Aus der Medizinischen Klinik und Poliklinik IV der
Ludwig-Maximilians-Universität München
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Einfluss mesenchymaler Stammzellen aus dem Fettgewebe auf das Invasionsverhalten muriner und humaner Brustkrebszellen

Dissertation zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität München

Vorgelegt von
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aus
Schrobenhausen
2012

Mit Genehmigung der Medizinischen Fakultät
der Universität München

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Tag der mündlichen Prüfung:	14.06.2012

meiner Mutter

und

meinem Vater

Teile dieser Arbeit wurden publiziert in:

Pinilla, S., Alt, E., Abdul Khalek, F.J., Jotzu, C., Muehlberg, F., Beckmann, C., Song, Y.H. (2009). Tissue resident stem cells produce CCL5 under the influence of cancer cells and thereby promote breast cancer cell invasion. Cancer Lett. *284*, 80-5.

Muehlberg, F., Song, Y.H., Krohn, A., **Pinilla, S.**, Droll, L., Leng, X., Seidensticker, M., Ricke, J., Altman, A., Devarajan, E., Liu, W., Arlinghaus, R., Alt, E. (2009). Tissue resident stem cells promote breast cancer growth and metastasis. Carcinogenesis. *30*, 589-9.

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1. Zusammenfassung

Einleitung:

Brustkrebs ist weltweit die häufigste Krebserkrankung bei Frauen. Insgesamt hat sich die Prognose für die Betroffenen in den letzten 30 Jahren deutlich verbessert. Gründe dafür sind zum einen eine verbesserte Prävention, zum anderen neuartige therapeutische Möglichkeiten wie die Behandlung mit monoklonalen Antikörpern.
Trotz dieser Fortschritte ist die Prognose für Patienten mit metastasierten Tumoren nach wie vor ungünstig und die Therapie der metastasierten Krebsformen immer noch eine Herausforderung.

Die Forschung der letzten Jahre hat gezeigt, dass das Tumor umgebende Stroma eine entscheidende Rolle für das Tumorwachstum im Ursprungsgewebe, aber auch für die Metastasenbildung und das Überleben der disseminierten Tumorzellen in ursprungsfernen Organen spielt.
Der Einfluss von Fettgewebe als endokrin aktives Organ auf Brustkrebs wurde in verschiedenen Studien untersucht. Allerdings ist bisher unklar, welche Rolle mesenchymale Stammzellen aus dem Fettgewebe für die Metastasierung von Brustkrebszellen spielen.
In der vorliegenden Arbeit wurde der Einfluss dieser Zellen auf die Invasivität muriner und humaner Brustkrebszellen untersucht und versucht, molekulare therapeutische Ziele zu identifizieren, um den Metastasierungsprozess zu reduzieren.

Methodik:

Der Einfluss mesenchymaler Stammzellen auf Brustkrebszellen wurde mit Hilfe von Proliferations-, Invasions-Assays und zweidimensionalen direkten Kokulturen untersucht. Mediatoren, die für diese Interaktion eine Rolle spielen könnten, wurden mit ELISA, Zymographie, Immunohistochemie und RT-PCR identifiziert. Um die

Relevanz des gefundenen Signalmoleküls für die Invasion zu evaluieren, wurde ein neutralisierender Antikörper eingesetzt.

Ergebnisse:

Humane Brustkrebszellen induzieren eine de novo Sekretion des Chemokines CCL5 (RANTES) in mesenchymalen Stammzellen und dieses steigert wiederum die Anzahl der invasiven Brustkrebszellen um 126%. Weitere Experimente zeigen, dass CCL5 die Sekretion der Matrixmetalloproteinase 9 in mesenchymalen Stammzellen steigert.

Schlussfolgerung:

Es konnte gezeigt werden, dass mesenchymale Stammzellen aus dem Fettgewebe einen signifikanten Einfluss auf die Invasivität muriner und humaner Brustkrebszellen haben. Das Chemokin CCL5 (RANTES) ist in diesem Zusammenhang verantwortlich für den proinvasiven Effekt und kann mithilfe eines neutralisierenden Antikörpers blockiert werden.
Weitere Studien mit in-vivo Tumormodellen können wichtige Hinweise zur Rolle dieser Stammzellen in der gesamten Tumorgenese liefern. Weiterhin sollte CCL5 (RANTES) als therapeutisches Ziel bei Brustkrebspatienten evaluiert werden.

2. Summary

Introduction:

Breast cancer is the leading form of cancer in women worldwide. Overall the prognosis for those patients has improved in the last 30 years. This is due to improved prevention and new therapeutic approaches like monoclonal antibody treatments.
However, the treatment of metastatic breast cancer remains challenging not only in breast cancer but all kinds of disseminating cancers. Therefore the prognosis for patients with metastases is still poor.

During the last decades of cancer research it became clear that the tumor stroma plays a crucial role in primary tumor growth, metastatic progression, and survival of disseminated tumor cells in distant organs. The influence of adipose tissue as an endocrine organ on breast cancer has been investigated in some studies. However it is still elusive what role adipose tissue derived stem cells (ASCs) play in breast cancer metastasis.

The present study investigates the effect of ASCs on the invasion of murine and human breast cancer cells and tries to identify candidate signaling molecules that might be therapeutic targets in metastatic breast cancer patients.

Methods:

The effect of ASCs on breast cancer cells has been studied with proliferation assays, invasion assays and 2-dimensional direct co-cultures. Molecules involved in this interaction have been measured with ELISA, Zymography, Immunohistochemistry and RT-PCR. To evaluate the relevance of the identified molecules, neutralizing antibody has been used.

Results:

We could show that human breast cancer cells induce a de-novo secretion of CCL5 (RANTES) in ASCs which then acts in an invasion promoting as well as paracrine manner on breast cancer cells (126% increase in number of invasive breast cancer cells).
Furthermore we found that CCL5 might induce a higher secretion of matrix metalloproteinase 9 in ASCs.

Conclusion:

Taken together, our results indicate that adipose tissue derived stem cells promote breast cancer cell invasion in a CCL5 (RANTES) dependent manner. A CCL5 neutralizing antibody could block the invasion promoting effect.

Further investigation with in-vivo models is necessary to clarify the role of CCL5 in breast cancer progression, and in order to evaluate the potential relevance of ASCs and CCL5 as therapeutic targets in breast cancer patients.

3. Abbreviations

AB	Antibody
AB-Ag complex	Antibody-antigen complex
ASCs	Adipose tissue-derived stem cells
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DiI	1,1',di-octadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
DiO	3,3'-dioladecyloxacarbocyanine perchlorate
DMEM/F12	Dulbecco modified Eagle's minimal essential medium/F12
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glycerine aldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
H_2O	Water
hASC	Human Adipose tissue-derived stem cells
HBSS	Hankes' balanced salt solution
HMEC	Human mammary epithelial cells
IBC	Inflammatory breast cancer
MRI	Magnetic resonance imaging
LABC	Locally advanced breast cancer
mASC	Murine adipose tissue-derived stem cells
MEM α1	Minimal essential medium alpha modification

mg	Milligram(s)
ml	Milliliter(s)
MMP	Matrix metalloproteinase
P	Passage
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
SCCM	Stem cell conditioned medium
TCM	Tumor conditioned medium
VEGF	Vascular endothelial growth factor
µg	Microgram(s)
µl	Microliter(s)

4. Introduction

EPIDEMIOLOGY

Breast cancer is the most common cancer in women worldwide and represents 23% of all cancers. In 2002 an estimated number of 1.15 million patients were diagnosed with breast cancer. Furthermore breast cancer is, despite having a generally rather good prognosis, one of the leading causes of cancer death in women (Jemal et al., 2008; Parkin et al., 2005).
Globally, the rates of breast cancer incidence vary. The highest rates are found in North America and northern Europe, whereas the lowest rates are observed in Asia and Africa. Reasons for these differences could be different genetic backgrounds as well as the corresponding environmental and biological circumstances that affect risk factors involved in facilitating the outgrowth of breast cancer (Parkin et al., 2005). Studies taking into consideration migration patterns consistently indicated that the incidence of breast cancer increases from generation to generation in migrant populations, thus supporting the hypothesis of societal factors influencing rates of breast cancer (Willett et al., 2004).

Interestingly, the mortality of breast cancer has been declining constantly since 1975 despite a significant rise of breast cancer incidence in the early 1980s, which was most likely due to the newly established mammography and its successful introduction as a diagnostic tool for screening purposes. This new technique, together with the use of aggressive adjuvant chemotherapies, led to the overall decrease in breast cancer mortality (Berry et al., 2005).

However, the management of the metastatic progression in breast cancer, as well as in other cancer types, remains extremely challenging due to lack of knowledge about the cellular and molecular components of this pathological process. Therefore, the five

year survival rates for the more aggressive subtypes of breast cancer such as locally advanced and inflammatory breast cancer range according to the lymph node status after neoadjuvant therapy from 20% to 30% (Buzdar et al., 1995).

RISK FACTORS

Various risk factors have been identified as relative contributors to the increased incidence of breast cancer. Age and gender are among the strongest risk factors, followed by factors such as genetics, personal history, benign breast disease, ethnicity and lifestyle, hormonal, and environmental factors. These different parameters add up to a multifactorial and personal risk profile that needs to be evaluated individually for each patient.

Interestingly body weight as a risk factor for breast cancer is controversially discussed since a high premenopausal body mass index (BMI) has been correlated with a lower breast cancer incidence (van den Brandt et al., 2000), whereas postmenopausal obesity is associated with a higher breast cancer risk (Ahn et al., 2007). In the latter case, adipose tissue as a converter of estrogen precursors to estrogen seems to be most likely explanation. However, the complex role of adipose tissue in breast cancer as a highly active endocrine organ remains elusive.

SYMPTOMS

Most patients in Western countries are diagnosed because of abnormal mammograms and do not show specific symptoms related to breast cancer at the time of diagnosis. Nevertheless, breast cancer also has a typical pathological manifestation that can be evaluated by means other than mammography.

Patients with locally advanced breast cancer (LABC, 30 to 50% in medically underserved populations) and inflammatory breast cancer (IBC, 0.5 to 2 % of invasive breast cancer) especially have a characteristic clinical presentation (Ezzat et al., 1999; Hance et al., 2005).

