roche Ltd
PBS	Cellegro
Penicillin solution	Cellegro
Polystyrene round bottom tubes	Becton Dickinson Labware
Steriflip filter; 0.45µm, 100µm	Millipore
Streptomyicin solution	Cellegro
Tissue culture flasks; T25, T75, T175	Greiner Bio One
Tissue culture plate; 24 well	Becton Dickinson Labware
Tissue culture plate; 6 well	Greiner Bio One
Trypsin- EDTA	Sigma-Aldrich

2.1.4 Antibodies

Alexa Fluor 488-conjugated goat anti-mouse AB	Invitrogen, Molecular Probes
Alexa Fluor 488-conjugated goat anti-rat AB	R&D Systems
Anti-phospho-Smad2; catalog # 3101	Cell Signaling
Anti-phospho-Smad3; clone EP823Y	Epitomics
Anti-Smad2; catalog # 3122	Cell Signaling
Anti-Smad3; clone EP568Y	Epitomics
Anti-tenascin-C; clone 578	R&D Systems
Anti-tenascin-C; clone BC-24	Sigma-Aldrich
Anti-TGFβ RII; clone H-567	Santa Cruz Biotechnology
Anti-TGFβ1	R&D Systems
Anti-α-SMA; clone 1A4	Sigma-Aldrich
Anti-α-SMA; clone E184	Abcam
Anti-β-Actin; clone AC15	Sigma-Aldrich
Fluorescein-conjugated anti-TGFβ RII	Santa Cruz Biotechnology
Normal chicken IgY	R&D Systems
Normal mouse IgG isotype-matched controls	eBioscience
Normal rat IgG isotype-matched controls	eBioscience
PE-conjugated anti-human CD105	eBioscience
PE-conjugated anti-human CD14	eBioscience
PE-conjugated anti-human CD29	eBioscience
PE-conjugated anti-human CD34	eBioscience
PE-conjugated anti-human CD90	eBioscience
PerCP-conjugated anti-human CD44	eBioscience
PerCP-conjugated anti-human CD44	eBioscience

2.1.5 Western blot materials

BioMax™ MR single-emulsion film; 5x7 inch	Sigma-Aldrich
Biotinylated protein ladder	Cell Signaling
Blotting device, semi-dry	BioRad
Cell lysis buffer	Cell Signaling
ECL™ detection reagents	GE Healthcare
Mini-Protean® Tetra Cell System	BioRad
Polyvinylindene difluoride membranes	Millipore
Protease inhibitor cocktail	Roche Diagnostics
Running buffer	BioRad
SDS Page gels	BioRad

2.1.6 Assays and kits

Human CCL5 Quantakine kit	R&D Systems
Human SDF-1α Quantakine kit	R&D Systems
Human TGFβ1 Quantakine kit	R&D Systems
Invasion chamber, pore size 8µm	BD Bioscience

2.1.7 Plasmid

pLOX/EW-iRES-EGFP lentivirus	Trono lab, Lausanne, CH

2.1.8 Chemicals and other reagents

2-Mercaptoethanol	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
DiI	Invitrogen, Molecular Probes
DMSO	Sigma-Aldrich
Glycine	Sigma-Aldrich
Goat Serum	Sigma-Aldrich
HCl	Sigma-Aldrich
Hoechst 33342	Sigma-Aldrich
Methanol	Sigma-Aldrich
NaCl	Sigma-Aldrich
Non-fat dried milk; bovine	Sigma-Aldrich
Paraformaldehyde Solution	Sigma-Aldrich
Polybrene transfection reagent	Millipore
Recombinant human TGFβ1	R&D Systems
SDS	MP Biomedicals
TGFβ1 receptor kinase inhibitor SB431542	Sigma-Aldrich
Tris base	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
Tween ® 20	Invitrogen

2.1.9 Software

FACSDiva version 6.1.1	BD Biosciences
Flow Jo 7.5.4	Tree Star, Inc.

Image J	http://rsbweb.nih.gov/ij/
NIS-Elements Basic Research	Nikon Instruments Inc.
SPSS version 16.0	SPSS Science

2.2 Cell biology methods

2.2.1 Isolation and culture of human adipose tissue derived stem cells

Unprocessed subcutaneous adipose tissue was obtained from patients undergoing elective body contouring procedures in accordance with the Institutional Ethical Review Board, Protocol Number ING_200601001 ("Human Subcutaneous Adipose Tissue for Cell Isolation Studies"). Freshly prepared adipose derived stromal cells were generously provided under Materials Transfer Agreement by Ingeneron, Inc., Houston, TX.

For isolation of human adipose tissue derived stem cells (hASCs), tissue was minced and dissociated using Liberase Blendzyme 3 (F.Hoffman-La Roche Ltd) containing a mixture of highly purified collagenase and neutral protease enzymes at a concentration of 2 units/g tissue in phosphate-buffered saline (PBS; Cellegro) for 60 minutes at 37°C with intermittent shaking at 50 rpm. The digested tissue was then passed through a 100-µm filter (Millipore) and the floating adipocytes were separated from the stromal-vascular fraction by centrifugation at 1,000 rpm for 10 minutes. The cell pellet containing the target cells was washed twice with Hank's balanced salt solution (HBSS; Cellegro) and finally re-suspended in alpha-modification of Eagle's medium (αMEM; Cellegro) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 2 mM L-glutamine (Cellegro), 100 U/ml penicillin and 100 µg/ml streptomycin (Cellegro). The plastic adherent adipose tissue derived stem cells were cultured on T75 tissue culture plates at 37°C in a humidified atmosphere (95%

humidity) containing 5% CO_2. Daily washings with PBS removed the non-attached as well as the red blood cells. After three to five days hASCs were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) and plated in tissue culture flasks at a density of 1,000 cells/cm^2. The complete process of cell isolation was carried out under sterile conditions in a laminar flow hood. The cell growth medium was changed every 3 days and hASCs were subcultured every 4 to 5 days. Cells passaged for 2-5 population doublings (PDs) were used for all experiments (Fig. 2.1).

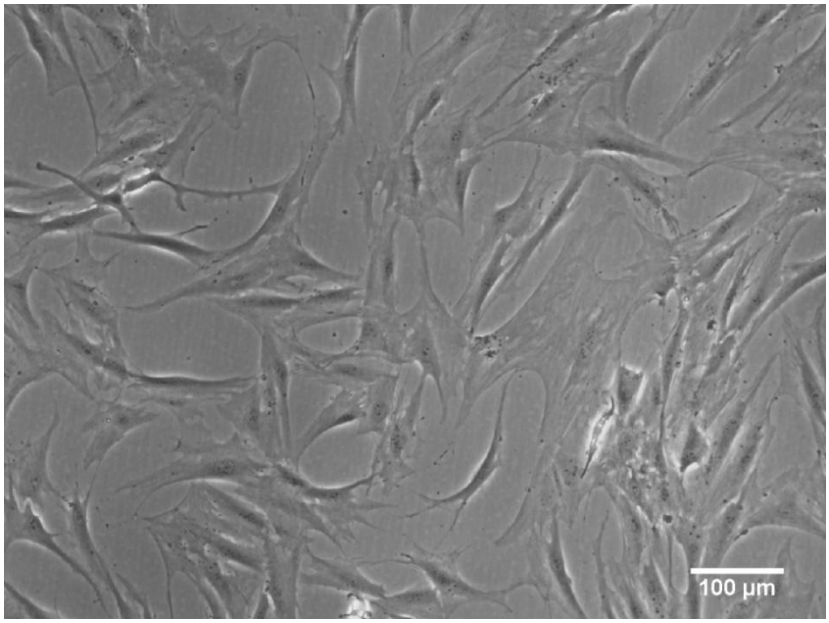

Figure 2.1 Human adipose tissue derived stem cells (hASCs) passage 3 in culture.

All experiments were performed with hASCs isolated from three different donors (N=3) and each experiment was repeated at least three times (n=3).

2.2.2 Flow cytometric analysis of CD surface markers on hASCs

In order to characterize human ASCs, cells were labeled with either phyocoerythrin (PE)-conjugated-antibodies or peridinin-chlorophyll (PerCP)-conjugated antibodies to target specific surface proteins. Subsequently the labeled cells were analyzed using flow cytometry and the distribution of the cells into different populations according to their specific emission intensity was displayed graphically.

For flow cytometric analysis of phenotype in hASCs, cells in passage 3 were treated with 0.05% trypsin-0.53 mM EDTA, washed twice with PBS and cell aliquots ($1x10^5$ cells/100µl) were stained with primary-conjugated antibodies at room temperature for 30 minutes in polystyrene round bottom tubes (Becton Dickinson Labware) in the dark. The conjugated antibodies used for these experiments were PE-conjugated anti-human CD14, CD29, CD34, CD90, CD105 and PerCP-conjugated anti-human CD44 and CD45 (all eBioscience). Normal mouse and rat IgG at the same concentrations as the primary antibodies were used as isotype-matched controls (all eBioscience). At least $1x10^4$ events were counted for each sample using the fluorescence-activated cell sorter FACSCalibur (BD Biosciences) and data analysis was performed with Flow Jo 7.5.4 software (Tree Star, Inc.).

2.2.3 Tumor cell lines

In order to study the interactions between human adipose tissue derived stem cells and breast cancer cells in vitro the two distinct breast cancer cell lines MDAMB231 and MCF7 were used in different settings. Both cell lines are of epithelial phenotype and originate from pleural effusion of adenocarcinoma of the breast.

The human breast cancer cell lines MDAMB231 and MCF7 were purchased from the American Type Culture Collection and cultured in αMEM supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin at 37°C in a 5% CO_2 containing chamber. The cell

growth medium was changed every 3 days and cancer cells were subcultured every 4 to 5 days.

2.2.4 Exposure of hASCs to tumor conditioned medium

Tumor conditioned medium (TCM) from either MDAMB231 or MCF7 cancer cells was collected for further experiments in which hASCs were exposed to the cancer cell conditioned medium.

TCM was collected from 90% confluent T175 flasks (8–10x10^6 cancer cells) of cancer cells after 24 h of incubation at 37°C with 25 ml of fresh, serum-free αMEM. The medium was then harvested, centrifuged at 3,000 rpm for 5 min at 4°C and passed through sterile 50 mL filtration system with 0.45-µm polyvinylidene difluoride membrane (Millipore). Conditioned medium (CM) was stored at -80°C in aliquots for subsequent use. hASCs were serum-starved with 0% FBS αMEM for 24 hours and subsequently exposed to conditioned medium and the CM was changed every second day for the entire culturing period (24 h to 4 days).

2.2.5 Direct co-culture of hASCs with cancer cells

The interaction of breast cancer cells with human adipose tissue derived stem cells was studied in a direct co-culture system *in vitro*.

The different cells were washed twice with PBS, digested with trypsin-EDTA and counted. Breast cancer cells (MDAMB231 or MCF7) and hASCs were co-seeded at a 2:1 ratio and grown to sub-confluence for immunofluorescence analysis (Fig. 2.2A and 2.2B).

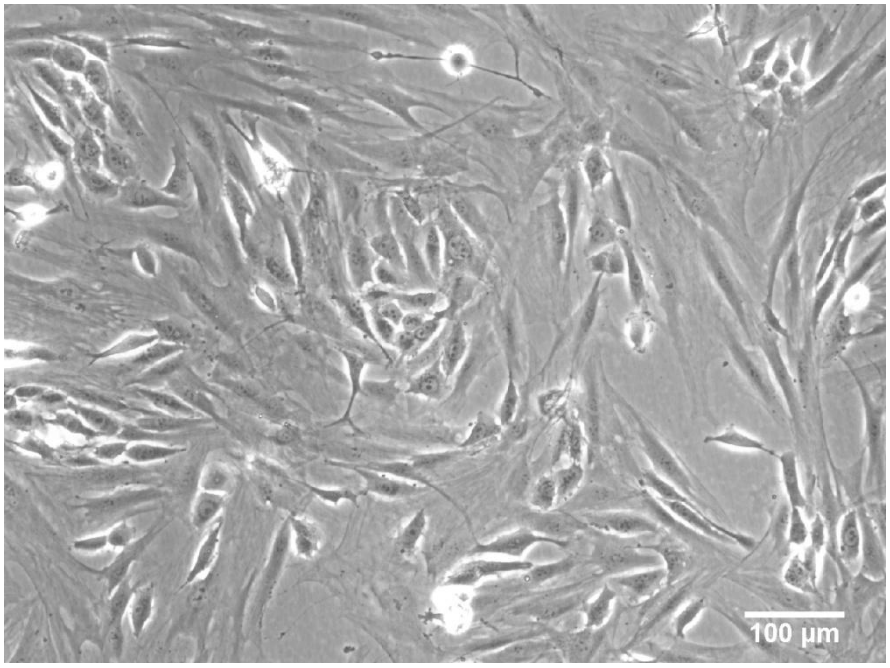

Figure 2.2 (A) Breast cancer cell line MDAMB231 in direct co-culture with hASCs passage 3.

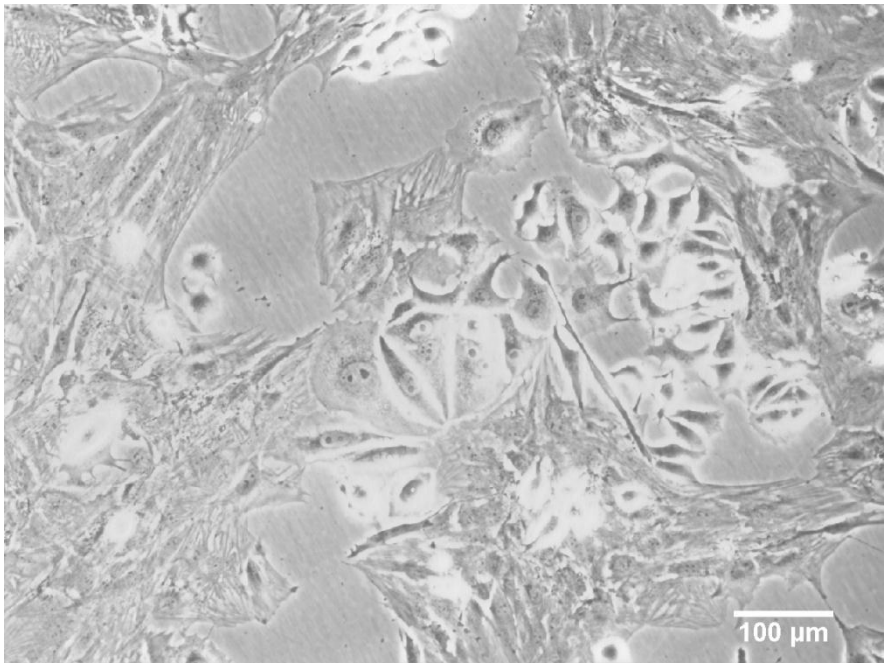

Figure 2.2 (B) Breast cancer cell line MCF7 in direct co-culture with hASCs passage 3.

2.2.6 TGFβ1 treatment of hASCs

In order to investigate the effect of human transforming growth factor beta 1 (TGFβ1) on the development of hASCs towards carcinoma-associated myofibroblasts, hASCs were stimulated with recombinant human TGFβ1 (rTGFβ1).

Lyophilized recombinant human TGFβ1 (R&D Systems) was reconstituted in sterile 4 mM hydrogen chloride (HCl) containing 0.1% BSA to prepare a stock solution 10 µg/mL and used for the treatment of hASCs. Before use, cells grown in regular medium containing 10% FBS were deprived of serum using serum-free medium for 24 hours. This was followed by incubation of the cells with serum-free medium containing 0.2 or 2 ng/ml rTGFβ1 for up to 4 days at 37°C in a humidified atmosphere containing 5% CO_2. The recombinant TGFβ1 treated hASCs were subsequently used for further experiments.

2.2.7 TGFβ receptor kinase inhibition and anti-TGFβ1 treatment

For inhibition of the TGFβ Type 1 receptor-like kinase, cells were pre-incubated with SB431542 (10 µM; Sigma-Aldrich) for 30 min. Pre-incubation with dimethylsulfoxide (DMSO) as a vehicle for the same time was used as a control.

To neutralize TGFβ1, cells were cultured in the presence of 0.2 µg/ml anti-TGFβ1 neutralizing-antibody (R&D Systems; Catalog Number: AF-101-NA). Normal chicken IgY (R&D Systems; Catalog Number: AB-101-C) at the same concentration was used as a control.