Different visual and haptic signs can indicate the development of breast cancer. Palpable and in some cases visible indurations are present, along with local pain and an increasingly tender, firm and enlarged breast. A spontaneous clear or bloody discharge from the nipple is often associated with a breast lump, along with the retraction or indentation of the nipple or any flattening or indentation of the skin over the. Further examination of the skin might show a warm and thickened “peau d’orange” (skin of an orange) with an alteration in color from pink to redness, or a purplish hue in case of IBC. Signs of LABC and IBC include a fixation of the breast to the chest wall, fixed or matted axillary nodes, or ipsilateral satellite skin nodules (Robertson et al., 2010).

DISEASE PROGRESSION

Breast cancer can arise from different cell types associated with the mammary gland and is therefore divided into the following molecular subtypes of breast cancer: basal-like, luminal A, luminal B, HER2+/ER- and normal breast-like. Natural history of breast cancer involves the progression from ductal hyperproliferation to subsequent ductal carcinoma in situ (DCIS), invasive carcinoma, and finally metastatic disease.

According to the angiogenic and angioinvasive properties of the tumor cells, they also invade lymphatic vessels and furthermore form so-called tumor emboli, which are responsible for both the local signs and symptoms as described above, and for the lethal development of distant metastases.

The organs most commonly affected by breast cancer metastases are bone, liver, lung, and brain, in this order. Secondary organ failure finally leads to exitus. Therefore, patients with metastatic breast cancer disease have the worst outcome and are unlikely to be treated in such a way that complete remission is achieved (Greenberg et al., 1996).

DIAGNOSIS

The leading diagnostic tool for breast cancer is mammography. Once a suspect radiologic correlate of breast malignancy has been detected, several diagnostic adjuncts to mammography are available. In order to differentiate between cystic and solid masses, ultrasound examination of the breast can provide important evidence.

Contrast-enhanced breast magnet resonance imaging (MRI) might prove as an ultimately better but also more expensive diagnostic test as compared to the ultrasound, since it provides the clinician with more accurate diagnostic information in patients where mammography and ultrasound cannot give clear results.

Finally, different techniques of biopsy such as surgical biopsy, fine needle aspiration biopsy, or core biopsy are available in order to gain more details about histologic composition of suspect lesions, and thus can provide important information to aid in designing the optimal individual treatment (Winchester et al., 2000).

PROGNOSIS

The overall prognosis of breast cancer is good due to well established screening methods, improvement in surgical intervention, and new approaches in combined chemotherapies.
However, the prognosis is dependent on the presence of prognostic factors such as age and menopausal status, tumor stage and histologic grade, clinical response, hormone receptor status, and lymph node status following induction (also termed neoadjuvant) therapy, the latter being most important for prognostic estimations.

In general, the prognosis for patients worsens according to the invasiveness of the breast cancer type. The study of Smart showed an average decrease in 20-year survival rate of nearly 30 percent when comparing In situ-breast cancer patients to lymph node positive invasive breast cancer patients (Smart et al., 1997).

TUMOR-STROMA INTERACTION IN BREAST CANCER PATHOLOGY

In the last decades, the concept of cancer changed in a way that cells which are not cancer cells per se turned out to be promising targets for cancer therapy since it became clear that the tumor microenvironment plays a crucial role in both primary tumor growth and metastatic spread. Although the “seed and soil” theory had already been developed in 1889 by Paget (Paget, 1989), the complex interplay of cancer cells and stromal or circulating cells leaves many unanswered questions.

The transformation from individual, polarized, structurally organized, quiescent epithelial cells anchored in extracellular matrix, to highly motile, matrix-digesting breast cancer cells capable of invading vessels, travelling with the circulation, and extravasating vessels at distant organ sites, is an extremely complex transformation.

This malignant progression, common to all lethal cancerous diseases, becomes even more intriguing when considering that invasive breast cancer cells, once present in their main metastatic targets such as bone, liver, and lung, need to recruit and manipulate local cells in order to initiate remodeling of extracellular matrix, release of different growth factors, and neovascularization to support the colonization of other organs and enable the outgrowth of metastases.

Another interesting approach to the cancer disease is the comparison of cancer with a “never healing wound” (Schafer and Werner, 2008). Recent studies outlined the parallels between the processes that occur during wound healing and the progression from a locally restricted tumor to a metastatic disease. Both require the crosstalk between epithelial cells and stromal cells and involve a wide variety of cytokines and matrix molecules.

Chemokines have been characterized for their myriad of functions in inflammatory processes, especially leukocyte chemotaxis and proliferation, but also hematopoiesis, cell-virus interactions, angiogenesis, neovascularization, and tumor metastasis (Allavena et al., 2008a; Homey et al., 2002; Balkwill, 2003; Challita-Eid et al., 1998).

The family of chemokines includes about 50 different members subdivided into groups according to their chemical structure.

CCL5, formerly known as RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted), belongs to the group of CC–Chemokines (Fig.1) and has become more and more interesting in the last ten years of breast cancer research for its strong pro-migratory effects as well as its function as a strong chemoattractant for inflammatory cells to wounds or tumor sites (Wigler et al., 2002; Eissa et al., 2005; Yaal-Hahoshen et al., 2006; Soria and Ben-Baruch, 2008).

CCL5, located on chromosome 17, is a β-chemokine and binds to three different G-protein linked transmembrane receptors (CCR1, CCR3 and CCR5), with CCR5 being the main receptor.

Several pathways activated by CCL5 subsequently enhance the expression of cell migratory effector molecules and also induce the transcription of molecules involved in the inflammatory signaling cascade (van Deventer et al., 2008; Ben-Baruch, 2008; Youngs et al., 1997; Mueller et al., 2006; Luo et al., 2002).

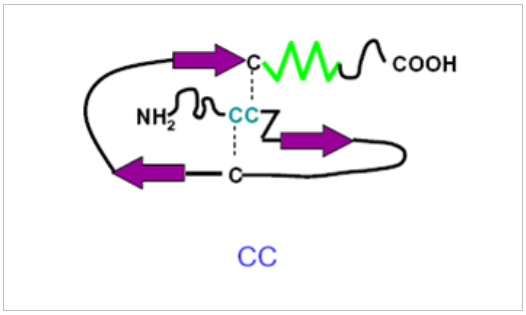

Fig. 1 Concept of chemical structure of CC-motif Chemokines. The position of the first two cysteines in the N-terminal part of the protein defines the group of chemokines (Ali and Lazennec, 2007)

In the process of invasion and metastasis, not only the chemotactic molecules play an important role for tumor and stromal cell mobilization and homing, but also enzymes

that degrade the extracellular matrix and thus might contribute to the release of sequestered factors that promote angiogenesis such as VEGF (Du et al., 2008; Zhao et al., 2008; Luo et al., 2006; Chabottaux and Noel, 2007).

Furthermore, they enable tumor cells to migrate through basement membranes in the context of intra- and extravasation. These enzymes are mostly matrixmetalloproteinases (MMPs). Two well-characterized MMPs responsible for digesting collagen IV, a major component of the basement membrane, are MMP-9 and MMP-2 (Figueira et al., 2009; Sun et al., 2009; Gonzalez et al., 2008).

Together with intrinsic alterations in the cancer cell comes an alteration in the tumor microenvironment. It has been shown that cytokines and chemokines are responsible for the recruitment of leukocytes to the tumor site (Mantovani et al., 2004).

We hypothesized that in breast cancer, the surrounding fat tissue, which contains multipotent stem and progenitor cells, might be used by tumor cells to create the inflammatory environment that eventually facilitates the transition towards malignancy, specifically by orchestrating an extracellular matrix structure and milieu that enhances processes like leukocyte recruitment, neovascularization, tumor cell invasion, and intravasation.

We carried out a series of candidate approach experiments in order to determine whether and how adipose tissue derived stem cells might be involved in the secretion of CCL5 and MMP-9.

4.1 Aim of the study

The aim of this study was to investigate whether tissue resident stem cells from the adipose tissue might play a role in breast cancer metastasis. Recent studies have shown that the process of breast cancer metastasis is significantly influenced by the surrounding tumor stroma and circulating cells. In order to elucidate the role of tumor-adjacent multipotent stem cells possibly representing early tumor response cells, we performed a series of experiments as shown in the flow chart of experiments (Fig. 5).
The main focus of this study was to evaluate the significance of human adipose tissue derived stem cells (hASCs) on breast cancer cell invasion and to identify mediators involved in the interaction of hASCs and breast cancer cells.

Therefore, we defined the following questions in the indicated order:

1) What is the effect of hASCs on breast cancer cell invasion in vitro?

2) What role does the chemokine CCL5 (RANTES) play in this context?

3) Is the matrixmetalloproteinase 9 (MMP-9) involved?

In addition, we wanted to elucidate whether there is a direct link between the de novo secretion of CCL5 and the upregulation of MMP-9.