2.2.8 Cell staining

2.2.8.1 Immunofluorescence analysis

The cells subject to immunofluorescence analysis were grown to subconfluence in 6-well plates with growth medium containing 10% FBS, and then deprived of serum by rinsing three times with PBS and incubating with serum-free medium (αMEM containing 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) for 24 hours. The serum-starved cells were treated under appropriate conditions, washed twice with PBS, fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature, washed twice with PBS and blocked with 10% goat serum (Sigma-Aldrich) at room temperature for 10 minutes.

Subsequently the cells were incubated with primary antibodies using anti-alpha smooth muscle actin (dilution 1:250; Clone 1A4; Sigma-Aldrich) or anti-tenascin-C (dilution 1:250; Clone BC-24) antibodies for 1 hour at room temperature or at 4°C overnight in the cold room. Following further washing the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen, Molecular Probes) for 1 h at a dilution of 1:500 in the dark.

For TGFβ type II receptor staining, the cells were stained with a fluorescein conjugated anti-TGFβ RII antibody (1:50; Clone H-567; Santa Cruz Biotechnology).

Finally all cells were washed twice with PBS, counterstained with the nuclear dye Hoechst 33342 (Sigma-Aldrich) and examined with a fluorescence microscope. Pictures were taken at a 10 or 20-fold magnification.

hASCs cultured under different conditions were quantified for the expression of alpha smooth muscle actin (α-SMA) and cells staining positive for α-SMA were counted as a fraction of the total cell number (at least 70 cells were counted per view field) in nine independent view fields in each group at a 10x magnification under the microscope. For each group at least 630 cells were counted. The experiments were repeated at least three times.

2.2.8.2 DiI staining

The hydrophobic cyanine dye DiI (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) allows fast and easy fluorescent cell labeling.

For cell staining, 80% confluent T25 flask of MDAMB231 cells were incubated with 3 ml of their regular culture medium containing 15 µl of DiI (Invitrogen, Molecular Probes) for 1 hour at 37°C in a humidified atmosphere containing 5% CO_2. Cells were washed twice with PBS and regular medium was added for 5 minutes. This procedure was repeated twice and after that cells were harvested and seeded for the following experiment.

2.2.8.3 GFP labeling of MDAMB231 cells

For stable green fluorescence protein (GFP) labeling of MDAMB231 cancer cells, $5x10^4$ cells per well were seeded in 6-well tissue culture plates in the presence of 2 ml of αMEM. Lentiviral GFP transfection was performed with a plox/EW-iRES-EGFP lentivirus (Trono lab, Lausanne, CH) and polybrene (hexadimethrine bromide) was used in a concentration of 8 µg per ml to increase the efficiency of transfection. The cells were incubated with transfection medium for 24 h at 37°C in a humified atmosphere containing 5% CO_2 whereupon the medium was replaced using 2ml of fresh αMEM per well. After three weeks of keeping the transfected cells in αMEM with weekly medium changes, fluorescence-activated cell sorting was performed at MD Anderson cancer center core facility. GFP-labeled MDAMB231 cancer cells were sorted using the BD FACSAria II cell sorter and FACSDiva Version 6.1.1 software. GFP-positive cells were subsequently cultured in 10% FBS αMEM until used for invasion assay.

2.2.9 Invasion assay

Invasion assays allow studying the potential of cells to invade extracellular matrix (ECM) and offer a valuable tool to quantify the invasion potential of cancer cells *in vitro*.

The invasion potential of MDAMB231 cancer cells was evaluated using a Boyden chamber with filter inserts (pore size 8 µm) coated with matrigel basement membrane matrix in 24-well dishes (BD Biosciences). The matrigel matrix, which consists mainly of laminin and collagen IV, functions as a reconstituted basement membrane *in vitro*. According to the manufacturer's protocol the invasion chambers were allowed to come to room temperature (RT) for an hour. Then PBS was added and the chambers were rehydrated for 2 h at 37°C. $35x10^3$ DiI-stained or GFP-labeled MDAMB231 cancer cells were seeded alone or in co-culture with hASCs ($70x10^3$ cells/well) pre-exposed to TCM or recombinant human TGFβ1 (0.2 ng/ml) for 4 days in 600 µl of αMEM 5% FBS in the upper chamber. The lower chamber contained αMEM 10% FBS. For invasion assays of MDAMB231 cancer cells with conditioned medium, $35x10^3$ cancer cells per well were seeded with CM from hASCs treated under appropriate conditions. For preparation of hASC-CM, cells were pretreated with 10 µM SB431542 or DMSO as a vehicle for 30 min and then cultured in serum free medium, 0.2 ng/ml recombinant human TGFβ1 (rTGFβ1; R&D Systems), or MDAMB231-CM over 4 days. The medium was then changed to αMEM 5% FBS and conditioned for 72h before use. For all invasion assays the chambers were incubated for 40 h at 37°C in 5% CO_2 and non-invaded cells were removed from the top surface of the insert by scrubbing with cotton tip swabs and the filters were rinsed with PBS. Invaded cells were fixed on the membrane in 5% PFA, washed twice with PBS and counterstained with Hoechst 33342 dye as a nuclear stain. Invaded DiI + Hoechst 33342 positive cancer cells on the underside of the filters were counted in five independent view fields at 20x magnification of each insert under the microscope. For invasion assays with GFP-labeled MDAMB231 the cells were not counterstained Invaded GFP-positive cells on the underside of the filters were equally counted under the microscope.

2.2.10 FACS analysis of hASCs

As a specialized type of flow cytometry, fluorescence-activated cell sorting (FACS) provides a fast method for sorting a heterogeneous mixture of cells. Based upon the specific

fluorescent and light scattering characteristics of each cell the heterogeneous cell-mixture can be sorted into two or more containers at a time.

For FACS analysis of tenascin-C in hASCs, cells were grown to subconfluence (60%) in T175 flasks with growth medium containing 10% FBS. Subsequently 5-7x10^5 cells were incubated with serum-free medium for 24 h. The serum-starved cells were afterwards treated for 4 days under appropriate conditions, harvested, washed twice with PBS and cell aliquots were incubated with 5 µg/ml anti-tenascin-C antibody (R&D Systems, clone 578) for 20 minutes. Following further washing the cells were incubated with Alexa Fluor conjugated goat anti-rat IgG secondary antibody (1:200; R&D Systems) for 20 min, washed twice with PBS and used for FACS analysis to separate tenascin-C positive from tenascin-C negative cells. The MD Anderson cancer center core facility was used to sort the cells using the BD FACSAria II cell sorter and FACSDiva Version 6.1.1 software. Positive and negative tenascin-C cells were then cultured separately in 10% FBS αMEM over 4 days. For further experiments conditioned medium was collected from approximately 5 million cells of each group and used for chemokine C-C motif ligand 5 (CCL5) ELISA using the CCL5 Quantikine kit (R&D Systems). Each experiment was repeated at least three times.

2.3 Molecular biology methods

2.3.1 Enzyme-linked immunosorbent assay

The quantitative sandwich enzyme immunoassay technique was used for the determination of the concentrations of human stromal cell-derived factor 1 alpha (SDF-1α), CCL5 and TGFβ1 in cell culture supernates.

Enzyme-linked immunosorbent assay (ELISA) was performed using the human TGFβ1, SDF-1α and CCL5 Quantikine kit (R&D Systems; Catalog Number: DB100B, DSA00 and DRN00B)

according to the manufacturer's description and experiments were repeated at least three times. Cells were treated under appropriate conditions and cell culture supernatants were collected, centrifuged and filtered through a 0.45-µm Steriflip Filter Unit (Millipore). Standards, samples and controls were pipetted into the wells of a pre-coated microplate containing monoclonal antibodies specific for SDF-1α, TGFβ1 or CCL5 respectively. Any SDF-1α, TGFβ1 or CCL5 present in the specific samples was thereby bound to the immobilized antibodies. After 2 hours of incubation any unbound substances were washed away and a specific enzyme-linked polyclonal antibody was added to the wells. Following further washing, a substrate solution was added to the wells in the dark and the intensity of the developing color was measured. The absorbance (450nm) for each sample was analyzed by an ELISA reader (Spectrophometer µQuant; BioTek Instrumentes) and interpolated with a standard curve.

2.3.2 Western blot analysis

The expression levels of Smad2, Smad3, phosphorylated Smad2 and Smad3, α-SMA and β-Actin in different samples of hASCs were determined by western blot analysis.

Serum-starved hASCs were treated under appropriate conditions, washed with ice-cold PBS and cells were re-suspended in lysis buffer (Cell Signaling), including protease inhibitor cocktail (Roche Diagnostics) according to the vendor´s protocol. The samples were heat-blocked at 95°C for 5 minutes and 24 µg protein-extract/sample was run on pre-cast SDS PAGE 4-15% Tris-HCl gels (BioRad) in electrophoresis running buffer 10xTris/glycine/SDS (BioRad) using the BioRad Mini-Protean® Tetra Cell system. Gels were run at 70V for 30 minutes and then for up to 1 hour at 100V until the loading dye had run out.

Subsequently the gels were transferred onto polyvinylindene difluoride (PVDF) membranes (Millipore) soaked in 1x transfer buffer (40 mM Tris-HCl, 39 mM Glycine, 1.3 mM SDS, 20% Methanol) using a semi-dry blotting device (BioRad) at 0.18A and 24V for 2 hours.

The membranes were washed briefly and after blocking with 5% nonfat milk in 1x Tris buffered saline (TBS) with Tween® 20 (polyoxyethylenesorbitan monolaurate) for 1 h at room temperature the primary antibody was applied. Primary antibodies were diluted in 5% nonfat milk/1x TBS with Tween® 20 (TBST) and membranes were incubated with antibody mixtures overnight at 4°C on a shaker. The primary antibodies used were anti-phospho-Smad2 (dilution 1:1000; Cell Signaling, catalog # 3101), anti-Smad2 (1:1000; Cell Signaling, catalog #3122), anti-phospho-Smad3 (1:1000; Epitomics, clone EP823Y), anti-Smad3 (1:1000; Abcam, clone EP568Y), anti- α-SMA (1:400, Abcam, clone E184) and anti-β-Actin (1:1000; Sigma-Aldrich, clone AC15). Membranes were probed with corresponding HRP-conjugated secondary antibodies (1:2000; Cell Signaling) in 5% nonfat milk /1x TBS with Tween® 20 and incubated for 1 h at RT under agitation. The membranes were washed with TBST six times for five minutes under agitation and then incubated in ECL™ detection solution (GE Healthcare) for 1 minute. Finally the membranes were transferred to a western blot cassette and exposed to BioMax™ MR single-emulsion film (Sigma-Aldrich). Image J software was used to determine the average density of western blot protein bands. The experiments were repeated at least three times independently.

2.4 Statistical analysis

All results are presented as means ± standard deviations (SD). All experiments were repeated at least three times independently from each other. For statistical analysis, the Student's t test was performed using SPSS 16.0 software. Probability values were calculated two-sidede and considered to be statistically significant with a value of $P \leq 0.05$.

3. Results

3.1 Flow cytometric analysis of phenotype in hASCs

Flow cytometric analysis was performed on three different samples in passage 3 in order to characterize the surface antigens of human adipose tissue derived stem cells (hASCs). The phenotype of hASCs was analyzed for different antibodies typically expressed by mesenchymal stem cells. Flow cytometric analysis revealed that hASCs were positive for the mesenchymal stem cell markers CD29, CD44, CD90 and CD105. The cells were negative for CD14, CD34 and CD45, which excludes contamination with hematopoietic cells (Fig. 3.1).

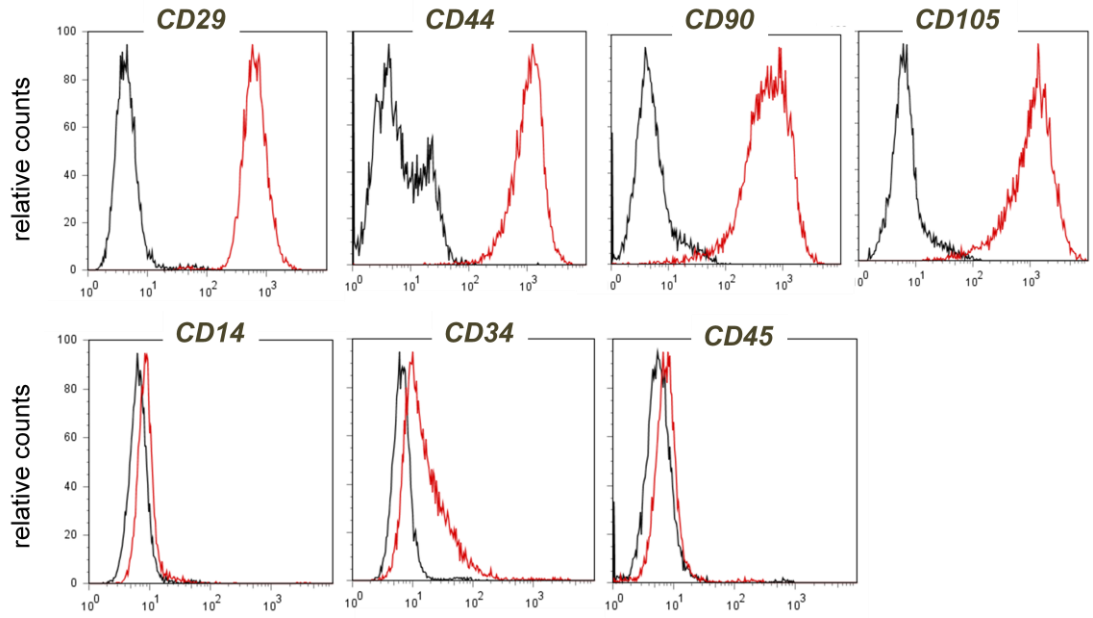

Figure 3.1 hASCs express CD surface markers typical for mesenchymal stem cells. Flow cytometric characterization of CD surface markers on hASCs. Black histograms indicate isotype-matched controls; red histograms show surface antigen expression level.

3.2 hASCs within the breast cancer tumor microenvironment

In order to explore whether hASCs could be a potential source of myofibroblasts within the tumor microenvironment of breast cancer, the direct and indirect interactions of hASCs with the two breast cancer cell lines MDAMB231 and MCF7 was studied *in vitro*. The question was addressed by looking at the phenotype of hASCs and if this initial phenotype might change to a more specific myofibroblast-like one under the influence of the tumor microenvironment.

3.2.1 hASCs express myofibroblast markers in co-culture with breast cancer cells

In order to explore whether hASCs could be a potential source of myofibroblasts, a direct co-culture system of hASCs with MDAMB231 or MCF7 breast cancer cells had been established as a starting point. Stem cells were always co-seeded with MDAMB231 or MCF7 at a 2:1 ratio and cultured in serum-free αMEM medium over 4 days (Fig. 3.2a and 3.2b). Myofibroblast-like cells were detected by immunofluorescence (IF) staining using an antibody against alpha smooth muscle actin (α-SMA) which is a specific myofibroblast marker. Immunofluorescence staining revealed that hASCs contained only a small number of α-SMA positive cells when grown in mono-culture over 4 days. MDAMB231 or MCF7 breast cancer cells alone did not contain any α-SMA positive cells after 4 days in culture. Interestingly, the number of α-SMA positive cells increased significantly after co-culturing hASCs with MDAMB231 or MCF7 cells for 4 days, suggesting that hASCs differentiated into myofibroblast-like cells in this co-culture system (Fig. 3.2c-3.2f).

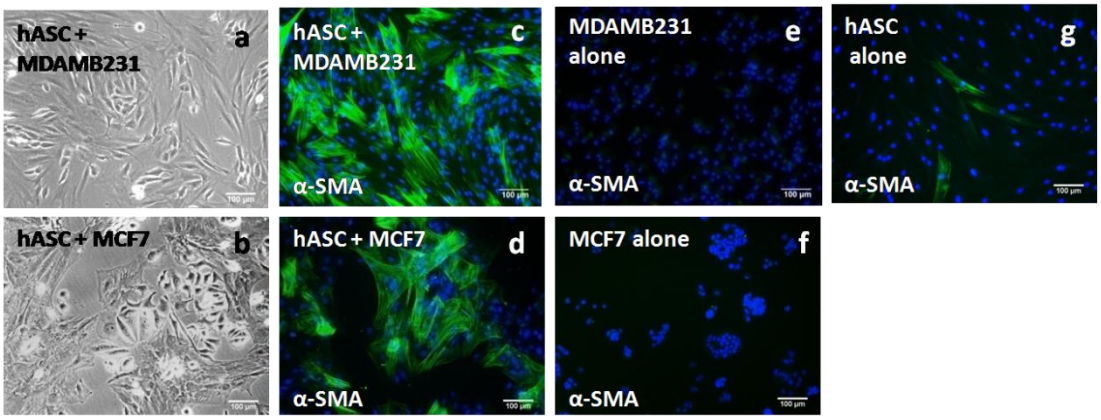

Figure 3.2 Direct co-culture of hASCs with breast cancer cells. (a, b) Brightfield pictures of direct co-culture of MDAMB231 cells or MCF7 cells with hASCs grown in serum-free medium over 4 days. The co-culture of hASCs with (c) MDAMB231 or (d) MCF7 and monocultures of either (e) MDAMB231, (f) MCF7 or (g) hASCs were grown 4 days in serum-free medium and the expression of α-SMA (green channel) was determined by immunostaining with an anti α-SMA antibody. Nuclei were counterstained with Hoechst 33342 and appear blue. Scale bar, 100 µm.