5. Materials and Methods

5.1 Materials

5.1.1 Animals

Balb/c mice, 6-8 weeks Ingeneron, Houston, TX, USA

5.1.2 Cells

Table 1 Cell types

Cell line/ cell type	Abbreviation	Transfection	Source
Human Adipose tissue derived stem cells	hASC	--	Established from patients undergoing elective liposuctions
Murine adipose tissue derived stem cells	mASC	--	Established from subcutaneous and kidney capsule fat of Balb/c mice
Murine breast cancer cells 4T1	4T1	--	ATCC, Manassas, VA, USA
GFP-labeled murine breast cancer cell line 4T1	GFP-4T1	EGFP	ATCC, Manassas, VA, USA
Human breast cancer cell line MDA MB 231	MDA MB 231	--	ATCC, Manassas, VA, USA
GFP-labeled human breast cancer cell line MDA MB 231	GFP-MDA MB 231	EGFP	ATCC, Manassas, VA, USA
Human breast cancer cell line MCF-7	MCF-7	--	Oesterreich Lab, Baylor College of Medicine, Houston, TX, USA
Human embryonic fibroblast WI-38	WI-38	--	Hung Lab, MDACC, Houston, TX, USA
Immortalized Human mammary epithelial cells	HMEC	Immortalized with Telomerase and SV40 large T-antigen	Weinberg Lab, Whitehead Institute, Boston, MA, USA

5.1.3 Tissue Culture

α-MEM	Mediatech Inc, Herndon, VA, USA
RPMI	Mediatech Inc, Herndon, VA, USA
L-15 Leibovitz	HyClone Lab., Inc., I South Logan, UT, USA
DMEM	Mediatech Inc, Herndon, VA, USA
Fetal bovine serum	Atlanta biologicals, Lawrenceville, GA, USA
Penicillin/Streptomycin	Mediatech Inc, Herndon, VA, USA
L-Glutamin	Mediatech Inc, Herndon, VA, USA
Trypsin-EDTA	Sigma, St. Louis, MO, USA
Hanks' balanced salt solution (1X)	Mediatech Inc, Herndon, VA, USA
Dulbecco's Phosphate-buffered saline (1X)	Sigma, St. Louis, MO, USA
Liberase Blendzyme 3	Roche Diagnostics, Indianapolis, IN, USA
Culture flasks T25, T75, T175	Greiner Bio-One, Monroe, NC, USA
Centrifuge tubes 15ml, 50ml	Greiner Bio-One, Monroe, NC, USA
6-well plate	Greiner Bio-One, Monroe, NC, USA

5.1.4 Cell staining

Paraformaldehyde (Fixation)	Sigma, St. Louis, MO, USA
DiI	Invitrogen Corporation, Carlsbad, CA, USA
DIO	Invitrogen Corporation, Carlsbad, CA, USA
DAPI	Invitrogen Corporation, Carlsbad, CA, USA
Goat-serum	Sigma, St. Louis, MO, USA

5.1.5 Antibodies

Anti-human RANTES Antibody	R&D Systems, Inc., Minneapolis, MN, USA
Normal Goat IgG Control Antibody	R&D Systems, Inc., Minneapolis, MN, USA
Rabbit anti-human CCR-5 Antibody	Abcam Inc., Cambridge, MA, USA
Goat anti-rabbit IgG (H+L)	Invitrogen Corporation, Carlsbad, CA, USA

5.1.6 Assays and Kits

Invasion Chambers	BD Biosciences, Bedford, MA, USA
Quantikine ELISA CCL5, TNFα	R&D Systems Inc., Minneapolis, MN, USA
Interleukin-6 (human) EIA Kit	Assay Designs, Ann Arbor, MI, USA
SYBR-Green Master Mix	Applied Biosystems, Foster City, CA, USA
RNAqueous®-Micro Kit	Applied Biosystems, Foster City, CA, USA
iScript cDNA Synthesis Kit	Bio-Rad Laboratories, Hercules, CA, USA

5.1.7 Antibiotics

Puromycin	Sigma, St. Louis, MO, USA

5.1.8 Zymography

Zymogram (Gelatin) Gel	Invitrogen Corporation, Carlsbad, CA, USA
Prestained Protein ladder	Invitrogen Corporation, Carlsbad, CA, USA
Renaturing buffer	Invitrogen Corporation, Carlsbad, CA, USA

Developing buffer	Invitrogen Corporation, Carlsbad, CA, USA

5.1.9 Real-Time PCR

Primer	Sigma, St. Louis, MO, USA
DNAse/RNAse free water	Invitrogen Corporation, Carlsbad, CA, USA
BlueJuice gel loading buffer	Invitrogen Corporation, Carlsbad, CA, USA
Agarose gel (UltraPure)	Invitrogen Corporation, Carlsbad, CA, USA

5.1.10 Plasmid

plox/EW-iRES-EGFP	Tronolab, Lausanne, CH

5.1.11 Chemicals

Ethanol	Sigma, St. Louis, MO, USA
DMSO	Sigma, St. Louis, MO, USA
Trypan Blue	Sigma, St. Louis, MO, USA
Bovine Serum Albumin (BSA)	Sigma, St. Louis, MO, USA
Antibody Diluent	Invitrogen Corporation, Carlsbad, CA, USA
Ethidium bromide	Sigma, St. Louis, MO, USA
Recombinant human CCL5	Sigma, St. Louis, MO, USA
Polybrene Infection/ Transfection Reagent	Millipore, Billerica, MA, USA
Coomassie Blue	Sigma, St. Louis, MO, USA

5.1.12 Equipment

Centrifuge, Model 5682	Forma Scientific Inc., Marietta, OH, USA
Steri-Cult Incubator	Forma Scientific Inc., Marietta, OH, USA
ChemiImager (Zymography)	Alpha Innotech Corp., San Leandro, CA, USA
Digital camera	Canon, Lake Success, NY, USA
Hemacytometer	Hausser Scientific, Horsham, PA, USA
Axiovert 25 (microscope)	Zeiss, Thornwood, NY, USA
Axiovert S100 (Fluorescence microscope)	Zeiss, Thornwood, NY, USA
Cool SNAP cf (Camera)	Photometrics, Tucson, AZ, USA
µ-Quant (Plate-Reader)	Bio-Tek Instr. Inc., Highland Park, VT, USA
i-Cycler (RT-PCR)	Bio-Rad Laboratories, Hercules, CA, USA
x-cell Sure Lock (Zymography)	Invitrogen Corporation, Carlsbad, CA, USA
FACSVantage SE cell sorter	Becton–Dickinson, Franklin Lakes, NJ, USA

5.1.13 Software

NIS-elements BR	Nikon Instruments Inc., Melville, NY, USA
ImageJ	http://rsb.info.nih.gov/ij/
SPSS (Version 16)	SPSS Science, Chicago, IL, USA

5.2 Cell biology methods

5.2.1 Isolation and culture of adipose tissue derived stem cells (ASCs)

5.2.1.1 murine ASCs

Murine adipose tissue derived stem cells were obtained from Balb/c mice (InGeneron, Inc.) following the guidelines of Veterinary Medicine & Surgery of the MD Anderson Cancer Center. The procedures were carried out at InGeneron Inc., Houston, TX. For primary culture of murine adipose tissue derived stem cells regular α-MEM supplemented with 20% FBS, penicillin (100 U/ml), 100 µg/ml streptomycin and 0.3 mg/ml L-Glutamin.

PROTOCOL

Each mouse was sacrificed by carbon dioxide inhalation. After performing a longitudinal cut from the umbilical area to the ventral neck, skin was further dissected without disturbing the muscular fascia. Subcutaneous fat was scratched off, collected in a sterile petri-dish, humidified with PBS and put on ice until digestion. The fat tissue was furthermore harvested from the renal capsules after opening the abdominal cavity. The collected fat mass was weighed and minced with a razor blade to less than 1 mm^3 pieces in a petri dish. Samples were processed from this step on as described above for human adipose tissue.

5.2.1.2 human ASCs

Adipose tissue was obtained from patients undergoing elective liposuction (Fig 2). After isolation, cells were grown in regular α-MEM supplemented with 20% FBS, penicillin (100 U/ml), 100 µg/ml streptomycin and 0.3 mg/ml L-Glutamin.

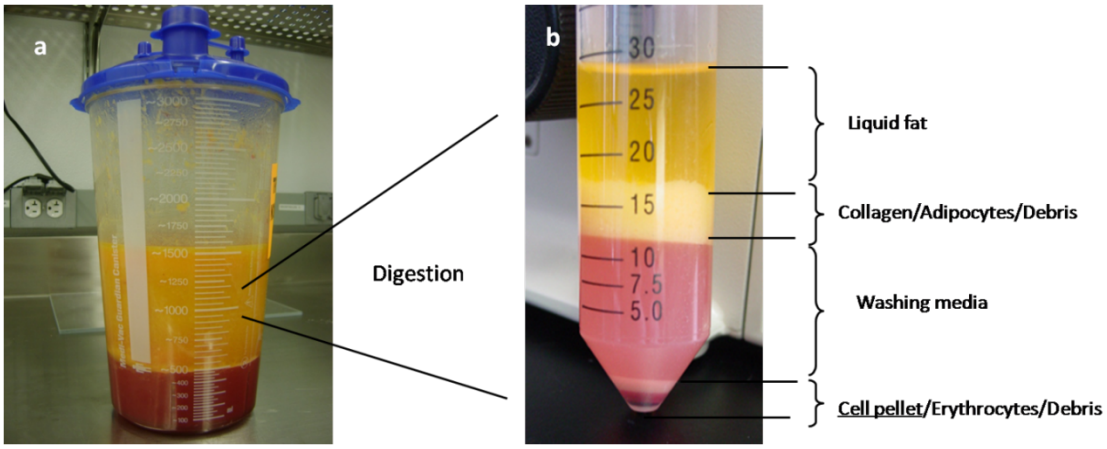

Fig. 2 a) Human fat obtained during surgical liposuction and processed for isolation of ASCs. b) Human fat tissue after digestion, filtration and centrifugation steps. The pellet contains the target cells which were subsequently cultured in T75-Flasks

PROTOCOL

After its weight was recorded, the fat tissue was washed with sterile Phosphate buffered saline (PBS) and subsequently minced thoroughly into pieces of less than 1 mm^3. The minced tissue was incubated with Dulbecco's Phosphate-buffered saline (10 ml per gram fat) containing two units Blendzyme 3 per gram fat for 30 minutes at 37 °C on a shaker at 50 rpm. Thereafter the suspension was disaggregated by pipetting it through a 25 ml serological glass pipette 10 times under sterile conditions. The incubation procedure was repeated for 10 minutes followed by pipetting. After a total digestion time of approximately 30 minutes the processed tissue was transferred into 50 ml plastic tube, followed by centrifugation at 1500 rpm for 10 minutes and 3 washing

steps with phosphate buffered saline (PBS). The cell pellet was resuspended in PBS and subsequently filtered through a 100 µm vacuum filtration system. The filtered cell suspension was then centrifuged at 1500 rpm for 10 minutes followed by two washing steps with PBS. The pelleted cells were resuspended in growth medium consisting of alpha modification of Eagle's medium (α-MEM), 20 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Plastic adherent adipose tissue derived stem cells (ASCs) were grown in cell culture flasks (Fig. 3) at 37 °C in a humidified atmosphere containing 5 % CO_2. Daily washings removed red blood cells and non-attached cells. Three to five days later, ASCs were plated in tissue culture flask at a density of 1,000 cells/cm2.