3.2.2 Tumor cell conditioned medium induces the expression of myofibroblast markers in hASCs

In order to investigate if the expression of myofibroblast markers in hASCs does only take place when direct cell-cell interactions are allowed (Fig. 3.2) or if humoral factors might be involved in this process through paracrine communications, hASCs alone were cultured in breast cancer cell conditioned medium (CM). Tumor cell conditioned medium (TCM) was obtained from either MDAMB231 or MCF7 cancer cells as described in the materials & methods section (see 2.2.4 for details). Briefly, TCM was collected from confluent flasks of cancer cells after 24 h of incubation in serum-free medium, filtered and directly used for further experiments or stored at -80°C. hASCs in culture (passage 2-4) were serum-starved with serum-free medium for 24 h, exposed to TCM for 4 days and subsequently stained with either α-SMA or tenascin-C, another specific myofibroblast marker. IF staining revealed that the majority of hASCs had acquired a myofibroblast phenotype (expression of α-SMA and tenascin-C) after culturing for 4 days in TCM, indicating that breast cancer cell derived humoral factors are involved in the differentiation (Fig. 3.3).

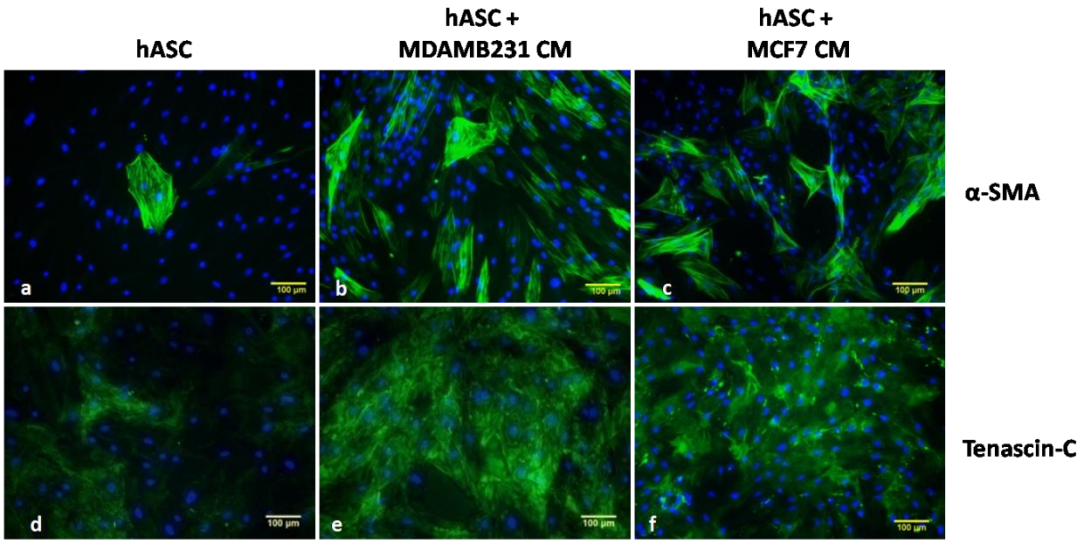

Figure 3.3 Immunofluorescence staining of hASCs for myofibroblast markers. (a, d) hASCs were cultured in serum-free medium or with serum-free tumor conditioned medium from either (b, e) MDAMB231 or (c, f) MCF7 cancer cells for 4 days and stained with (a-c) anti α-SMA and (d-f) anti tenascin-C antibodies, respectively. Scale bar, 100 µm.

3.2.3 The induction of α-SMA in hASCs is time-dependent

In order to quantify the expression of α-SMA in TCM-stimulated hASCs the percentage of cells expressing α-SMA was determined at different time points. Immunostaining was performed as described above (see 2.2.8.1 in materials & methods section) and α-SMA positive cells were counted as a fraction of the total cell number in nine randomly chosen, independent view fields in each group at a 10x magnification under the microscope.

The differentiation of hASCs towards myofibroblast-like cells was time-dependent. After culturing hASCs with TCM for 1 day, only 2.0% ± 0.3% (hASCs cultured in MDAMB231 CM) and 3.0% ± 0.8% (hASCs cultured in MCF7 CM) of the cells expressed α-SMA, whereas 52.0% ± 11.2% (hASCs cultured in MDAMB231 CM) and 50.5% ± 3.7% (hASCs cultured in MCF7 CM) of the cells expressed α-SMA after 4 days (Fig. 3.4).

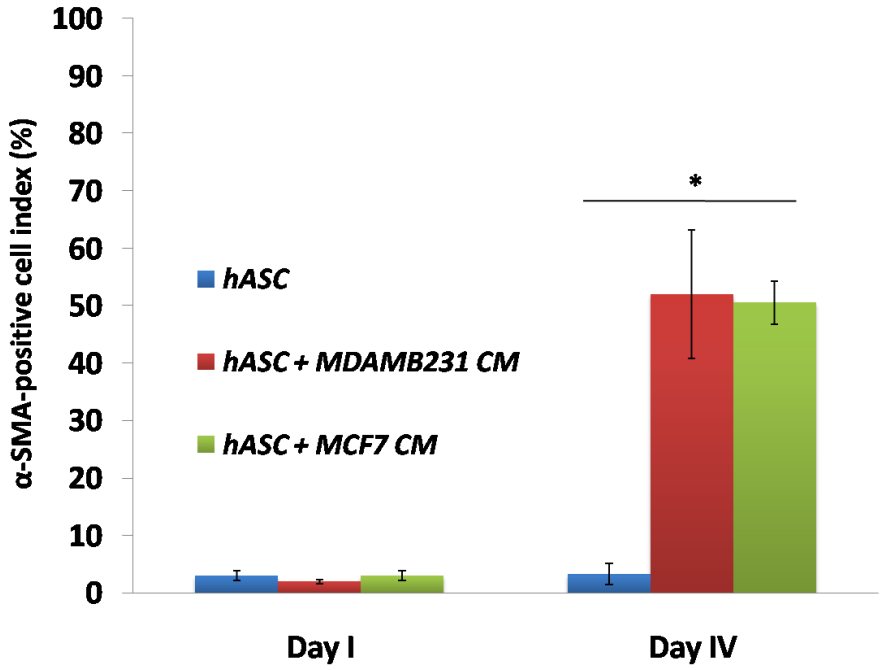

Figure 3.4 Quantitative analysis of the expression of α-SMA in hASCs. α-SMA in hASCs cultured for 1 or 4 days in either MDAMB231 or MCF7 serum-free conditioned medium or serum-free medium alone was determined by immunofluorescence analysis (see materials & methods for details).* indicates P<0.0001 as compared to hASC alone on day IV.

3.3 Influence of breast cancer-cell derived TGFβ1 on the differentiation of hASCs

hASCs stimulated with tumor conditioned medium expressed a significant number of α-SMA positive cells (Fig. 3.4) which is a main marker for myofibroblasts. Furthermore it was investigated which breast cancer cell derived humoral factors could be involved in the differentiation of hASCs towards myofibroblast-like cells.

3.3.1 MDAMB231 and MCF7 breast cancer cells secrete TGFβ1

It is known that α-SMA expression can be induced by stimulation with exogenously added transforming growth factor beta 1 (TGFβ1) in quiescent human breast gland fibroblasts (Ronnov-Jessen and Petersen, 1993). Hence the possible involvement of TGFβ1 in the signaling pathway of human adipose tissue derived stem cells towards α-SMA positive cells was studied.

In a first step the question was raised whether the two breast carcinoma cell lines MDAMB231 and MCF7 secrete TGFβ1, which could be involved in myofibroblast differentiation of hASCs. Hence MDAMB231 and MCF7 were cultured in αMEM without addition of FBS (which might by itself contain TGFβ) and conditioned medium from these cells was collected after 0, 24 and 48 hours for the detection of TGFβ1 protein levels by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 3.5, a significant amount of TGFβ1 (204.9 ± 31.9 pg/ml in CM from MDAMB231 and 283.3 ± 24.8 pg/ml in CM from MCF7) was detected in the medium after 24 hours conditioning time and TGFβ1 secretion from MDAMB231 and MCF7 reached a maximum level after 48 hours in culture (291.1 ± 7.3 and 666.7 ± 47.2 pg/ml respectively). On the other hand TGFβ1 was not detected in conditioned medium from hASCs cultured for 24 and 48 hours (Fig. 3.5). Serum-free αMEM medium was used as a negative control for ELISA and did not contain any TGFβ1.

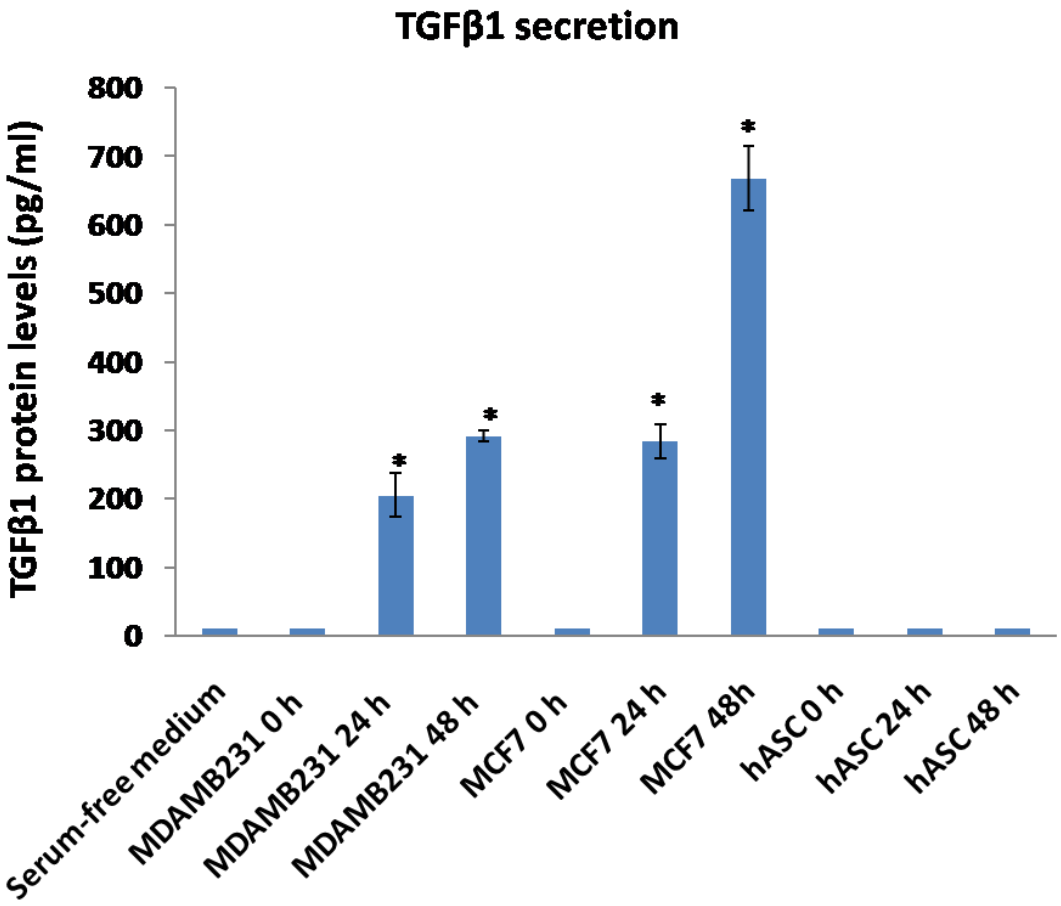

Figure 3.5 Tumor conditioned medium contains TGFβ1. The amount of TGFβ1 in serum-free medium or serum-free conditioned medium from MDAMB231, MCF7 and hASCs was measured by ELISA at different time points. Data represent mean ± SD. (n=3). *P indicates <0.0001 as compared to serum-free medium.

3.3.2 TGFβ type II receptor is expressed in hASCs

It is known that TGFβ1 induces α-SMA expression through binding to its type II receptor and subsequent phosphorylation and activation of Smad2 and Smad3 (Hu et al., 2003; Massague, 2008). Since TCM contained significant amounts of TGFβ1 (Fig. 3.5) the next step was to investigate whether hASCs might express the corresponding receptor. Immunofluorescence staining of hASCs with a fluorescein conjugated anti-TGFβ type II receptor antibody (TGFβ RII) revealed that hASCs do indeed expresse the receptor (Fig. 3.6)

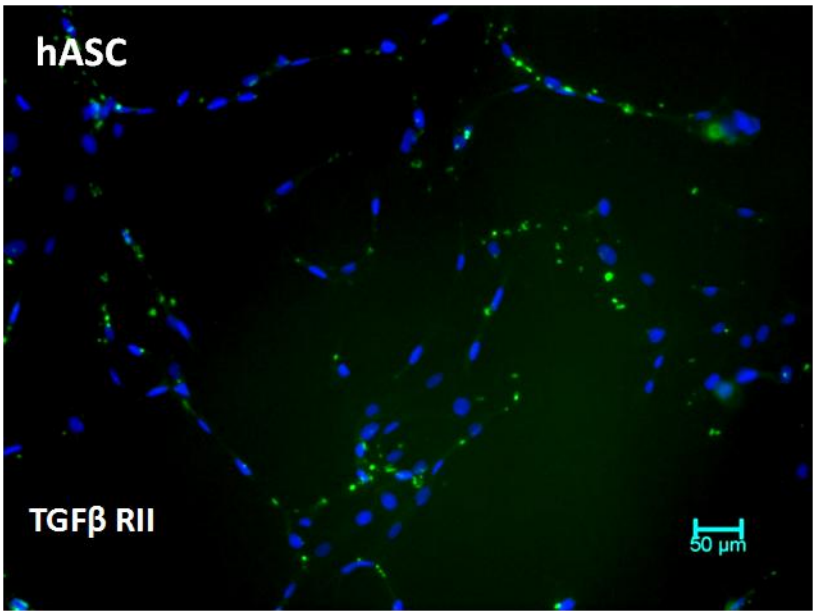

Figure 3.6 Immunofluorescence staining of hASCs with a fluorescein conjugated anti-TGFβ type II receptor antibody (green channel). Nuclei were counterstained with Hoechst 33342 and appear blue in the photograph. Scale bar, 50 µm

3.3.3 Breast cancer cell-derived TGFβ1 induces α-SMA expression in hASCs through Smad2/3 signaling

Phosphorylation of the transcription factors Smad2 and Smad3 are key components in intracellular signaling of TGFβ1 (Shi and Massague, 2003). To further prove whether TGFβ is involved in myofibroblast differentiation of hASCs, protein levels of phosphorylated Smad2 and Smad3 (p-Smad2, p-Smad3) and α-SMA in stimulated hASCs were measured using western blot analysis. In a first set of experiments protein levels of Smad3 (molecular weight 58kDa), p-Smad3 (58kDa), α-SMA (42kDa) and β-Actin (42kDa, used as a loading control) were measured in TCM-activated or TGFβ1-stimulated hASCs. Non-stimulated hASCs cultured in serum-free medium over 4 days were used as a control. Western blot analysis demonstrated that hASCs expressed higher protein levels of p-Smad3 and α-SMA after exposure to CM from MDAMB231 (0%FBS; 24 h conditioning time) over 4 days compared to hASCs cultured in regular, serum-free medium (Fig. 3.7A). Similarly, hASCs cultured in serum-free medium supplemented with 0.2 ng/ml recombinant TGFβ1 over 4 days revealed strong phosphorylation of Smad3 and high protein levels of α-SMA. The different protein expression levels of p-Smad3 in stimulated and non-stimulated hASCs were confirmed by measuring the average density of the western blot protein bands of p-Smad3 with Image J software. The up-regulation of p-Smad3 in stimulated hASCs as compared to non-stimulated control hASCs was graphically displayed as fold induction of p-Smad3 and is shown in Figure 3.7B.