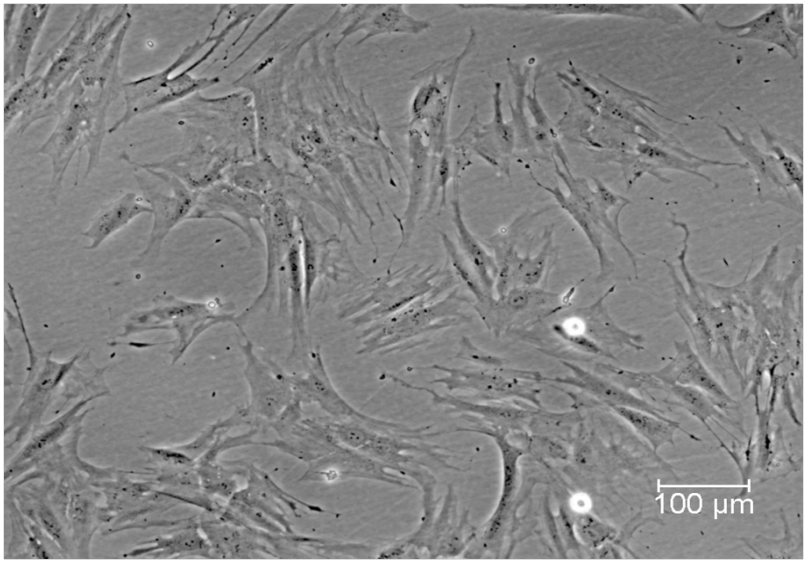

Fig. 3 Human adipose tissue derived stem cells in culture. Phase contrast picture of hASCs at passage 3, brightfield and 10x magnification

5.2.2 Cell freezing and thawing

To maintain a stock of each cell line, 10^6 cells/freezing vial were stored in a liquid nitrogen tank.

PROTOCOL

Cells to be stored were trypsinized, counted and centrifuged at 1400 rpm for 5 minutes. The cell pellet was then resuspended in freezing medium (FBS containing 10% DMSO). 1 ml of freezing medium and 10^6 cells was pipetted into freezing vials and stored for 24h at -70°C. After 24h at -70°C freezing vials were transferred to the liquid nitrogen tank.
To thaw cells, the specific cell-culture medium had been preheated in a T75 flask in the incubator. The freezing vial was warmed to 37°C after being removed from the nitrogen tank and complete content was transferred into 37°C warm medium of T75 flask. Medium was changed 24h after thawing procedure.

5.2.3 Labeling of cells

5.2.3.1 GFP-labeling

To clearly identify the counted cells on the lower surface of invasion assay membranes, cells were either labeled with green fluorescent protein (GFP), DiI or DiO. Nuclei were stained with DAPI.

PROTOCOL (GFP-LABELING)

MDA MB 231 cells or 4T1 cells respectively grown in bulk (in T75 flasks) for 2 weeks were detached by trypsin-EDTA treatment and replated in 6-well plates at a density of 5 x 10^4 cells in 2 ml of α-MEM (MDA MB 231) or RPMI (4T1) per well. Transductions with

a plox/EW-iRES-EGFP lentivirus were carried out in the presence of 8 µg of Polybrene per ml. After incubation at 37°C for 24 h, the transduction medium was replaced with fresh α- MEM (RPMI respectively). The cells were kept for 3 more weeks with medium changes at weekly intervals before FACS analyses were done. After 3 weeks GFP-positive cells were analyzed and sorted using FACSVantage SE cell sorter**.** GFP-positive cells were sub cultured in α-MEM/RPMI (both 10% FBS) until used for invasion assays.

5.2.3.3 DiI, DiO and DAPI staining

PROTOCOL (DII)

Cells that were 80% confluent in a T25 flask were incubated at 37^{o}C in 5% CO_2 with 3ml of their regular Culture-Medium, containing 15 µl of DiI (Dilution of DiI 1/200) for 1h. In the next step cells were washed 2x with PBS, before regular medium was added for a 5 minutes incubation time. This step was repeated and cells were then harvested and seeded for the following experiment.

PROTOCOL (DIO)

The procedure applied was analogue to the one described for DiI-Staining. 21µl of DiO stock solution were diluted in 3 ml of corresponding culture medium.

PROTOCOL (DAPI)

Cells were fixed with 4% PFA (Volume was adapted to experimental setting) for 10 minutes at room temperature. After rinsing 2x with PBS cells were exposed to a 1/1000 diluted DAPI solution (1µl of DAPI stock solution in 1 ml of PBS) for 15 min at room temperature protected from light. Cells were rinsed again and pictures were taken.

5.2.3.3 Immunostaining

The cells subject to immunostaining were seeded in a density of 3 to 5 x 10^4 in 12-well plates. The staining procedure was carried out according to the following protocol:

1. Washing of cells twice with PBS.
2. Adding of 4 % paraformaldehyde for 10 min at RT.
3. Washing of cells twice with PBS.
4. Adding of serum blocking solution (10% goat serum), incubation at RT for 10 minutes
5. Adding of primary antibody (dilution 1:100 in antibody diluent), incubation at 37 ^{o}C for 1 hour, or incubation overnight at 4 ^{o}C
6. Washing of cells twice with PBS.

The following steps were carried out in the dark.

7. Adding of secondary antibody (dilution 1:1000 in antibody diluent); incubate at 37 ^{o}C in moist chamber for 40 minutes
8. Washing of cells twice with PBS.
9. Adding of DAPI (Dilution of 1:1000 in PBS), incubation at RT for 15 mins
10. Washing of cells twice with PBS.

Cells were viewed under the fluorescent microscope and pictures were taken at a 10/20 fold magnification.

5.2.4 Conditioning of medium

Media of hASCs or MDA MB 231 were conditioned to elucidate whether humoral factors secreted by the cells would induce CCL5 secretion in other cell lines.

PROTOCOL

Cells were seeded in 6-well plates at a confluence of 40-50% in 5% FBS containing medium. After 48h the medium was harvested, centrifuged at 1500 rpm for 5 minutes. The supernatant was passed through a Millipore sterile 50 mL filtration system with a 0.45-µm polyvinylidene difluoride membrane. Conditioned medium was stored at -20^{o}C until used for ELISA or Proliferation Assay. Conditioned medium from MDA MB 231 cells (Tumor-conditioned medium, TCM) or from hASCs (stem cell conditioned medium, SCCM) was also used to stimulate other cell lines for 48h before stored at -20^{o}C and analyzed with ELISA.

5.2.5 Proliferation Assay

To see whether the milieu created by hASCs provides a proliferation friendly environment for MDA MB 231 cells a proliferation assay was conducted by documenting viable cell numbers over four days in the control group and the treatment group respectively.

PROTOCOL

10^4 MDA MB 231 cells were seeded in 6-well plates in 2 ml of MEM (10% FBS). After 24h medium was exchanged to MEM (5% FBS) in the control group and to stem cell conditioned medium (conditioning time 48h) in the treatment group. Cell number and viability was assessed after day 1, day 2, day 3 and day 4 with Trypan Blue exclusion test.

5.2.6 Direct co-culture Assay

Breast cancer cells (MDA MB 231/MCF-7) were given the possibility to interact directly with stromal (hASCs/WI-38) or epithelial cells (HMECs). The supernatant of direct co-cultures was used for chemokine/interleukin analysis by ELISA.

PROTOCOL

After washing cells with PBS they were digested with Trypsine and counted. Breast cancer cells and stromal/epithelial cells were always seeded at a ratio of 1:2 (BCC:stromal/epithelial cell). When seeded in a 6-well plate the absolute seeding number for Breast cancer cells was $5x10^4$ and for stromal/epithelial cells 10^5 per well.

5.2.7 Invasion Assay

In order to assess the ability of cells to invade extracellular matrix, Boyden Chamber Invasion assays were used. The membranes of the inserts were covered with Matrigel (main components being laminin and collagen IV) and thus represent a valuable tool to evaluate invasion in vitro which can be correlated to metastatic behavior of tumor cells in vivo.

PROTOCOL

The chemoinvasion assay was performed using Boyden chambers with filter inserts (pore size, 8 μm) coated with Matrigel in 24-well dishes (BD Biosciences Bedford, MA) according to the manufactures instructions. Before performing the invasion assay, cells were mixed together in 5% FBS containing MEM when seeded in co-culture. Approximately, 3 x 10^4 cells or 6 x 10^4 cells respectively were placed in 600μl of 5% FBS containing MEM in the upper chamber, and 750μl of the same medium containing 10% FBS was placed in the lower chamber. The plates were incubated for 48 h at 37^0C

in 5% CO_2. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were rinsed with PBS. Cells on the lower side of the filters were examined and counted under a microscope. Fluorescent cell signal of GFP/DiI/DAPI-labeled 4T1 or MDA MB 231 cells was counted in five randomly chosen view fields at a 10x magnification of every insert (Fig 4).

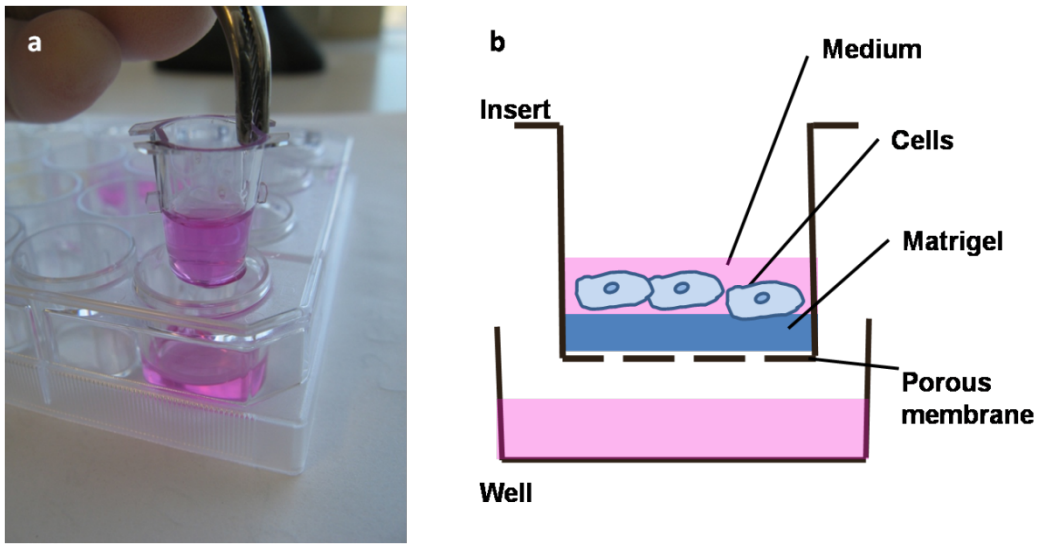

Fig. 4 a) Insert and 24-well plate containing medium as used for invasion assays. b) Schematic illustration of invasion assay

5.2.8 CCL5 treatment

Breast cancer cells (MDA MB 231) and hASCs were stimulated with human recombinant CCL5 in different concentrations to see whether CCL5 induces a higher expression of MMP-2 /MMP-9 both on mRNA and active protease level.

PROTOCOL

Cells were grown in 6-well plates to 70-80% confluence. When the experiment was started, medium was changed to 5% MEM containing 1ng/ml, 5ng/ml, 10ng/ml, 20ng/ml and 100ng/ml of recombinant human CCL5. Cells were incubated at 37^{o}C, 5% CO_2 and 95% humidity for 1h, 2h and 48h. At the end of the treatment, medium was harvested, centrifuged at 1500 rpm for 5 minutes and supernatant-aliquots stored at -20^{o}C until used. Cells in each well were counted (Trypan Blue) and either disposed or used for RNA extraction.

5.3 Molecular biology methods

5.3.1 ELISA

Enzyme-linked Immunosorbent assay is a common and widely used technique designed for detecting and quantitating substances such as peptides, proteins, antibodies, antigens and hormones.

The plate surface is coated with an antibody (capture antibody) which specifically binds to one particular type of protein. After adding the sample to the plate, the proteins of interest adhere to the antibody-coated surface. In the next step, detection antibody specific to the protein of interest is added to all wells. The detection antibody binds to the capture antibody-linked proteins; any unbound detection antibodies are removed by a subsequent washing step. Thereafter, a secondary enzyme-conjugated antibody is added which recognizes the detection antibody. In the final step, a substrate is added and converted by the antibodylinked enzyme to a detectable form. In order to quantify the sample proteins, the generation of a standard curve with known protein concentration is required which is typically a serial dilution of the protein. Protein concentration of CCL5/IL-6/TNFα in the supernatant of cell cultures was achieved by ELISA according to the manufacturer's protocol.

5.3.2 RNA extraction

In order to evaluate the mRNA levels of different genes of interest under different conditions, RNA was extracted from cells, transcribed into cDNA stored at -20^{0}C and subsequently used for real-time PCR analysis.

PROTOCOL

Total RNA was extracted from hASCs and MDA MB 231 after CCL5 treatment for 1h, 2h, and 4h using RNAqueous kit (Applied Biosystems) according to the manufacturer's instructions. The final concentration of RNA was determined with a spectrometer at wavelengths of 260 nm and 280 nm.
The RNA was then used right away for subsequent reverse transcription into complementary DNA (cDNA) or stored at -80^{o}C.

5.3.3 cDNA synthesis

The extracted total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit according to the manufacturer's instructions. The added amount of RNA was standardized between samples to the concentration of 1 µg/µl.

Table 2 cDNA reaction mix

Component	Total volume: 20 µl
RNA	x µl
Reaction mix	4 µl
Reverse transcriptase	1 µl
RNA	y µl

x = volume of RNA that equals 1 µg after measuring conc. with spectrometer
y = volume of water
to add reaction volume up to 20 µl

PROTOCOL

The complete reaction mix was incubated using following cycles with the iCycler (BioRad): 5 minutes at 25^{o}C, 30 minutes at 42^{o}C, 5 minutes at 85^{o}C, and kept at 4^{o}C until stored at -20^{o}C and subsequently used for real-time PCR.

5.3.4 Real time PCR

Detection of gene transcripts in MDA MB 231 and hASCs was determined by qRT-PCR (quantitative real time polymerase cheain reaction). All samples were prepared and quantified as described above. mRNA expression level was determined with RT-PCR using SYBRGreen assay according to manufacturer's instructions. 12.5 µl of the assay reaction mix, 1 µl of each primer (Table 2) and 1 µl of cDNA were mixed with water to a total volume of 25 µl and placed into BioRad PCR cycler. For relative quantification,
glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) served as reference and data were analyzed according to Pfaffl method.
The sequences (5'-3') for the primers were the following:

GAPDH-*forward*	5'- GAA GGT GAA GGT CGG AGT C -3'
GAPDH-*reverse*	5'- GAA AGA TGG TGA TGG GAT TTC -3'
MMP-2-*forward*	5'- TGGCGATGGATACCCCTTT -3'
MMP-2-*reverse*	5'- TTCTCCCAAGGTCCATAGCTCAT -3'
MMP-9-*forward*	5'- CCTGGGCAGATTCCAAACCT -3'
MMP9-*reverse*	5'- GCAAGTCTTCCGAGTAGTTTTGGAT -3'
CCR-5-*forward*	5′-CAAAAAGAAGGTCTTCATTACACC-3′
CCR-5-*reverse*	5′-CCTGTGCCTCTTCTTCTCATTTCG-3′

5.3.5 Zymography

In order to detect catalytically active MMP-9 and MMP-2 respectively zymography was performed with supernatants of cell cultures.

PROTOCOL

MMP-9 and MMP-2 activity was determined using a 10% zymogram (gelatin) precast gel (Invitrogen cat no. EC6175). Preparation of cell lines was conducted by seeding the appropriate amount of cells (50 000) into each respective well of a 6-well plate. Cells were cultured at 37^{o}C (5% CO2 atmosphere) until 80% confluency. Upon confluence media was changed to serum-free media and cells were cultured for an additional 48 hours. After 48 hours conditioned media was collected and mixed with equal volumes of 2X SDS (15 µl conditioned media: 15 µl 2X SDS). (Note: Preparation of conditioned media was not heated or reduced for detection of MMP-9 activity using zymography). The electrical running apparatus was then prepared containing the 10% zymogram (gelatin) pre-cats gel and 1X running buffer (12g Tris, 57.6g Glycine, 10% SDS, 4l distilled water). Samples were then loaded accordingly (15 µl Invitrogen pre-stained protein ladder-cat no. 10748010) into the 10% zymogram (Gelatin) pre-cat gel and allowed to run at room temperature at 125V for 1.5 hours. After electrophoresis the zymogram gel was removed and incubated (30 minutes with gentle agitation) at room temperature in a zymogram renaturing buffer (1:9 with deionized water). Upon incubation, the renaturing buffer was decanted and further incubated at room temperature in 1X developing buffer (1:9 with deionized water) for 30 minutes. After 30 minutes the developing buffer was then decanted and replaced with fresh developing buffer. After equilibration with fresh developing buffer the zymogram gel was allowed to incubate overnight at 37^{o}C. After overnight incubation the developing buffer was decanted and replaced with coomassie staining solution (25% MeOH, 10% Acetic Acid, and 0.1% Coomassie Brillant Blue-dissolved in water before adding) for two hours at room temperature. Upon staining the zymogram gel was destained (10% Acetic acid, 15% MeOH) at room temperature using gentle agitation. Once destained the

zymogram gel was photographed (ChemiImager 5500) to determine the areas of protease activity (clear bands).

5.4 Statistical analysis

Data are presented as means ± standard deviation (SD). Analysis was performed using the Statistical Program for Social Science (SPSS) for Windows (SPSS Inc., Chicago, IL, USA). A level of $p \leq 0.05$ was considered to be statistically significant.

5.5 Flow chart of experiments

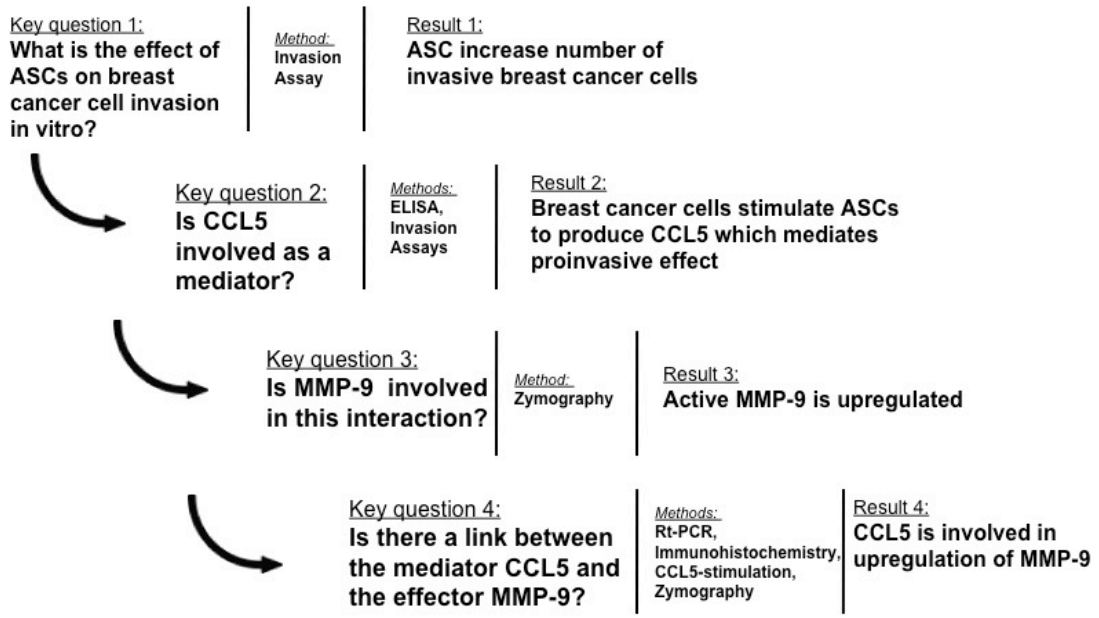

Fig. 5 Flowchart of experiments

The experimental outline to address the questions standing in the focus of this work is shown in Figure 5.