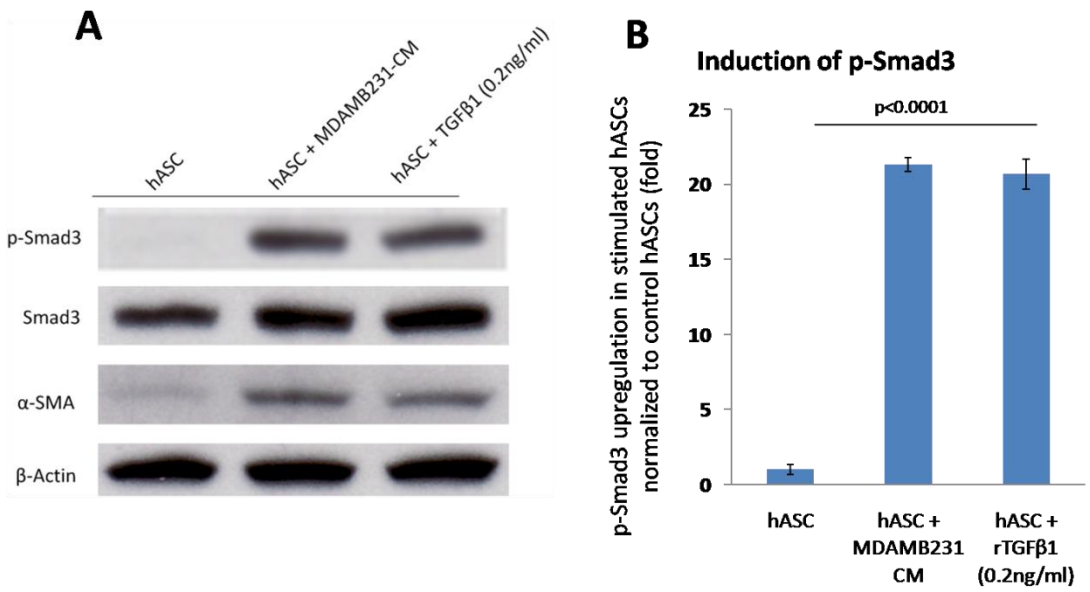

Figure 3.7 Induction of p-Smad3 in stimulated hASCs. **(A)** hASCs were exposed to either serum-free medium alone, MDAMB231 CM (0% FBS; 24 h conditioning time), or serum-free medium containing 0.2 ng/ml recombinant TGFβ1 over 4 days. The expression levels of Smad3, phosphorylated Smad3 (p-Smad3), α-SMA and β-Actin were determined by western blot analysis using anti-Smad3, anti-p-Smad3, anti-α-SMA and anti-β-Actin antibodies. β-Actin was used as a loading control. **(B)** The induction of p-Smad3 in stimulated hASCs was analyzed by measuring the density of p-Smad3 western blot protein bands and displayed as fold induction normalized to non-stimulated control hASCs. P<0.0001 as compared to control hASCs.

These experiments illustrate that phosphorylation of Smad3 can be induced by exogenously added TGFβ1 as well as by breast cancer cell conditioned medium, indicating that TGFβ1 present in TCM is responsible for the activation of p-Smad3 in TCM-stimulated hASCs.

In a further step it was explored if the inhibition of the TGFβ1 signaling pathway in hASCs would result in a reduced phosphorylation of the transcription factors Smad2 and Smad3 and would consequently lead to lower protein levels of α-SMA. For inhibition of the TGFβ1 activin receptor-like kinase receptors in hASCs, cells were pretreated for 30 minutes with dimethylsulfoxide (DMSO) as a vehicle or 10 µM SB431542 which is a selective and potent inhibitor specifically of activin receptor-like kinase 4, 5 and 7 (ALK4, ALK5 and ALK7). The

cells were then exposed to serum-free medium as a control group or MDAMB231 CM (0%FBS; 24 h conditioning time). To neutralize TGFβ1, cells were cultured in MDAMB231 CM (0%FBS; 24 h conditioning time) over 4 days in the presence of 0.2 µg/ml anti-TGFβ1 neutralizing-antibody. The protein levels of Smad2 (molecular weight 60kDa), p-Smad2 (60kDa), Smad3, p-Smad3, α-SMA and β-Actin were measured by western blot analysis. Both TGFβ activin receptor-like kinase receptor inhibition with SB431542 as well as anti-TGFβ1 treatment with the neutralizing antibody markedly reduced the phosphorylation of Smad2 (Fig. 3.8A) and Smad3 (Fig. 3.8B). Notably, the inhibition of the TGFβ1 signaling pathway did also significantly reduce protein levels of α-SMA in hASCs (Fig. 3.8).

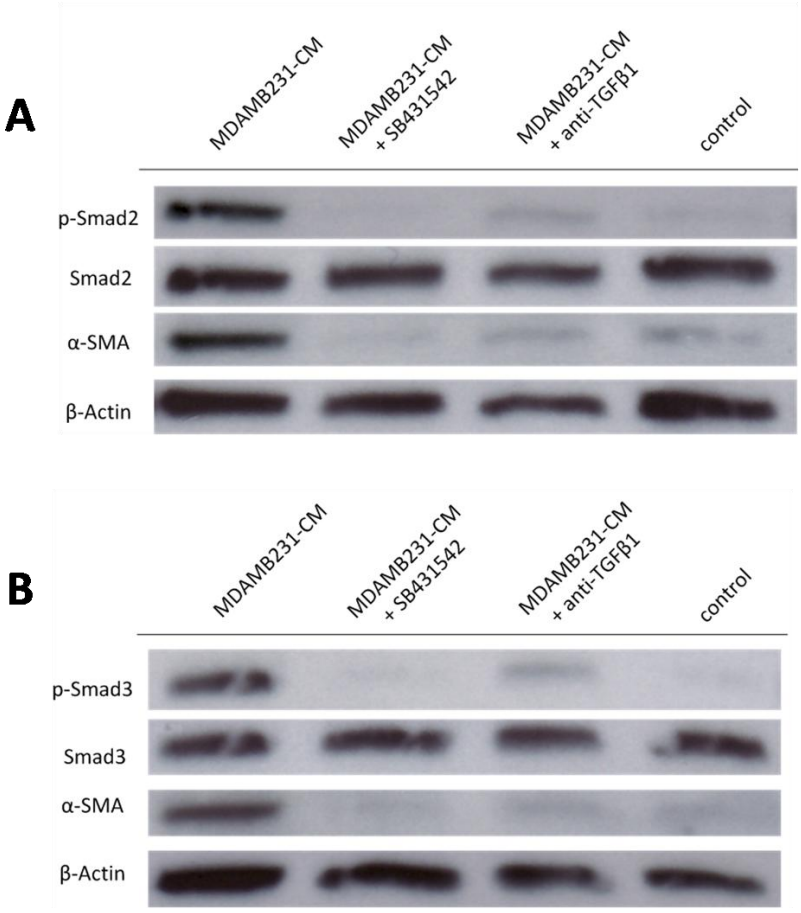

Figure 3.8 Inhibition of Smad2/3 phosphorylation and α-SMA expression. hASCs were pretreated with DMSO as a vehicle or 10 µM SB431542 (TGFβ1 activin receptor-like kinase receptor inhibitor) for 30 minutes and then exposed to serum-free medium (control) or MDAMB231 CM for 4 days. To neutralize TGFβ1, cells were cultured in TCM for 4 days in the presence of anti-TGFβ1 neutralizing-antibody (0.2 µg/ml). The expression levels of **(A)** Smad2, phosphorylated Smad2, **(B)** Smad3, phosphorylated Smad3, α-SMA and β-Actin were determined by western blot analysis.

To further confirm the role of TGFβ1 in hASC differentiation towards myofibroblasts, hASCs were treated with different concentrations of recombinant human TGFβ1 or TCM from MCF7 and MDAMB231 cancer cells for 4 days and the percentage of cells expressing α-SMA was quantified by immunofluorescence analysis as described in previous experiments (see 3.2.3). After stimulation with 0.2 ng/ml rTGFβ1 50.6% ± 3.2% of the cells expressed α-SMA, whereas after stimulation with 2 ng/ml rTGFβ1 75.2% ± 7.5% of the cells expressed the myofibroblast marker, indicating that exogenously added TGFβ1 dose-dependently increased the expression of α-SMA in hASCs (Fig. 3.9). Furthermore, it was shown that treatment with 0.2 ng/ml recombinant TGFβ1 over 4 days, which is approximately the amount of TGFβ1 secreted by MDAMB231 cells after 24 hours (as determined by ELISA within the established experimental setup [see Fig. 3.5], elicited a stem cell response indistinguishable from that obtained with TCM (0% FBS; 24 h conditioning time) (Fig. 3.9).

Additionally, abrogation of TGFβ1-dependent activity was achieved using a neutralizing antibody against TGFβ1 (0.2 µg/ml) in both hASCs cultured with 0.2 ng/ml rTGFβ1 and in hASCs cultured in MDAMB231 CM (Fig. 3.9). Cells treated with rTGFβ1 in the presence of anti-TGFβ1 over 4 days expressed only 3.7% ± 2.0% α-SMA positive cells. Cells cultured in MDAMB231 CM in the presence of anti-TGFβ1 over 4 days expressed only 8.2% ± 1.2% α-SMA positive cells. hASCs cultured in MDAMB231 CM in the presence of normal chicken IgY over 4 days were used as a control group and expressed 50.3% ± 3.5% α-SMA-positive cells in line with previous experiments.

Furthermore, the percentage of TCM-induced expression of α-SMA (both by MDAMB231 CM as well as MCF7 CM) was markedly reduced by pretreatment of hASCs with the TGFβ1 activin receptor-like kinase receptor inhibitor (Figure 3.9). Cells pretreated with 10 µM SB431542 for 30 minutes and consequently stimulated with TCM over 4 days expressed significantly less α-SMA positive cells than those pretreated with a vehicle (DMSO) for 30 minutes in the corresponding control group.

These immunofluorescence analysis results confirm the findings of the previous western blot analysis (Fig. 3.7 and 3.8) and together clearly indicate the involvement of cancer-cell derived TGFβ1 in the differentiation of hASCs towards myofibroblast-like cells.

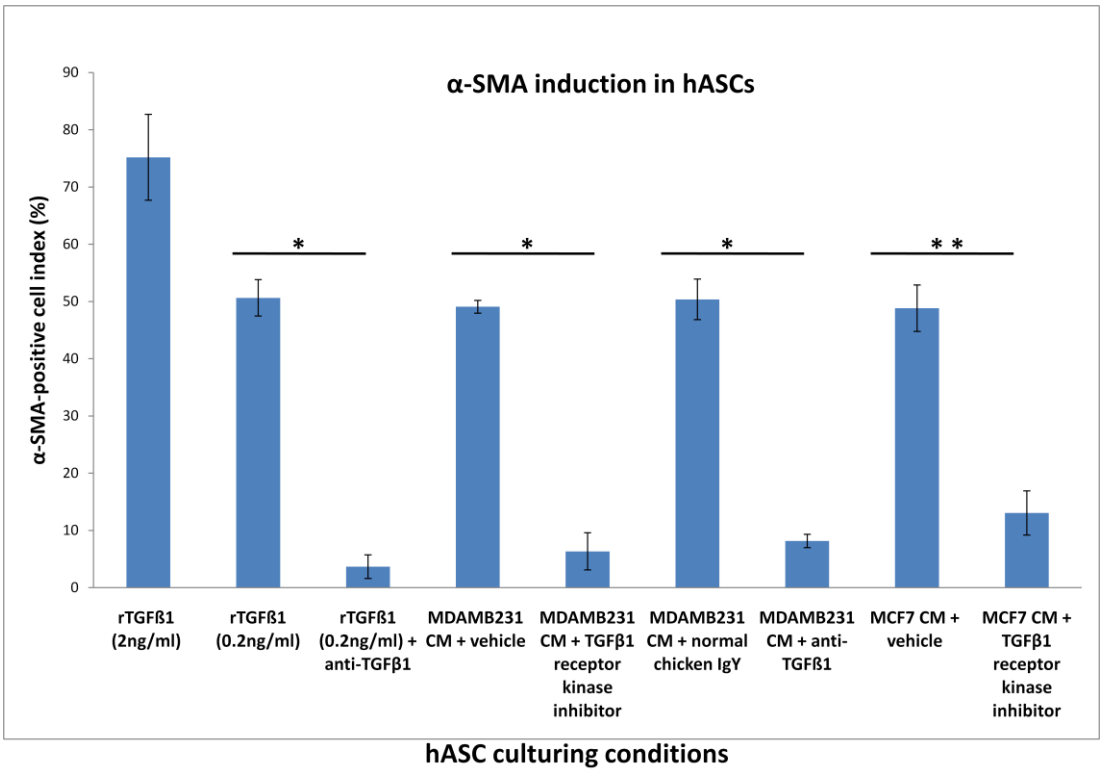

Figure 3.9 TGFβ1 is critical to hASC-differentiation towards myofibroblast-like cells. Serum-starved hASCs were treated with different concentrations of recombinant TGFβ1 (0.2 and 2 ng/ml) or cultured in MDAMB231 conditioned medium (0% FBS; 24 h conditioning time) for 4 days in the presence of anti-TGFβ1 neutralizing antibody (0.2 µg/ml) or control normal chicken IgY (0.2 µg/ml). For inhibition of TGFβ1 receptor kinases hASCs were pretreated with 10 µM SB431542 or DMSO as a vehicle for 30 minutes and then exposed to CM from either MDAMB231 or MCF7 cancer cells for 4 days. The quantitative expression of α-SMA in hASCs was analyzed by immunofluorescence staining and cells positive for α-SMA were counted as a fraction of the total cell number in each group. Data represent average values ± SD. (n=3). * indicates P<0.0001, ** indicates P<0.0004.

3.4 Secretion of SDF-1α from stimulated hASCs

In breast cancer a key feature of tumor activated myofibroblasts is their potential to stimulate tumor growth and to promote angiogenesis partially through their ability to secrete stromal cell-derived factor 1 (SDF-1α) (Orimo et al., 2005). To further prove that hASCs acquire a myofibroblast-like profile under the influence of breast carcinoma cell-derived chemokines it was examined whether stimulation with MDAMB231 and MCF7 CM could induce SDF-1α protein production in hASCs.

3.4.1 Secretion of SDF-1α from hASCs increases upon stimulation with TCM

For the detection of stromal cell-derived factor 1 alpha, hASCs were cultured in TCM (0%FBS, 24 h conditioning time) of MDAMB231 or MCF7 cancer cells. Cell culture supernatant from these hASCs was analyzed by ELSIA after 12, 24, 48 and 72 hours. ELISA testing indicated that the secretion of SDF-1α from TCM-activated hASCs was time-dependent, reaching a maximum level after 72 hours in culture for both hASCs cultured in MCF7 and MDAMB231 CM (Fig. 3.10). Stimulation with conditioned medium from MDAMB231 cells caused a significantly higher production of SDF-1α than stimulation with MCF7 CM (742.3 ± 37.6 pg/ml as compared to 176.1 ± 7.8 pg/ml after 72 h). As shown in Figure 3.11, the increase in SDF-1α protein-level after 72h was 5.2-fold for hASCs cultured in MDAMB231 CM as compared to the control group of hASCs cultured in serum-free regular medium ($P<0.001$).

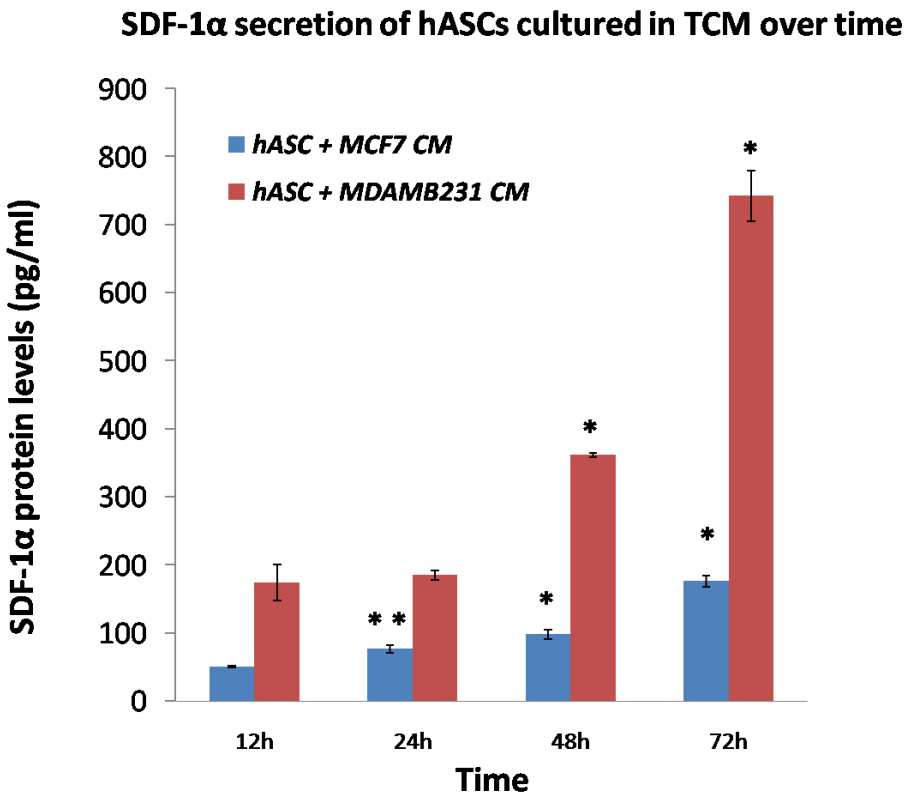

Figure 3.10 TCM stimulates the secretion of SDF-1α from hASCs. Protein levels of SDF-1α secreted from hASCs exposed to either MCF7 (blue bars) orMDAMB231 (red bars) conditioned medium (0%FBS, 24 h conditioning time) over time was measured by ELISA. The experiment was repeated three times. *P<0.0001 and **P<0.002 versus 12 h TCM (MCF7 and MDAMB231 CM respectively).

3.4.2 Influence of TGFβ1 on SDF-1α secretion

Additionally the involvement of TGFβ1 in the production of SDF-1α was analyzed. Serum-starved hASCs were incubated with the TGFβ1 receptor kinases inhibitor SB431542 or DMSO as a vehicle for 30 minutes, cultured in MDAMB231 conditioned medium (0%FBS, 24 h conditioning time) or serum-free αMEM for 72 h and subsequently cell culture supernatants were subjected to SDF-1α ELISA. As shown in Figure 3.11, the increased

expression of SDF-1α in TCM-activated hASCs was significantly reduced by pretreatment with SB431542 (417.8±74.5 pg/ml as compared to 792.3±129.8 pg/ml; P<0.01). Interestingly, 72 h stimulation with recombinant TGFβ1 did not affect the secretion of SDF-1α from hASCs (Fig. 3.11), suggesting that TGFβ1 alone cannot be sufficient for the TCM-induced production of SDF-1α, although the TGFβ1 signaling pathway seems to be involved in SDF-1α secretion.

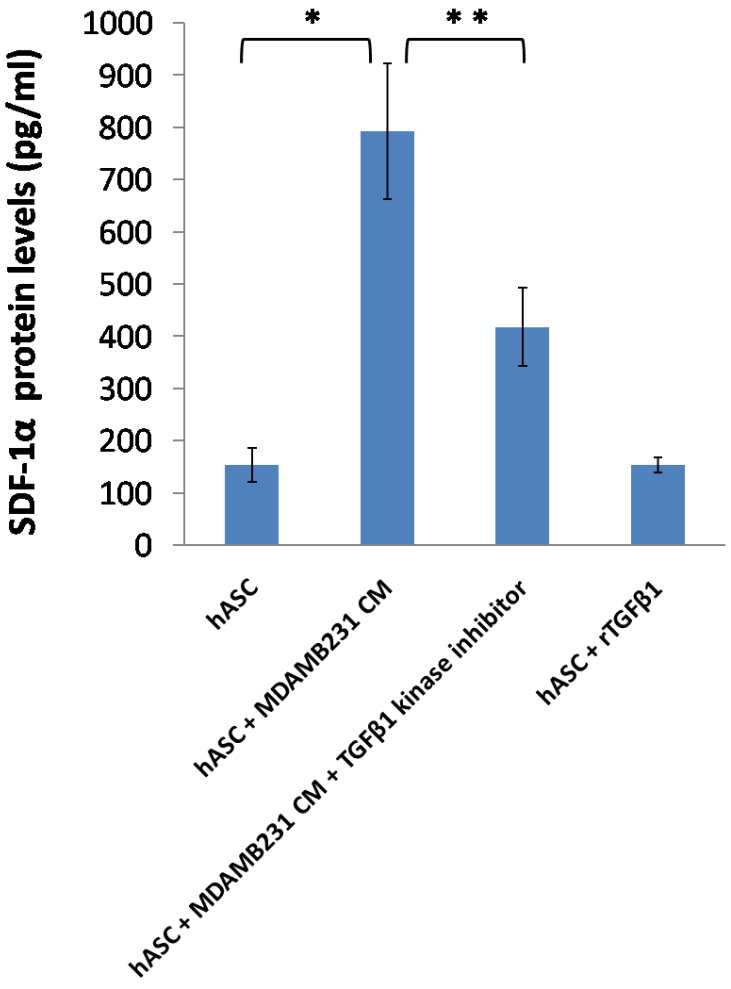

Figure 3.11 Effect of TGFβ1 on SDF-1α in hASCs. Serum-starved hASCs were pretreated with a vehicle (DMSO) or 10 μM SB431542 (TGFβ1 kinases inhibitor) for 30 min and then cultured in serum-free medium, MDAMB231 CM or exposed to 0.2 ng/ml rTGFβ1 for 72 hours. Cell culture supernatants were subjected to ELISA for the measurement of SDF-1α protein levels. *P<0.001, **P<0.01.

3.5 Secretion of CCL5 from TCM-activated hASCs

In a further step the question was raised whether the secretion of the chemokine CCL5 which is known to be involved in breast cancer progression (Soria and Ben-Baruch, 2008) was increased in hASC-derived myofibroblasts. CCL5 had already been identified as a hASC-derived humoral factor that can promote breast cancer cell invasion *in vitro* in a previous study (Pinilla et al., 2009).

For detection of hASC-derived myofibroblast-like cells the expression level of tenascin-C in TCM-activated and normal hASCs was determined using fluorescence-activated cell sorting (FACS). Hence hASCs were cultured in either MDAMB231 CM (0% FBS; 24 h conditioning time) or serum-free regular growth medium over 4 days, incubated with 5 µg/ml anti-tenascin-C antibody and Alexa Fluor conjugated secondary antibody and subsequently separated by fluorescence-activated cell sorting. FACS sorting analysis revealed that after 4 days in tumor conditioned medium 24.8% of the cells expressed tenascin-C whereas only 0.4% of the cells cultured in serum-free regular medium for the same time expressed this myofibroblast marker (Fig. 3.12b and 3.12c).

The separated cell fractions of tenascin-C positive and negative cells previously cultured in MDAMB231 conditioned medium over 4 days were subsequently cultured in regular growth medium (αMEM containing 10%FBS) over 4 days and cell culture supernatants were analyzed by ELISA for CCL5 protein levels using the human CCL5 Quantakine kit. Interestingly, cells positive for tenascin-C produced significantly more CCL5 than the tenascin-C negative cell fraction (744.13 ± 67.95 as compared to 217.38 ± 21.95 pg/500,000 cells; Figure 3.13). These results confirmed that hASCs cultured in tumor conditioned medium partially differentiate into tenascin-C positive myofibroblasts as shown by IF staining (see Fig. 3.3) and additionally demonstrated that these differentiated cells produce elevated levels of the chemokine CCL5.

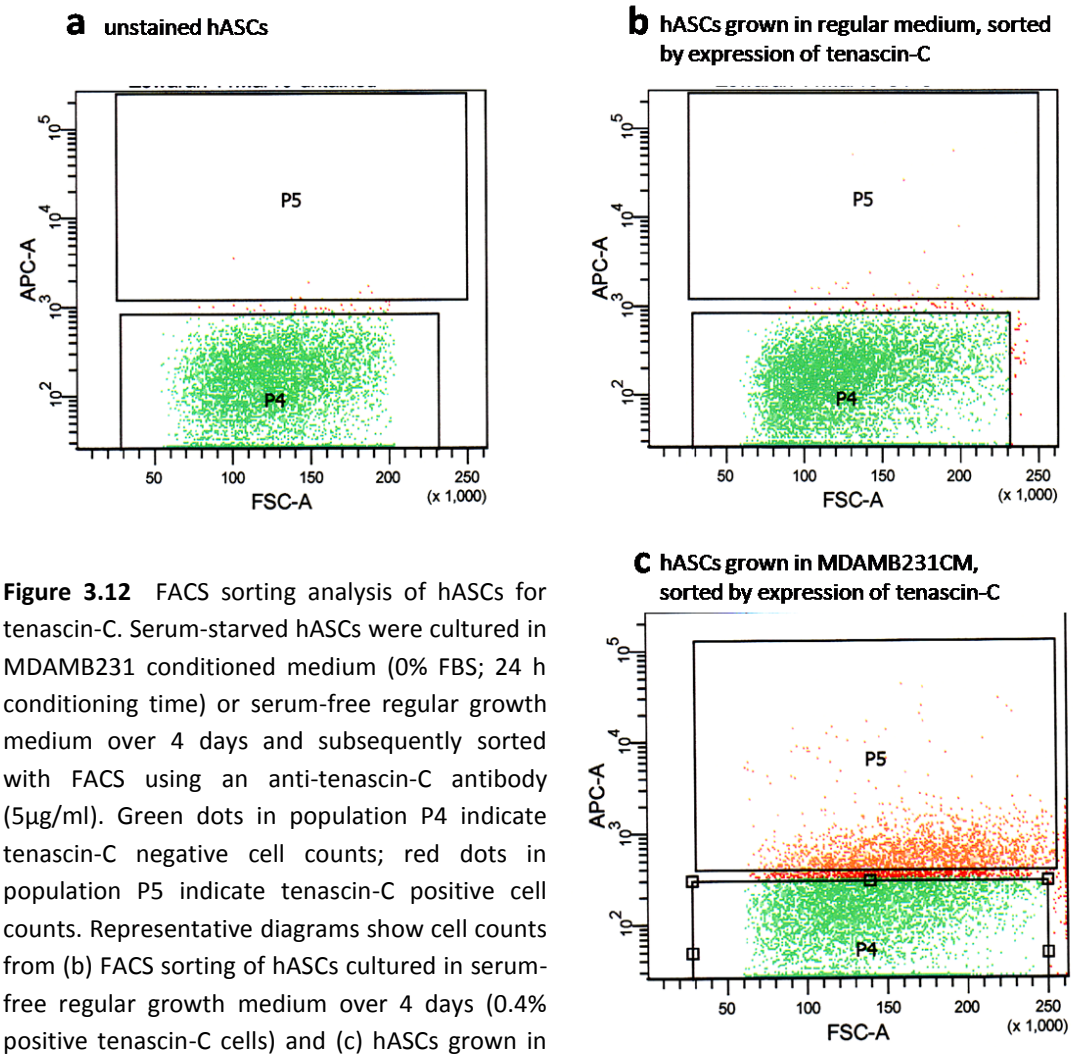

Figure 3.12 FACS sorting analysis of hASCs for tenascin-C. Serum-starved hASCs were cultured in MDAMB231 conditioned medium (0% FBS; 24 h conditioning time) or serum-free regular growth medium over 4 days and subsequently sorted with FACS using an anti-tenascin-C antibody (5µg/ml). Green dots in population P4 indicate tenascin-C negative cell counts; red dots in population P5 indicate tenascin-C positive cell counts. Representative diagrams show cell counts from (b) FACS sorting of hASCs cultured in serum-free regular growth medium over 4 days (0.4% positive tenascin-C cells) and (c) hASCs grown in MDAMB231 CM over 4 days (24.8% positive tenascin-C cells). (a) Unstained hASCs were used as negative control for cell sorting.

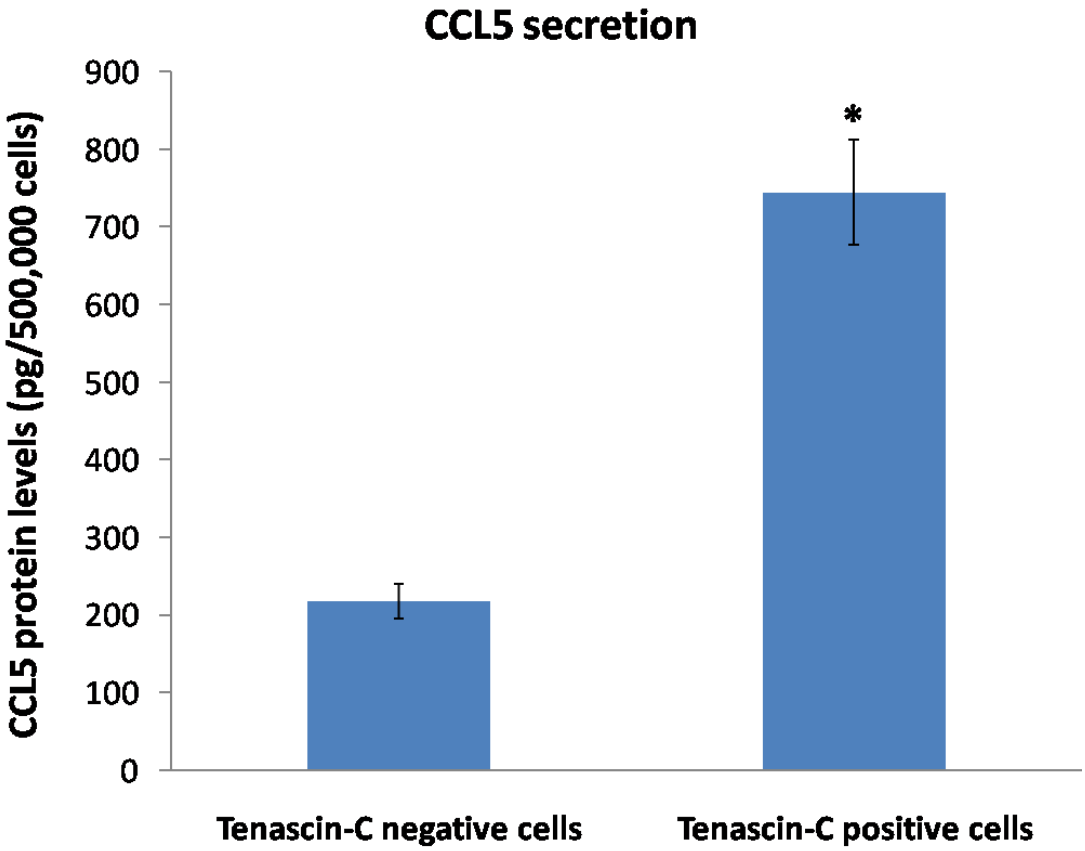

Figure 3.13 Tenascin-C positive hASC-derived myofibroblasts produce elevated levels of CCL5. Cell fractions of tenascin-C positive and negative cells previously cultured in MDAMB231 CM as sorted by FACS were cultured in regular growth medium over 4 days and cell culture supernatant from these cells was used for the detection of CCL5 protein levels by ELISA. Data represent mean ± SD. Experiments were repeated three times. * indicates $P<0.0003$.

3.6 Invasion of MDAMB231 cancer cells *in vitro*

An important feature of tumor-activated myofibroblasts is their ability to promote cancer cell invasion (De et al., 2004). An *in vitro* invasion assay was established in order to assess the impact of hASC-derived myofibroblasts on the invasion of breast cancer cells. The matrigel matrix of the invasion assay consists mainly of laminin (56%) and collagen IV (31%) and functions as a reconstituted basement membrane *in vitro*. In order to invade this membrane, cells have to degrade the matrigel through secretion of ECM digesting enzymes. Since MDAMB231 is known to be among the most invasive breast cancer cell lines, this cell line was used for all invasion assays rather than using MCF7 cancer cells which tend to grow in colonies and are less invasive.