6. Results

6.1 Proliferation of MDA MB 231 cells in stem cell conditioned medium (SCCM)

As a starting point to investigate the interaction of adipose tissue derived stem cells and breast cancer cells the effect of soluble factors secreted by hASCs on the proliferation of the human breast cancer cell line MDA MB 231 was studied. Therefore 5% FBS containing cell culture medium (α-MEM) of human ASCs was conditioned for 48h and MDA MB 231 cells were exposed to the conditioned medium to evaluate their growth rate in this environment in order approach the question whether and how hASCs interact with MDA MB 231 cells.

10^4 cells/well of MDA MB 231 cells were seeded in a set of 6-well plates and exposed either to stem cell conditioned medium (SCCM) or to regular 5% FBS containing α-MEM as a control.

The factors secreted by the hASCs had no negative impact on the growth rate of the MDA MB 231 cells. Notably a higher proliferation rate could be observed in the group with SCCM compared to the control group with regular growth medium on day four (Fig. 6).

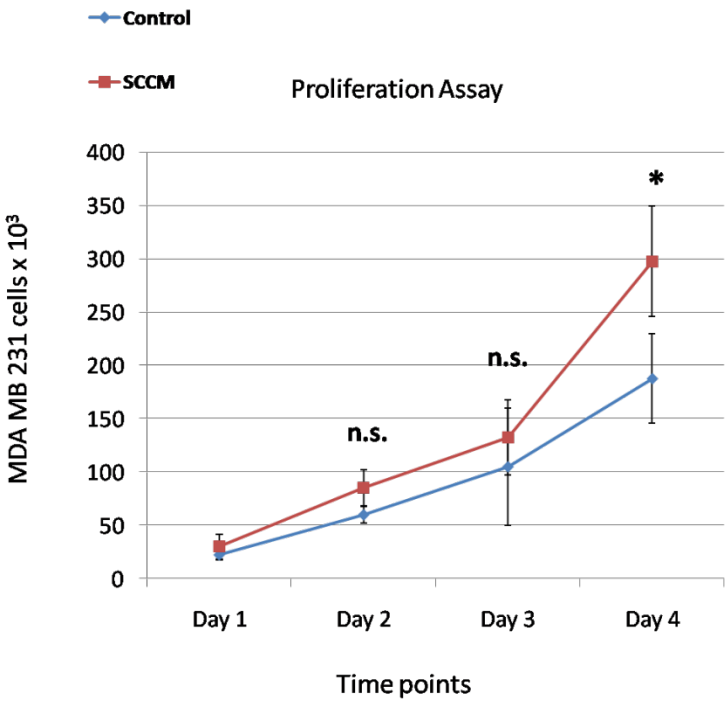

Fig. 6 Stem cell conditioned medium enhances proliferation of MDA MB 231. MDA MB 231 cells were grown in 6-well plates. The SCCM group was exposed to stem cell conditioned medium (hASCs). The control was exposed to regular growth medium containing 10 % FBS. The SCCM group shows a higher proliferation at day 4. * $P < 0.05$, n.s. not significant.

6.2 ASCs increase invasiveness of breast cancer cells

To characterize the effect that mesenchymal stem cells derived from the adipose tissue exert on the invasive behavior of breast cancer cells invasion assays were performed. One murine breast cancer cell line (4T1) and two human breast cancer cell lines (MCF-7 and MDA MB 231) differing in their invasive behavior (MCF-7 cells tend to be less invasive than MDA MB 231 cells) were seeded alone or together with ASCs on matrigel coated membranes imitating extracellular matrix. The matrigel on the chamber

membranes mainly contains Laminin (56%), Collagen IV (31%) and Entactin (8%). Therefore cells attempting to migrate through this membrane have to secrete extracellular matrix digesting enzymes like Matrixmetalloproteinase 2 (MMP-2) and MMP-9 besides of being stimulated to migrate.

Following a gradient from 5% FBS containing medium (upper chamber) to 10% FBS containing medium (lower chamber) the cells had to degrade the matrigel to invade towards the lower surface of the membrane. Initially a roughly doubling of invasive murine breast cancer cells could be detected. To see whether the observed effect in the context of murine cells interacting with murine adipose-tissue derived stem cells also applies for human cells a series of invasion assays was performed to elucidate the effect of hASCs on MCF-7 and MDA MB 231 cells. In parallel invasion assays were carried out with 24h, 36h and 48h incubation time. It turned out that an incubation time of 48h for the murine cell lines and 36h for the human cell lines represented the ideal time frame to evaluate the difference in number of invasive cells. The ratio between seeded breast cancer cells and additional cell line (hASCs, WI-38 or HMECs) was kept at 1:2 for all experiments.

6.2.1 mASCs and 4T1

$3x10^4$ GFP-labeled 4T1 cells and $6x10^4$ non-labeled mASC (Passage 2-4) were seeded on the upper surface of a matrigel coated membrane to evaluate the number of invasive cells without any further conditions. After 48h the non invasive cells were scrubbed off the upper surface by using a moistened q-tip. The remaining cells were counted under the microscope in five randomly chosen view fields at a 10x magnification. With the overlay of the brightfield image and the GFP-Signal it could be confirmed that the GFP-Signal clearly correlates with the cells and is not an artifact of the membrane (Fig. 7).

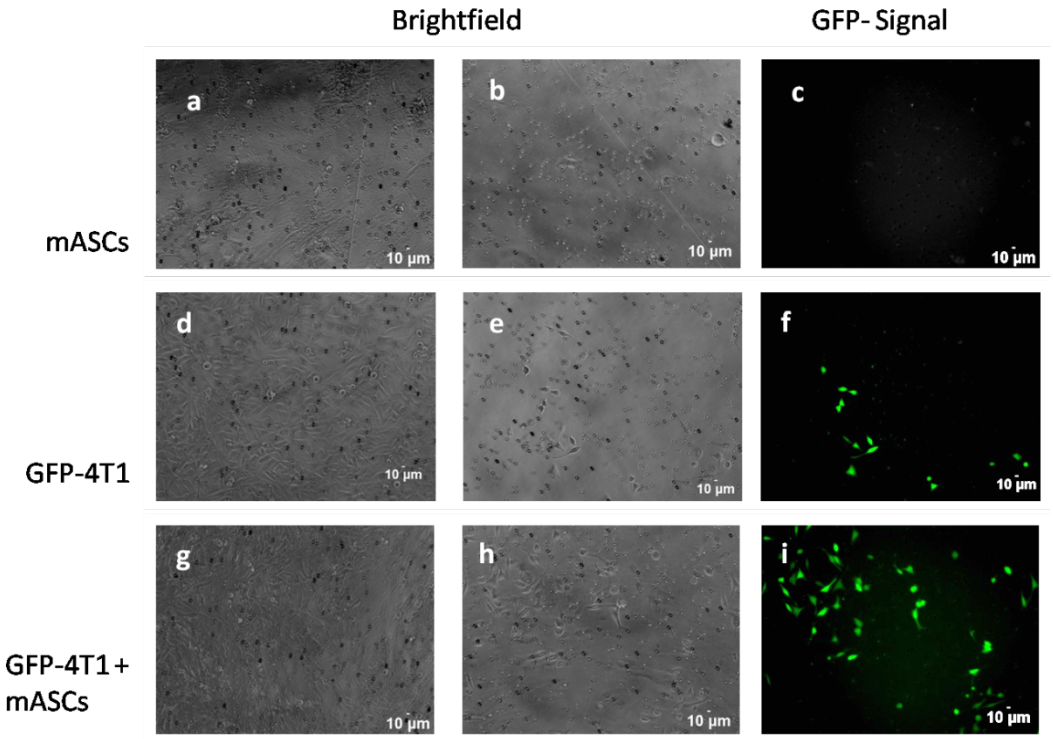

Fig.7 Murine adipose tissue derived stem cells promote GFP-4T1 breast cancer cell invasion. Pictures a,d,g show the matrigel coated membrane with the indicated cells seeded on the surface after 48h incubation time before scrubbing off the non-invasive cells. a) 6 x 10^4 mASCs, d) 3 x 10^4 GFP-labeled 4T1, g) 3 x 10^4 GFP-labeled 4T1 coseeded with 6 x 10^4 mASCs. Pictures b,e,h show the membranes after removing all non-invasive cells. b) mASCs, e) GFP-4T1, h) GFP-4T1 with mASCs. Pictures c,f,i show the GFP-Signals of invasive GFP-labeled 4T1 cells.

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To evaluate the effect of mASCs ($6x10^4$) on 4T1 ($3x10^4$) invasion the two different cell lines were co-seeded on the same membrane. After removing again the non-invasive cells only the GFP-Signal was counted in five view fields (Fig.8). Co-seeding lead to an increase of 137 % of invasive 4T1 cells (27.6 ± 6.98 as compared to 11.6 ± 5.12 per view field).

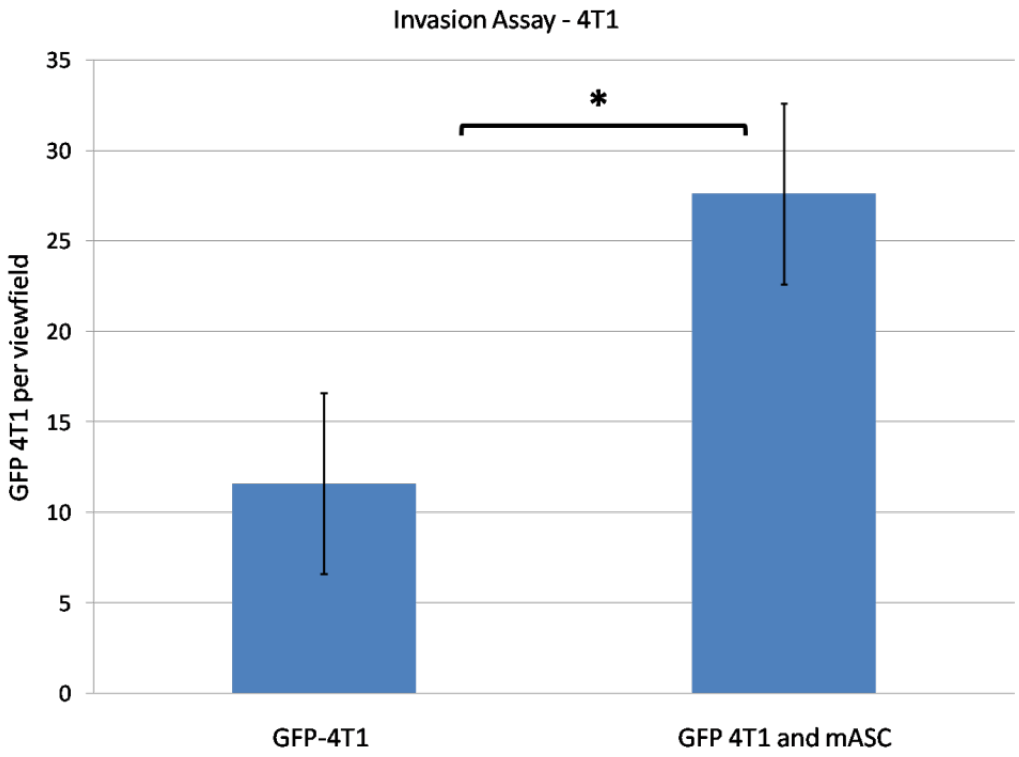

Fig.8 Coseeding of GFP-4T1 and mASCs resulted in 2.38 fold higher number of invasive GFP-4T1 cells as compared to seeding 4T1 cells alone. Results are expressed as the mean ± SD. *P < 0.005

6.2.2 hASCs and MCF-7

$5x10^4$ DiI-labeled MCF-7 cells alone and $5x10^4$ DiI-labeled MCF-7 cells together with 10^5 hASCs (Passage 2-4) were seeded on the Matrigel surfaces. After removing all non-invasive cells the nuclei of all invasive cells on the lower surface of the membranes were counterstained with DAPI.

Since MCF-7 is a less invasive breast cancer cell line and tends to grow in an epithelial cell like manner it was not possible to clearly identify single cells. Therefore, the visual impression of an increase in invasive number of MCF-7 cells could not be quantified as for the 4T1 and MDA MB 231 cells.

Interestingly, the staining of the nuclei with DAPI revealed that a lot more cells were invasive than the DiI (MCF-7) staining showed alone (Fig. 9) indicating that hASCs have a strong invasive capacity.

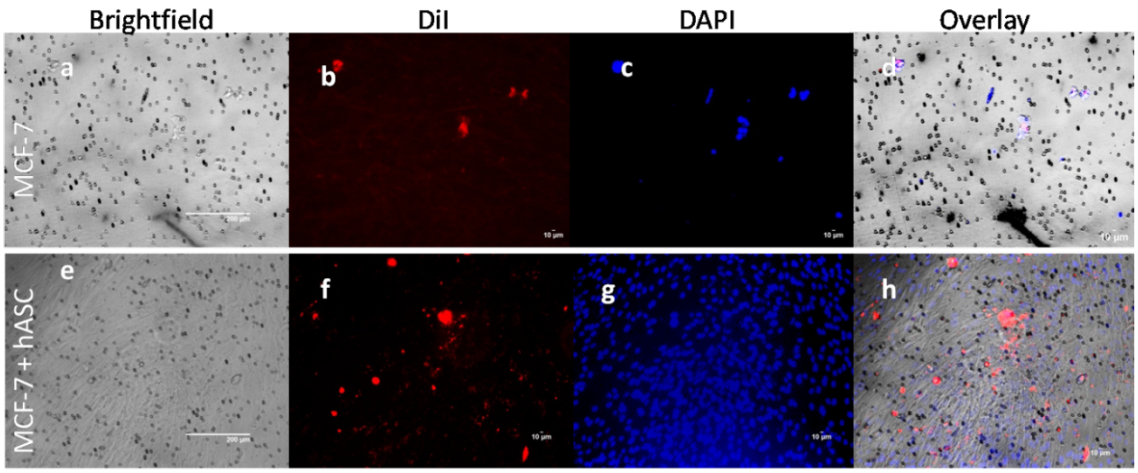

Fig.9 hASCs promote invasion of MCF-7. Coseeding of MCF-7 along with hASCs resulted in more DiI labeled MCF-7 colonies on the lower surface of invasion chambers. a,b,c,d show different stainings and overlay of MCF-7 cells alone. e,f,g,h show different stainings of MCF-7 coseeded with hASCs. The dye is indicated above each column.

With the MDA MB 231 human breast cancer cell line we chose one of the most invasive and metastatic cell lines that have been established. Therefore these cells represent a powerful source to study mechanisms of invasion in vitro and in vivo.
MDA MB 231 alone ($3x10^4$/insert) or MDA MB 231 ($3x10^4$/insert) together with hASCs ($6x10^4$/insert) were seeded on the matrigel surfaces. Furthermore, two more non-tumorigenic cell lines were coseeded with MDA MB 231 cells to elucidate whether the observed effect is indeed due to hASCs or simply a result of a higher seeding density. The human embryonic lung fibroblast cell line WI-38 has been used as control for the mesenchymal cell type and the immortalized human mammary epithelial cell line HMEC has been used as control for the epithelial cell type (Fig.10).
Remarkably the increase in invasive breast cancer cells could be only observed when MDA MB 231 cells were given the possibility to interact with hASCs (105 ± 16.97 as

compared to 46.4 ± 21.54). Whereas there was no increase in invasive MDA MB 231 cells when seeded together with WI-38 or HMEC cells (Fig. 11).

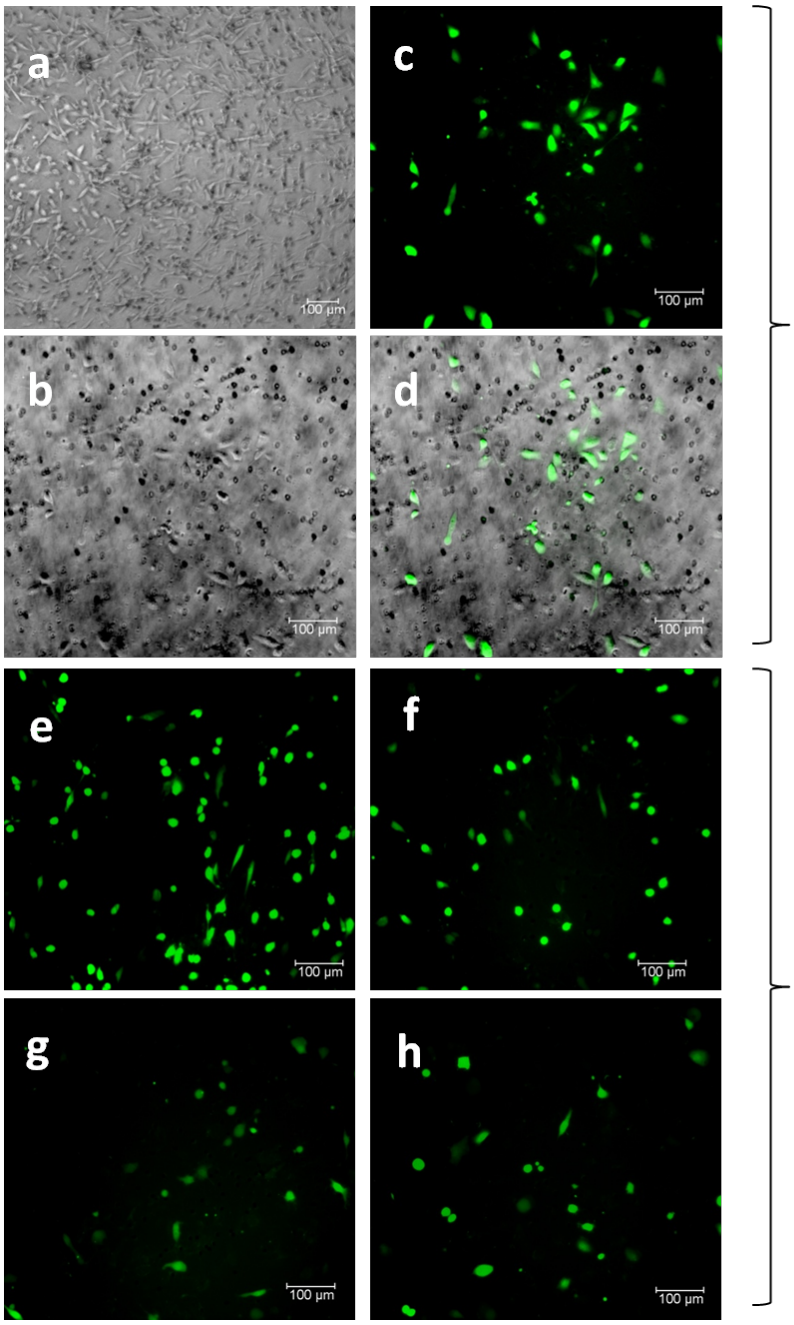

Fig.10 Human adipose tissue derived stem cells promote invasion of MDA MB 231.

a) Brightfield image of MDA MB 231 (35 x 10^3 cells) seeded on matrigel of invasion chamber.

b) Phase contrast image of matrigel after scrubbing the upper surface with a cotton swab to remove all non-invasive cells.

c) GFP signal of invaded MDA MB 231 cells.

d) Overlay of b) and c).

e) Green signal shows invaded GFP-MDA MB 231 when coseeded with hASCs.

f) Green signal shows invaded GFP-MDA MB 231 when Co-Cultured with hASCs in the presence of neutralizing CCL5-antibody (3µg/ml).

g) Green signal shows invaded GFP-MDA MB 231 when coseeded with WI-38 cells.

h) Green signal shows invaded GFP-MDA MB 231 when coseeded with HMECs.

The seeding ratio was always 1:2 (GFP-MDA MB 231 : hASCs/WI-38/HMEC).

To exclude that the alteration of invasive behavior is associated with the cell staining procedures the invasion assays for the MDA MB 231 series was carried out with GFP-labeled MDA MB 231 cells as well as with DiI-labeled MDA MB 231 cells. The invasion promoting effect of hASCs on MDA MB 231 cells could be observed in a similar pattern in both conditions.