3.6.1 hASC-derived myofibroblasts promote cancer cell invasion

On the surface of a matrigel coated insert DiI-stained MDAMB231 cells were seeded either alone ($35x10^3$ cancer cells/well) or in co-culture with hASCs ($70x10^3$ hASCs/well) previously grown in regular medium, MDAMB231 conditioned medium (0%FBS, 24 h conditioning time) or regular medium containing 0.2ng/ml TGFβ1 for 4 days, respectively. Since MDAMB231 cancer cells grow faster than mesenchymal stem cells the cell ratio of MDAMB231 to hASCs was kept at 1:2 for all experiments. All cells were seeded in 600 µl of αMEM containing 5%FBS in the upper chamber of the filter insert. In order to create a serum gradient that would stimulate cell invasion the lower chamber was filled with αMEM containing 10%FBS. After 40 h of incubation all non-invaded cells were removed from the matrigel coated insert by scrubbing the top surface with cotton tip swabs and remaining cells on the lower side of the insert were additionally stained with the nuclear dye Hoechst 33342 and analyzed under the microscope (representative images are shown in Fig. 3.14).

The remaining, invaded DiI + Hoechst 33342 positive cells were then counted under the microscope in five independent view fields at a 20x magnification.

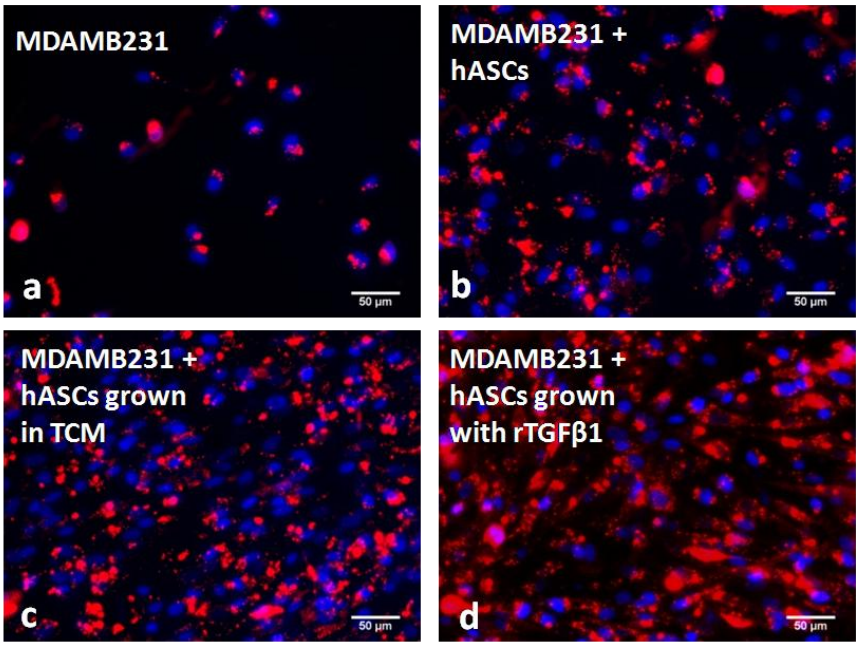

Figure 3.14 Representative images of DiI stained MDAMB231 cancer cells (red channel) that invaded into the matrigel after 40 h. The nuclei were counterstained with Hoechst (blue channel). Overlay of red with blue signal shows invaded cancer cells when seeded (a) alone or co-cultured with hASCs previously grown in (b) regular medium, (c) MDAMB231 tumor conditioned medium (TCM) or with (d) 0.2 ng/ml rTGFβ1 for 4 days, respectively. Scale bar, 50 µm.

When counting the cancer cells that invaded into the matrigel after 40 hours, significantly more cancer cells invaded the coated membrane when co-seeded with hASCs (96.9 ± 23.2 invaded cancer cells/view field) as compared to the mono-culture control group (33.6 ± 14.9 invaded cancer cells/view field, Fig. 3.15). Furthermore, MDAMB231 cells became even more invasive when co-cultured with hASCs that had previously been cultured with either TCM (0%FBS, 24 h conditioning time) or 0.2 ng/ml TGFβ1 for 4 days (Figure 3.15).

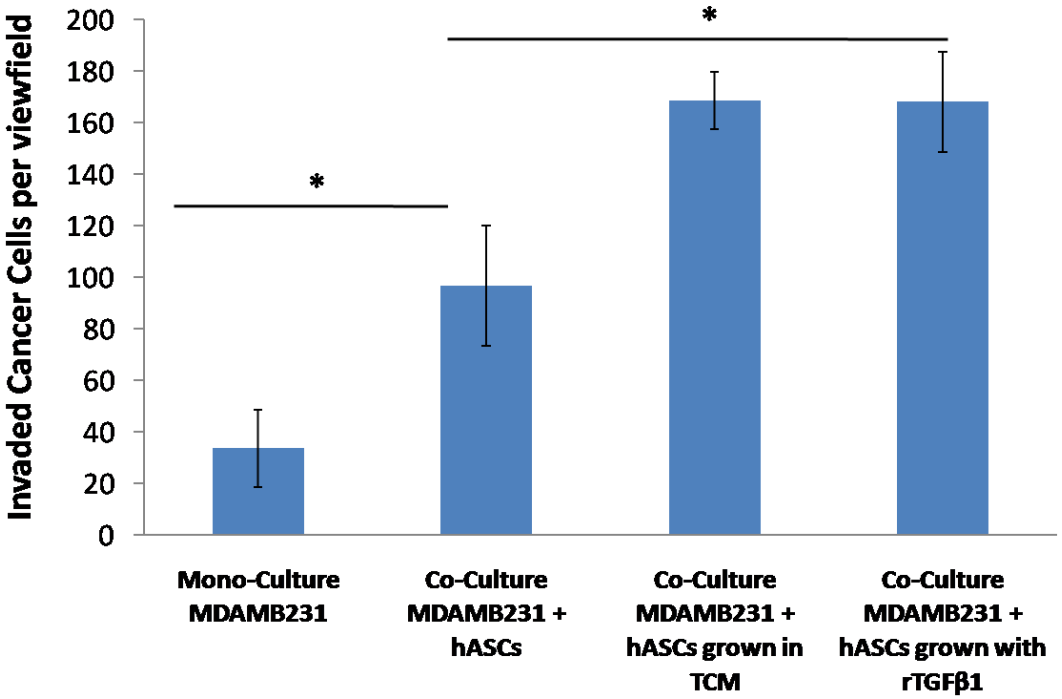

Figure 3.15 TCM-activated hASCs and recombinant TGFβ1 treated hASCs promote tumor cell invasion. Invaded DiI+Hoechst33342-positive MDAMB231 cancer cells were counted in five independent view fields at 20x magnification in each group and are shown as mean ± SD with experiments being repeated three times. * indicates P<0.001.

To confirm these results invasion assays were partly repeated using GFP-labeled MDAMB231 cells. As shown in Figure 3.16 significantly more GFP-labeled MDAMB231 cells invaded the matrigel membrane when co-seeded with TCM-activated hASCs as compared to invaded GFP-MDAMB231 cells co-seeded with regular hASCs (104 ± 10.3 as compared to 71 ± 5.6 cancer cells/view field). The invasion of GFP-MDAMB231 co-seeded with TCM-activated hASCs was 1.46 times higher than that of GFP-MDAMB231 co-seeded with regular hASCs. Consequently these results confirm the previous experiments performed with DiI-stained MDAMB231 cells, where the invasion was 1.7 times higher when comparing these two groups.

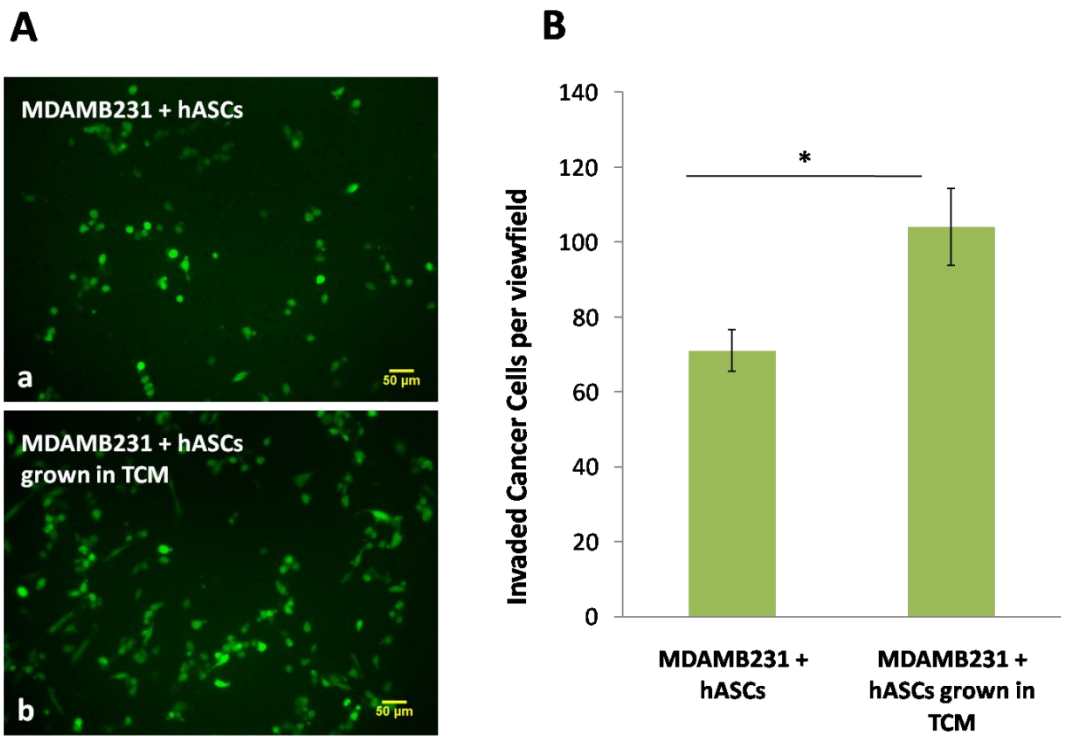

Figure 3.16 Invasion assay with GFP-labeled MDAMB231. **(A)** Representative images of GFP-labeled MDAMB231 cells that invaded into matrigel after 40 h. Green channel shows GFP-MDAMB231 when seeded in co-culture with hASCs previously grown in (a) regular medium and (b) MDAMB231 tumor conditioned medium (TCM) for 4 days, respectively. Scale bar, 50 µm. **(B)** Invaded GFP-positive MDAMB231 cancer cells were counted in five independent view fields at 20x magnification in each group and are shown as mean ± SD with each experiment being repeated three times. * indicates P<0.0001.

3.6.2 Influence of hASC conditioned medium on breast cancer cell invasion

To exclude that the observed increased invasion of breast cancer cells in co-culture with TCM-activated hASCs was merely an effect of higher cell density in the co-culture experiments and in order to assess the influence of conditioned medium from hASC-derived myofibroblasts on the invasion of breast cancer cells, another series of invasion assays with MDAMB231 cells was performed.

For this assay $35x10^3$ cancer cells per well were seeded alone on the surface of the coated inserts and exposed to conditioned medium from hASCs, TGFβ1-treated hASCs, TCM-

activated hASCs and TCM-activated hASCs pre-incubated with 10 µM of the TGFβ1 receptor kinases inhibitor SB431542 for 30 minutes (see materials & methods 2.2.9 for details). After 40h the assay was stopped, the top surface of the matrigel was scrubbed with cotton tip swabs and invaded cancer cells were stained with the nuclear dye Hoechst 33342. The remaining, invaded cells were counted in five independent view fields (see Fig. 3.17a-e for representative pictures of invaded MDAMB231).

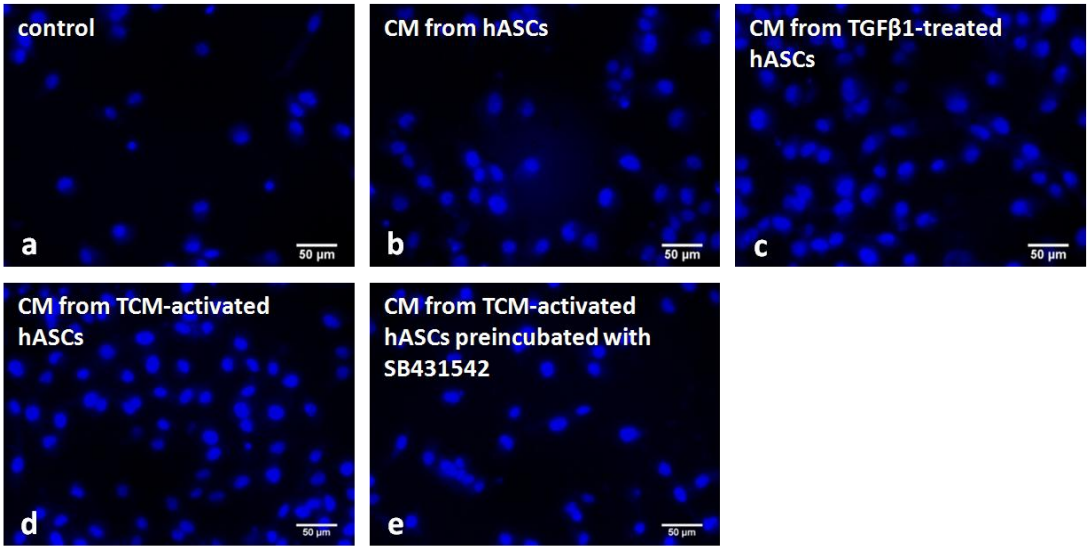

Figure 3.17 Representative images of Hoechst33342 stained MDAMB231 cancer cells that invaded into matrigel after 40 h. The nuclei of the invaded cells were stained with Hoechst 33342 (blue channel). Images show invaded cancer cells when cultured in (a) αMEM containing 5%FBS, (b) CM from hASCs, (c) CM from hASCs treated with 0.2 ng/ml rTGFβ1, (d) CM from TCM-activated hASCs and (e) CM from TCM-activated hASCs pretreated for 30 minutes with 10 µM SB431542 (TGFβ1 receptor kinases inhibitor). Scale bar, 50 µm.

As shown in Figure 3.18, the invasion assay revealed that both CM from TCM-activated hASCs as well as CM from TGFβ1-treated hASCs significantly increased the invasion of MDAMB231 cancer cells as compared to CM from normal hASCs (74.2 ± 8.2 and 67.8 ± 10.5 as compared to 46.2 ± 6.7 cancer cells/view field; $P<0.005$). Nevertheless CM from normal hASCs did already notably increase the invasion of MDAMB231 compared to cancer cells cultured with 5%FBS αMEM (control group). Moreover, the invasive capacity of cancer cells grown in CM from TCM-activated hASCs pretreated with the TGFβ1 receptor kinases

inhibitor SB431542 was considerably reduced when compared to the invasive rate of cancer cells grown in CM from TCM-activated cells for the same time (P<0.0001).

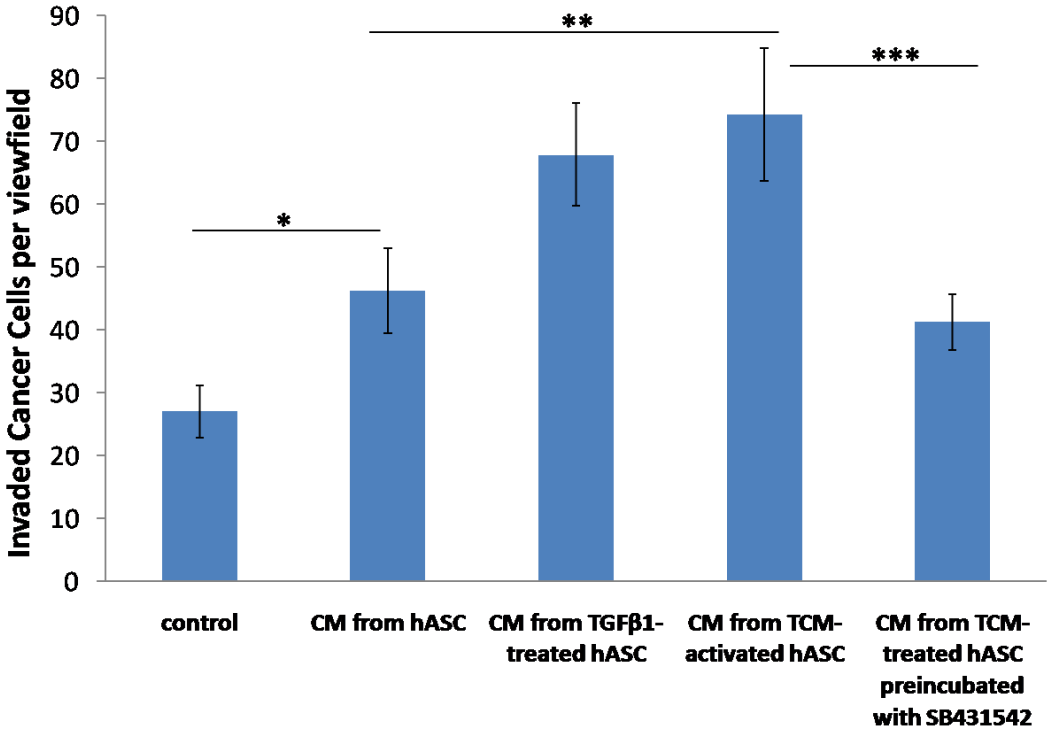

Figure 3.18 Conditioned medium from stimulated hASCs promotes tumor cell invasion. Hoechst 33342-positive MDAMB231 cancer cells were counted in five independent view fields in each group and are shown as mean ± SD. Experiments were repeated three times. *P<0.001; ** P<0.005; *** P<0.0001.

Hence these results confirm the influence of conditioned medium from hASC-derived myofibroblasts on the invasion of breast cancer cells indicating that humoral factors secreted from TCM-activated or TGFβ1-treated hASCs must be involved in this process. Furthermore, the experiments demonstrate that inhibition of the TGFβ1 signaling pathway in hASCs reduces the potential of these cells to enhance the invasion of breast cancer cells *in vitro*.

4. Discussion

The aim of the study was to assess whether human adipose tissue derived stem cells (hASCs) can develop into carcinoma-associated myofibroblasts under the influence of breast cancer cells *in vitro*.

The tumor stroma and in particular stromal myofibroblasts play a crucial role in promoting many aspects of tumor development. However, the origin of these cells and the mechanisms by which they develop is still uncertain. Bone marrow derived mesenchymal stem cells (BMSCs) have recently been found to integrate into the tumor-associated stroma and to differentiate into myofibroblasts which act in a paracrine fashion on cancer cells to enhance their invasion (Spaeth et al., 2009). However, the potential of adjacent tissue resident stem cells from the fat tissue, which might represent early response cells within breast cancer, to differentiate into myofibroblasts and support breast cancer cell invasion had not been investigated so far.

In this study it was shown that hASCs exhibit increased expression of the myofibroblast markers tenascin-C and α-SMA, when exposed to tumor cell conditioned medium from the two breast cancer cell lines MDAMB231 and MCF7. Further it was shown that tumor conditioned medium from MDAMB231 and MCF7 cells contains significant amounts of TGFβ1 and that the differentiation of hASCs towards myofibroblasts is dependent on TGFβ1 signaling via Smad2 and Smad3 in hASCs. Additionally, hASCs secrete increased protein levels of SDF-1α and CCL5 when cultured in tumor cell conditioned medium (TCM) and TCM-activated hASCs promote the invasion of cancer cells *in vitro*.

4. 1 Myofibroblast phenotype in TCM-activated hASCs

In a first set of experiments it was proven that the isolated human ASCs express mesenchymal stem cell markers. Using flow cytometric analysis it was shown that the cells are positive for CD surface markers CD29, CD44, CD90 and CD105 indicative of a broad differentiation potential. Cells are negative for CD34 and CD45 precluding contamination with hematopoietic cells. The expression profile of surface markers for hASCs as shown in these experiments is in line with previous reports from our (Bai et al., 2007a; Bai et al., 2007b) and other groups (Schäffler and Büchler, 2007).

For immunofluorescence experiments of myofibroblast-markers the focus was set on tenascin-C and α-SMA expression, since the relevance of both proteins as specifically important markers of carcinoma-associated myofibroblasts in reactive stroma of different types of cancer had been demonstrated in various studies. Tenascin-C, which physiologically modulates cell adhesion and migration and in cancer facilitates tumor cell invasion and metastasis, was found to be weakly expressed in normal tissue whereas both myofibroblasts in tumor stroma (Tuxhorn et al., 2002) as well as experimentally generated myofibroblasts (Kojima et al., 2010) showed strong staining. α-SMA expressing myofibroblasts are abundant in most invasive human breast cancers (Sappino et al., 1988) and many other epithelial carcinomas like that of the prostate, colon, lung and uterus (Orimo et al., 2001). In consequence it seemed of interest to focus on these markers in the context of the present study.

Certainly there are many other markers for carcinoma-associated fibroblasts such as vimentin, fibroblast surface protein and fibroblast-activation protein (Orimo and Weinberg, 2007) but as these markers have not been reported to be particularly specific for

carcinoma-associated myofibroblasts they were not used for the experiments performed in this study.

Although the expression of myofibroblast markers in hASCs alone was very weak and breast cancer cells did not express any of these markers, increased expression levels of the typical myofibroblast protein α-SMA could be observed in co-cultures with either MDAMB231 or MCF7 breast cancer cells. Furthermore, hASCs did also acquire a myofibroblast phenotype (expression of α-SMA and tenascin-C) after exposure to TCM from MDAMB231 or MCF7, indicating that physical contact between the cells was not required and breast cancer cell derived humoral factors were sufficient for the differentiation. The differentiation of hASCs towards myofibroblast-like cells was time-dependent with the majority of cells expressing α-SMA after 4 days of culturing in TCM from either MDAMB231 or MCF7 as determined by immunofluorescence analysis.

Similar experiments using bone marrow derived stem cells (BMSCs) and CM from various cancer cell lines were carried out by Mishra et al., who found that CM from breast cancer cells could induce α-SMA expression in BMSCs under defined tissue culture conditions (Mishra et al., 2008). Interestingly, in these experiments stimulation with TCM from MDAMB231 was performed for an entire period of 30 days after which most BMSCs expressed myofibroblast markers. In the experiments performed in this study the majority of hASCs did already differentiate into α-SMA positive cells after only 4 days of stimulation with MDAMB231 conditioned medium, showing that under defined tissue culture conditions mesenchymal stem cells from the adipose tissue respond faster to TCM-activation than those from the bone-marrow. It is therefore conceivable that also in *in vivo* breast cancer models, cells from the adipose tissue might respond faster to cancer signaling and be of special interest in the process of understanding the early cancer-stroma differentiation.

4. 2 Role of TGFβ in hASC-conversion towards myofibroblast-like cells

TGFβ1 is considered to have a central role in inducing the myofibroblastic phenotype, because it is capable of up-regulating fibroblast α-SMA both *in vitro* and *in vivo* (Tuxhorn et al., 2001; Kojima et al., 2010). In many types of cancers, TGFβ1 is overexpressed by carcinoma cells (Teicher, 2001), and it has been proposed previously that the expression of this cytokine by prostate carcinoma cells induces reactive stroma (Webber et al., 1999). Moreover, bone marrow derived mesenchymal stem cells undergo myofibroblast differentiation, including increased production of α-SMA in response to TGFβ1 (Wang et al., 2004).

Both supernatants from MDAMB231 and MCF7 cancer cells contained significant amounts of TGFβ1 after 24 hours of culturing in serum free medium, whereas hASCs did not secrete any TGFβ1 as determined by ELISA. Additionally, IF staining revealed that hASCs express the TGFβ type II receptor. Upon binding of TGFβ1 this receptor can phosphorylate the type I receptor, activating the TGFβ-complex which eventually signals through the Smad pathway and elicits a broad range of gene responses (Massague, 2008). Western blot analysis confirmed that both hASCs cultured in MDAMB231 conditioned medium and hASCs stimulated with 0.2 ng/ml recombinant TGFβ1 (the same concentration of TGFβ1 as present in TCM from MDAMB231 as used for experiments) activate the Smad pathway in hASCs as demonstrated by high expression levels of p(hosphorylated)-Smad2, p-Smad3 and α-SMA. The up-regulation of p-Smad3 was quantified by western blot protein band density measurements and revealed over 20-fold higher induction of p-Smad3 in TCM-activated or rTGFβ1-stimulated hASCs as compared to control hASCs. Consequently the increased expression of α-SMA in hASCs in response to exposure to TCM appears to be influenced through TGFβ1 secretion by breast cancer cells in a paracrine fashion.

Jeon et al. showed that lysophosphatidic acid (LPA) which is enriched in ascites of ovarian cancer patients stimulates the production of TGFβ1 in hASCs (Jeon et al., 2008). Jeon further showed that TGFβ1 activates its receptor and stimulates the expression of α-SMA through an autocrine mechanism. In this present study it was confirmed that hASCs indeed express the TGFβ type II receptor, however, breast cancer cell derived TGFβ1 was responsible for the induction of the myofibroblast-like phenotype in a paracrine fashion and hASCs themselves did not produce TGFβ1. It is therefore conceivable that different types of cancer can use different mechanisms to induce the expression of α-SMA in cells of the surrounding stroma.

After having identified TGFβ1 secreted from breast cancer cells as a potent agent in the differentiation of hASCs towards myofibroblasts, the question was raised if the observed effect could be inhibited by targeting TGFβ or its receptor on hASCs. Western blot analysis of hASCs cultured in TCM revealed that both, pretreatment of hASCs with 10 µM SB431542 (a potent and selective inhibitor of TGFβ type I receptor kinases ALK4, ALK5 and ALK6 (Inman et al., 2002)), as well as anti-TGFβ1 treatment with 0.2 µg/ml of a neutralizing antibody markedly reduced the phosphorylation of Smad2 and Smad3, and significantly reduced protein levels of α-SMA in hASCs. These findings were additionally verified by performing immunofluorescence analysis experiments, where it was shown that addition of either TGFβ1 neutralizing antibody or pretreatment of hASCs with SB431542 completely blocked the phenotypic switch of hASCs cultured in TCM towards α-SMA expressing myofibroblasts. It was confirmed that only specific inhibition and blocking of TGFβ was responsible for this effect using DMSO and normal chicken IgY as negative controls, respectively. It was also shown that exogenously added TGFβ1 dose-dependently increased the expression of α-SMA in hASCs.

Considering these results, it would be very interesting to perform *in vivo* studies which explore the possibility to reduce the generation of tumor-promoting myofibroblasts in breast cancer through direct targeting and disrupting TGFβ signaling in ASCs. One difficulty of this approach could be the complex nature of TGFβ in cancer. While TGFβ acts as a tumor-suppressor in normal or premalignant cells it “switches sides” during cancer

progression and promotes tumor growth in a paracrine and/or autocrine manner (Massague, 2008). These contextual differences do also apply for the role of TGFβ within normal and tumor stroma. In normal stroma TGFβ has anti-tumorigenic effects and can suppress the expression of fibroblast-derived mitogenic factors that influence the adjacent epithelial cells. Bhowmick et al. generated mouse models in which the TGFβ RII gene was inactivated in fibroblasts of the prostate and forestomach which subsequently expressed elevated levels of mitogenic factors resulting in prostate and gastric carcinoma (Bhowmick et al., 2004). Hence, reducing the number of tumor-promoting myofibroblasts in cancer through inhibition of TGFβ signaling in stromal hASCs could involve other dangers which need to be taken into consideration.

4. 3 Secretion of tumor-promoting factors from TCM-activated hASCs

In a further step it was explored if myofibroblast-like cells derived from TCM-activated hASCs express tumor-promoting soluble factors. Carcinoma-associated myofibroblasts within tumor stroma have been shown to support cancer progression via the secretion of a huge variety of tumor-promoting chemokines. Out of these numerous tumor-promoting soluble factors the importance of SDF-1 and CCL5 in breast cancer had lately been demonstrated in various studies (Mishra et al., 2011; Karnoub et al., 2007; Orimo et al., 2005). The secretion of SDF-1α and CCL5 was significantly increased in breast cancer TCM-activated hASCs.

4.3.1 Stromal cell-derived factor 1

Apart from its function in hematopoiesis and the development of the immune system the chemokine stromal cell-derived factor 1 (SDF-1), which signals primarily through its receptor CXCR4, plays a crucial role in the mobilization of metastatic cancer cells from various origins including cancer of the breast, ovary, kidney, lung, pancreas, colon and prostate (Sun et al., 2010), as well as for retention of acute leukemic cells in the bone marrow (Teicher and Fricker, 2010). This is achieved by promoting the migration of the CXCR4-expressing cancer cells towards a SDF-1 gradient produced by the stroma of these target organs (Burger and Kipps, 2006). Recent studies indicate that stromal cells in the primary tumor are an important source of SDF-1 (Mishra et al., 2011). The α-SMA protein which is present on myofibroblasts in tumor stroma is largely co-localized with the SDF-1 protein, in contrast fibroblast-like cells positive for SDF-1 could not be detected in non-cancer stroma in a study performed by Orimo et al. (Orimo et al., 2005). In a further study it was shown that LPA which is enriched in ascites of ovarian cancer patients stimulated the production of SDF-1 from human ASCs through a TGFβ1-Smad-dependent pathway (Jeon et al., 2008).

For the detection of SDF-1α cell culture supernatants from hASCs were analyzed and it was shown that TCM-activation with both conditioned medium from MDAMB231 and MCF7 significantly increased the secretion of SDF-1α reaching a maximum level after 72 h in culture. The increase in SDF-1α protein-level after 72h was 5.2-fold for hASCs cultured in MDAMB231 CM as compared to control hASCs.

It was shown that murine ASCs can promote tumor growth in a SDF1/CXCR4 dependent manner (Muehlberg et al., 2009). In this model co-injection of murine ASCs with the murine breast cancer cell line 4T1 into nude male Balb/c mice caused tumors to grow significantly faster and knockdown of CXCR4 in 4T1 cells inhibited this effect. Moreover, murine ASCs that were isolated from the fat tissue surrounding the tumor, expressed significantly more SDF-1 than control ASCs. These results obtained in a murine breast cancer model *in vivo* are

in line with the data generated *in vitro* in this study and prove that both human and murine ASCs secrete elevated levels of SDF-1 when interacting with breast cancer cells.

Furthermore these findings suggest that tumor cells produce specific factors that induce the production of SDF-1α by myofibroblasts. Since the present data demonstrated that TCM-derived TGFβ1 could generate myofibroblast-like cells from hASCs it was hypothesized that TGFβ1 was also responsible for the increased secretion of SDF-1α. Although inhibition of TGFβ1 receptor kinases with 10µM SB431542 significantly reduced the secretion of SDF-1α in TCM-activated hASCs, direct stimulation of hASCs with 0.2 ng/ml rTGFβ1 for 72 h did not influence the secretion of SDF-1α. These data suggest that the TGFβ signaling pathway is involved in SDF-1α secretion from tumor-activated hASCs but not sufficient by itself to induce SDF-1α production and most likely a complex interplay between several factors is causing the secretion of SDF-1α.

Contrary to the data of this present study, it was shown that myofibroblast-like cells, experimentally generated from fibroblasts using rTGFβ1, did show a 4-fold higher induction of SDF-1 in PCR analysis (Kojima et al., 2010). This differing data could be due to the fact that Kojima et al. performed cell-stimulation for 24 h using 10 ng/ml rTGFβ1, while in this study cells were stimulated over 72 h using only 0.2 ng/ml, which is a 50-fold lower concentration. 0.2 ng/ml rTGFβ1 is approximately the corresponding amount of TGF β1 secreted by 8-10x10^6 MDAMB231 cells cultured for 24 h in 25 ml of medium. Since most experiments in this study were performed using TCM from 8-10x10^6 MDAMB231 cultured for 24 h in 25 ml of serum free medium, it seemed appropriate to use this concentration in order to best mimic the effect of TCM.

Nevertheless, the present finding that inhibition of the TGFβ signaling pathway in TCM-activated hASCs significantly reduced the secretion of tumor-promoting SDF-1α points out the essential role of TGFβ in the genesis of CAFs and indicates that targeting this cytokine in breast cancer could be of therapeutic interest.

4.3.2 CCL5

BMSCs admixed with human breast cancer cells have been shown to produce high levels of the chemokine CCL5 which caused cancer cells to increase their migration, invasion and metastasis potential *in vivo*. The effects of CCL5 were dependent on mediation by its receptor CCR5 expressed on cancer cells and could be abolished by inhibition of CCR5 on cancer cells through small hairpin RNA (shRNA) knockdown (Karnoub et al., 2007). In that same study it was demonstrated that the induction of CCL5 required close physical contact between BMSCs and breast cancer cells. In a study performed by our group it was shown that CCL5 could also be produced in co-culture of hASCs with breast cancer cells, however physical contact between these cells was not required (Pinilla et al., 2009). While neither hASCs nor MDAMB231 secreted CCL5 when cultured alone, conditioned medium from MDAMB231 induced a "de novo" secretion of CCL5 from hASCs, suggesting that humoral factors present in TCM are responsible for CCL5-induction. In that study it was also shown that hASC-derived CCL5 was responsible for enhanced tumor cell invasion *in vitro*.

In this present study experiments were conducted to find out more about the cellular sources of CCL5 in TCM-activated hASCs. For this reason hASCs were cultured in MDAMB231 conditioned medium, the cells were sorted for the expression of the myofibroblast marker tenascin-C using FACS and subsequently the secretion of CCL5 in tenascin-C negative and tenascin-C positive cell populations was determined. The results showed that CCL5 is mainly produced by tenascin-C positive cells. While tenascin-C negative cells do also secrete CCL5 to some extent, the secretion from tenascin-C positive cells is 3.4-fold higher. Hence these data suggest that CCL5 is produced by tenascin-C positive myofibroblasts that developed from TCM-activated hASCs.

4.4 Breast cancer cell invasion *in vitro*

90% of cancer deaths from solid tumors are being caused by metastasis which is preceded by local invasion (Gupta and Massague, 2006). A key feature of carcinoma-associated myofibroblasts is their ability to actively promote the invasion of cancer cells thereby paving the way for metastasis (De et al., 2008). In breast cancer these myofibroblasts show extensive gene expression changes in genes encoding invasion-associated factors and receptors (Allinen et al., 2004).

Hence, it was investigated if experimentally generated, hASC-derived myofibroblasts do also resemble carcinoma-associated myofibroblasts in this regard. It had previously been demonstrated that hASCs promote cancer cell invasion through matrigel (a mixture of basement membrane proteins) in co-culture with breast cancer cells (Pinilla et al., 2009). These results were confirmed in the present study and it was further shown that breast cancer cell invasion in co-culture with hASCs was much more pronounced when hASCs had previously been stimulated with either TCM or recombinant TGFβ1 (~ 1.7 fold higher invasion of MDAMB231). These results were confirmed using both DiI-stained as well as GFP-labeled MDAMB231 cancer cells and indicate that hASC-derived myofibroblasts are responsible for enhanced invasion.

Additionally conditioned medium from hASC-derived myofibroblasts alone already significantly increased the invasion of MDAMB231 as compared to CM from normal hASCs. This invasion promoting effect could be abolished by inhibition of the TGFβ1 signaling pathway in hASCs, indicating that TGFβ was an indirect proinvasive factor for MDAMB231 cancer cells as it converts hASCs into myofibroblast-like cells that strongly stimulate invasion. These results do also indicate that cancer invasion can be promoted indirectly by the release of tumor-induced host factors from hASC-derived myofibroblasts and CCL5 had been identified as one possible factor in a previous study (Pinilla et al., 2009).

These findings support the results of previously performed *in vitro* studies which demonstrate that cancer-cell derived TGFβ can sustain the production of pro-invasive factors from myofibroblasts in various types of cancer (Lewis et al., 2003; Casey et al., 2008; De et al., 2004).

Together with the findings that TCM-activated hASCs express myofibroblast-like immunocytochemical markers, these results further corroborate the assumption that hASCs differentiate into functional carcinoma-associated myofibroblasts and prove the essential role of carcinoma-derived TGFβ1 in the interactions between breast cancer cells and mesenchymal stem cells from adipose tissue.

4.5 Conclusion and perspective

The essential feature of the present study, which was designed to reproduce the interactions of epithelial breast cancer cells with adipose tissue derived mesenchymal stem cells *in vitro*, is that under the influence of tumor cell conditioned medium, hASCs can differentiate into myofibroblasts. The differentiation of hASCs towards myofibroblasts expressing α-SMA and tenascin-C is dependent on TGFβ1 secreted from breast cancer cells and can be abolished using a neutralizing antibody to TGFβ1 as well as by pretreatment of hASCs with SB431542, a selective TGFβ1 receptor kinases inhibitor. More importantly, these hASC-derived myofibroblasts exhibit functional properties of carcinoma-associated myofibroblasts including the ability to produce the tumor-promoting chemokines SDF-1α and CCL5 and support tumor cell invasion as shown by an *in vitro* invasion assay. Together, these findings are of special interest, since human mesenchymal stem cells derived from adipose tissue are particularly abundant in breast tissue and might therefore be the most potent early response cells during cancer expansion.

The treatment of metastatic breast cancer remains extremely challenging and unsatisfactory. The problem might be that for many years the main approach in treating cancer was trying to target and kill only proliferating cancer cells. However cancer cells regularly acquire therapeutic resistance presumably because of their innate genomic instability (Martin et al., 2010; Casey et al., 2008) and the existence of dormant, drug resistant cells that persist for many months can lead to metastatic relapse and death despite aggressive chemotherapy (FEHM et al., 2008). Lately the importance of the microenvironment´s role in cancer has been widely recognized. With the use of new drugs in metastatic breast cancer such as bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), which among many other factors is important in the development of reactive stroma (Brown et al., 1999), attempts are being made towards targeting the cancer microenvironment. Several clinical trials have reported that with the use of bevacizumab significant improvements in progression-free survival of patients with metastatic breast cancer can be achieved (Hamilton and Blackwell, 2011). In order to further improve the treatment of this deadly cancer, additional pharmacological approaches targeting stromal cells need to be taken.

The present findings identify TGFβ1 secreted from breast cancer cells as a key factor in the differentiation of tissue resident stem cells towards myofibroblasts and suggest that inhibition of the associated TGFβ1 signaling pathway in hASCs can offer a way to prevent the formation of these tumor-promoting cells *in vitro*. It would now be necessary and interesting to investigate if the effects of TGFβ inhibition in hASCs *in vitro* can also be observed in an *in vivo* mouse model. As mentioned above, the potential danger of this approach, such as carcinoma-formation itself (Bhowmick et al., 2004), needs to be considered and critically investigated when following this approach.

Future *in vitro* and *in vivo* studies on adipose tissue derived stem cells within the breast cancer microenvironment under special consideration of TGFβ1 could offer a very promising tool to further study the progression of hASCs towards myofibroblasts and eventually pave the way for the development of more efficient therapeutic strategies against breast cancer.

5. Summary

Major advances in understanding and treating breast cancer have been made in the last two decades, yet it remains a significant problem with breast cancer being the most commonly identified cancer and the leading cause of cancer death among women worldwide. For many years breast cancer research has mainly focused on genetically changed cancer cells. However, recently the importance of the stromal compartment surrounding cancer cells in facilitating tumor growth, invasion and metastasis has been widely recognized. Cumulating evidence suggests that in particular carcinoma-associated myofibroblasts play a key role within the tumor stroma and influence many aspects of carcinogenesis. Nevertheless, the cell type of origin as well as the precise mechanisms by which these cells develop has not been conclusively established and remains controversial. The role of human adipose tissue derived stem cells (hASCs) in this context has not been studied so far. hASCs are locally adjacent to epithelial breast cancer cells and might represent early response cells within the tumor stroma. Hence, the aim of this study was to investigate whether carcinoma-associated myofibroblasts may originate from hASCs.

The present study revealed that a significant percentage of hASCs differentiate into myofibroblast-like cells expressing alpha smooth muscle actin (α-SMA) and tenascin-C when exposed to conditioned medium from the human epithelial breast cancer cell lines MDMAB231 and MCF7. This process is induced by transforming growth factor beta 1 (TGFβ1) secreted from breast cancer cells. It was shown that conditioned medium from MDMAB231 and MCF7 contains significant amounts of TGFβ1. It could further be demonstrated that the differentiation of hASCs towards myofibroblasts is dependent on TGFβ1 signaling via phosphorylation of Smad2 and Smad3 in hASCs. The induction of myofibroblasts can be abolished using a neutralizing antibody to TGFβ1 as well as by pretreatment of hASCs with SB431542, a selective inhibitor of the TGFβ1 activin receptor-like kinases 4, 5 and 7. Additionally, hASC-derived myofibroblasts exhibit functional

properties of carcinoma-associated myofibroblasts such as the increased secretion of the tumor-promoting soluble factors SDF-1α and CCL5. Furthermore hASC-derived myofibroblasts as well as conditioned medium from these cells promote the *in vitro* invasion of MDAMB231 breast cancer cells. Moreover inhibition of the TGFβ1 signaling pathway in hASCs reduces the potential of these cells to enhance the invasion of breast cancer cells.

Overall, the data of the present study suggest that human adipose tissue derived stem cells can differentiate into carcinoma-associated myofibroblast under the influence of TGFβ1 secreted from breast cancer cells *in vitro*. The differentiation of hASCs towards these tumor-promoting cells can be abolished by targeting the TGFβ1 signaling pathway. Hence, inhibition of the TGFβ1 signaling pathway may prove to be an interesting target for breast cancer therapies. *In vivo* studies on the cancer microenvironment under special consideration of the interactions between hASCs and cancer cells should be of interest for breast cancer research in the future.

6. Zusammenfassung

In den letzten Jahrzehnten konnten bedeutende Forstschritte bezüglich des Verständnisses und der Behandlung von Brustkrebs erzielt werden. Dennoch stellt Brustkrebs bei Frauen die weltweit am häufigsten diagnostizierte Krebserkrankung dar und ist auch die häufigste Krebstodesursache bei Frauen. Über viele Jahre hinweg konzentrierte sich die Brustkrebsforschung hauptsächlich auf die Untersuchung genetisch veränderter Krebszellen. Allerdings hat sich gerade in den letzten Jahren gezeigt, dass das den Tumor umgebende Stroma eine wesentliche Rolle für die Tumorprogression spielt und sowohl das Tumorwachstum im Ursprungsgewebe als auch die Tumorzellinvasion in das umgebende Gewebe sowie die Metastasenbildung erheblich erleichtert. Innerhalb des Tumorstromas spielen insbesondere tumor-assoziierte Myofibroblasten eine Schlüsselrolle und beeinflussen zahlreiche Aspekte der Kanzerogenese. Dennoch ist es bisher weder hinreichend gelungen den Ursprung dieser Zellen, noch die genauen molekularen Mechanismen durch welche diese Zellen sich entwickeln, zu identifizieren. Die Rolle von humanen mesenchymalen Stammzellen des Fettgewebes (human adipose tissue derived stem cells [hASCs]) wurde in diesem Zusammenhang bisher nicht untersucht. Innerhalb des Tumorstromas befinden sich hASCs in nächster Nähe zu Brustkrebszellen und können somit frühzeitig durch diese beeinflusst werden. Das Ziel der vorliegenden Studie war es daher zu untersuchen, ob sich tumor-assoziierte Myofibroblasten von humanen mesenchymalen Stammzellen des Fettgewebes ableiten können.

In der vorliegenden Studie konnte gezeigt werden, dass eine signifikante Anzahl von hASCs unter dem Einfluss von konditioniertem Medium der humanen Mammakarzinom-Zelllinien MDAMB231 und MCF7 zu Myofibroblasten differenzieren, die alpha smooth muscle actin (α-SMA) und tenascin-C exprimieren. Dieser Prozess wird durch das von MDAMB231 und MCF7 Krebszellen sezernierte TGFβ1 (transforming growth factor beta 1) induziert. Es wurde demonstriert, dass die Differenzierung mittels der Phosphorylierung von Smad2 und

Smad3 im Rahmen der TGFβ1-Signalübertragung in hASCs abläuft. Die Entstehung von Myofibroblasten konnte durch den Einsatz eines neutralisierenden Antikörpers sowie durch die Vorbehandlung von hASCs mit SB431542, einem selektiven Inhibitor der TGFβ1 Aktivin-Rezeptor-like-Kinasen 4, 5 und 7, unterbunden werden. Zusätzlich konnte gezeigt werden, dass von mesenchymalen Stammzellen abstammende Myofibroblasten funktionelle Eigenschaften tumor-assoziierter Myofibroblasten aufweisen, unter anderem die vermehrte Sekretion der Chemokine SDF-1α und CCL5, welche die Tumorprogression vorantreiben. Weiterhin fördern die experimentell generierten Myofibroblasten sowie konditioniertes Medium dieser Zellen die Invasion von MDAMB231 Brustkrebszellen *in vitro*. Die beobachtete, verstärkte Invasivität der Brustkrebszellen konnte durch die Hemmung der TGFβ1-Singalübertragung in mesenchymalen Stammzellen mittels SB431542 signifikant vermindert werden.

Zusammenfassend konnte gezeigt werden, dass humane mesenchymale Stammzellen des Fettgewebes (hASCs) in Interaktion mit Brustkrebszellen unter dem Einfluss von TGFβ1, zu tumor-assoziierten Myofibroblasten differenzieren können. Die Differenzierung von hASCs zu Myofibroblasten, welche wesentlich zur Tumorprogression beitragen, kann mittels der Hemmung des TGFβ1-Signalweges verhindert werden. Demnach könnte die Hemmung des TGFβ1-Signalweges im Tumorstroma eine interessante Option für zukünftige Brustkrebstherapieformen darstellen, indem dadurch die Homöostase der Tumoren gestört würde. Weiterführende *in vivo* Studien zum Tumorumfeld, die im Besonderen die Interaktionen von hASCs mit Brustkrebszellen untersuchen, könnten somit für die Brustkrebsforschung zukünftig von Bedeutung sein.

7. Index of abbreviations

αMEM	Alpha-modification of Eagle´s medium
α-SMA	Alpha smooth muscle actin
AB	Antibody
ALK	Activin receptor-like kinase
ASC	Adipose tissue derived stem cell
BMSC	Bone marrow derived stem cell
BRCA1/2	Breast cancer 1/2
BSA	Bovine serum albumin
CAF	Carcinoma-associated (myo)fibroblast
CCL5	Chemokine (C-C motif) ligand 5
CD	Cluster of differentiation
CM	Conditioned medium
CXCR4	C-X-C chemokine receptor 4
DiI	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
EndMT	Endothelial to mesenchymal transition
ER	Estrogen
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fig	Figure
GFP	Green fluorescent protein
h	Human

HBSS	Hank´s balanced salt solution
HCl	Hydrochloric acid
HER2	Human epidermal growth factor receptor 2
IF	Immunofluorescence
LPA	Lysophosphatidic acid
MCF7	Human breast cancer cell line MCF7
MDAMB231	Human breast cancer cell line MDAMB231
PBS	Phosphate-buffered saline
PD	Population doubling
PE	Phyocoerythrin
PerCP	Peridinin-chlorophyll
PR	Progesterone
p-Smad2/3	Phosphorylated Smad2/3
r	Recombinant
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SDF-1α	Stromal cell-derived factor 1 alpha
SDS	Sodium dodecyl sulfate
Smad2/3	Mothers against decapentaplegic homolog 2/3
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween® 20
TCM	Tumor conditioned medium
TGFβ RI/II	Transforming growth factor beta type I/II receptor
TGFβ	Transforming growth factor beta
Tris	Tris(hydroxymethyl)aminomethane
VEGF	Vascular endothelial growth factor

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9. References

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10. Publications and awards

PUBLICATIONS

Welte G, Alt EU, Devarajan E, Krishnappa S, **Jotzu C**, Song YH. Interleukin-8 derived from local tissue-resident stromal cells promotes tumor cell invasion. *Molecular Carcinogenisis **2012**; 51(11):861-868*

Jotzu C, Alt E, Welte G, Li J, Hennessy BT, Devarajan E, Krishnappa S, Pinilla S, Droll L, Song YH. Adipose tissue derived stem cells differentiate into carcinoma-associated fibroblast-like cells under the influence of tumor derived factors. *Cellular Oncology **2011**; 34(1):55-67.*

Pinilla S, Alt EU, Abdul Khalek FJ, **Jotzu C**, Muehlberg F, Beckmann C, Song YH. Tissue resident stem cells produce CCL5 under the influence of cancer cells and thereby promote breast cancer cell invasion. *Cancer Letters **2009**; 284(1):80-5.*

AWARD

First Place Graduate Student Award at the M.D. Anderson Cancer Center Trainee Research Day 2009 for the abstract “Breast Cancer Tumor-Conditioned Media Induced Differentiation of Tissue Resident Mesenchymal Stem Cells to Carcinoma-Associated Fibroblast-Like Cells

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12. Eidesstattliche Versicherung

Hiermit bestätige ich, Constantin Jotzu, dass ich die vorliegende Dissertation mit dem Thema «Differentiation of mesenchymal stem cells from the adipose tissue into carcinoma-associated myofibroblasts in interaction with human breast cancer cells » selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annährend übernommen sind, als solche kenntlich gemacht habe und nach ihrer Herkunft unter Bezeichung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

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Ort, Datum Constantin Jotzu