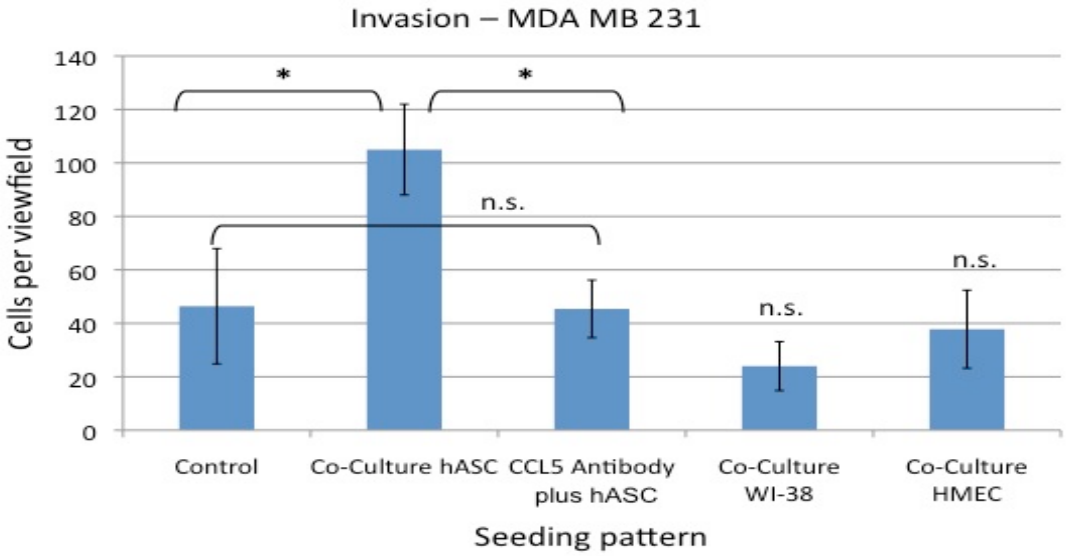

Fig.11 Coseeding of GFP-MDA MB 231 and hASCs resulted in 2.26 fold higher number of invasive GFP-MDA MB 231 cells as compared to seeding GFP-MDA MB 231 cells alone. The CCL5 neutralizing antibody blocked the invasion promoting effect of hASCs. WI-38 and HMECs did not show the invasion promoting effect as compared to hASCs. Quantitative data of invaded GFP-MDA MB 231 cells per view field is shown as mean ± SD. * $P<0.005$, n.s. not significant.

6.3 MDA MB 231 induce de-novo secretion of CCL5 (RANTES) in hASCs

In order to identify potential mediators of the interaction between breast cancer cells and ASCs the chemokine CCL5 (RANTES) has been measured with ELISA as a candidate mediator that has been shown to be involved in breast cancer metastasis.

Therefore breast cancer cells, ASCs or control cell lines have been seeded in systems of direct co-cultures in 6-well plates as shown below.

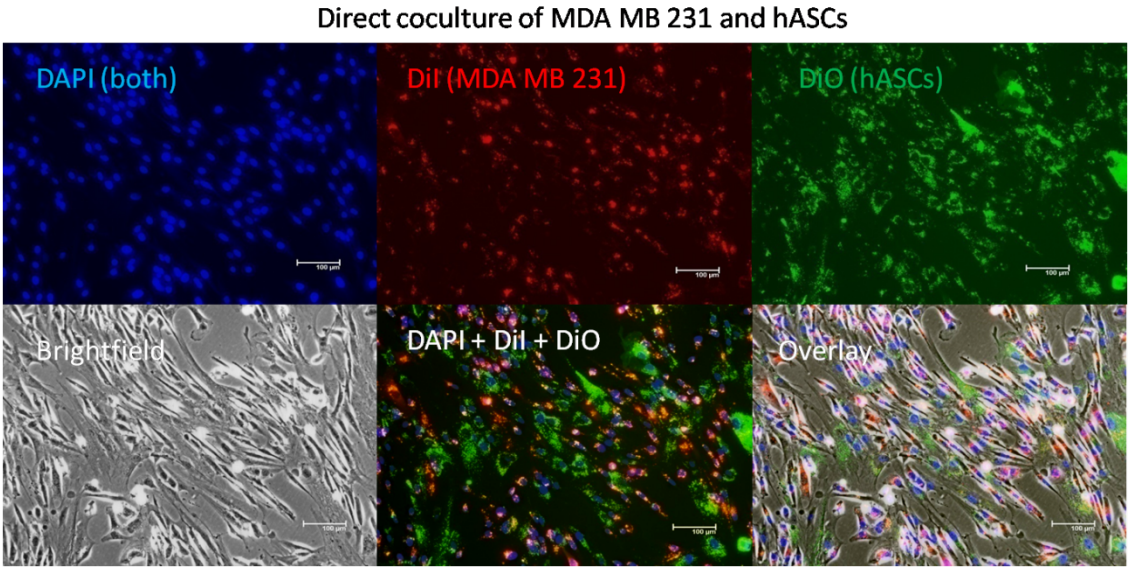

Fig.12 Direct Co-Culture of MDA MB 231 and hASCs. 5 x 10^4 DiI-MDA MB 231 (red) cells were seeded together with 10^5 hASCs (Passage 4, green) in 6-well plates. After 48h incubation direct Co-Cultures were fixed and nuclei were stained with DAPI (blue) before taking pictures.

Figure 12 shows a direct co-culture of MDA MB 231 cells and hASCs (Passage 4). Both cell lines were labeled either with DiI (red, MDA MB 231) or DiO (green, hASCs) respectively prior to seeding cells. Before taking the pictures all cells were counterstained with DAPI (blue, nuclei). In the lower row starting from the left the brightfield picture, overlay of DAPI, DiI and DiO and the overlay of all is shown. The MDA MB 231 showed a regular distribution between the hASCs without building colonies of cancer cells.

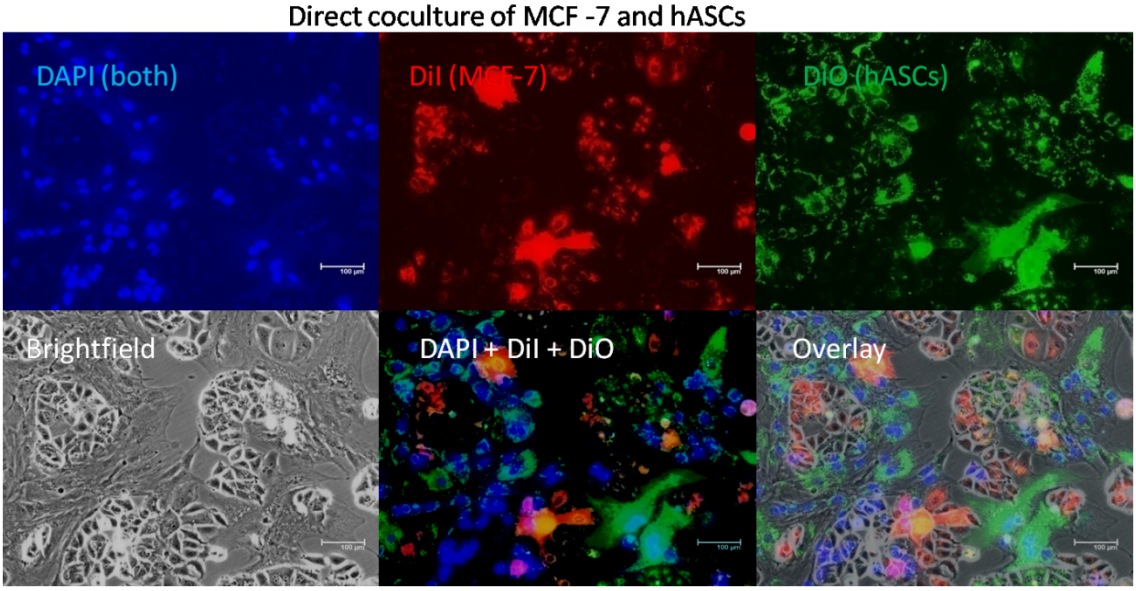

Fig.13 Direct Co-Culture of MCF-7 and hASCs. 5 x 10^4 DiI-MCF-7 (red) cells were seeded together with 10^5 hASCs (Passage 4, green) in 6-well plates. After 48h incubation direct Co-Cultures were fixed and nuclei were stained with DAPI (blue) before taking pictures.

Figure 13 shows a direct co-culture of MCF-7 cells and hASCs (Passage 4). Both cell lines were labeled as described above for the co-culture of MDA MB 231 and hASCs. In the lower row starting from the left the brightfield picture, overlay of DAPI, DiI and DiO and the overlay of all is shown. The MCF-7 formed round shaped islands of cancer cell colonies without penetrating the hASCs. The hASCs organized themselves around those islands.

Interestingly these two human breast cancer cell lines seem to differ not only in their in vitro invasion capacity but also in their way of organizing in a direct co-culture with hASCs. The MDA MB 231 cells deriving from the more invasive cell line as compared to the MCF-7 cell line showed a more equal distribution of individual breast cancer cells between the hASCs. Whereas the MCF-7 cells did not leave their source colony and thus formed growing islands of monolayer breast cancer cells surrounded by hASCs indicating that signaling between these cell lines affects the growth pattern of hASCs in a specific way according to their invasive capacity.

6.3.1 CCL5 is produced in direct co-culture of hASCs and breast cancer cells

The concentration of CCL5 was measured in the supernatants of cell culture by harvesting the medium, centrifuging it at 1,500 rpm for 5 min and the supernatant was passed through a Millipore sterile 50 mL filtration system with a 0.45-µm polyvinylidene difluoride membrane.

CCL5 could only be detected in the supernatants of the direct co-culture, whereas MDA MB 231 alone and hASCs alone did not produce any CCL5, highlighting this chemokine as potential target for further investigation (Fig.14a). By measuring the timepoints of 12h, 24h, 36h, 48h, 60h and 72h the kinetics of the chemokine showed a plateau starting between the 60h and the 72h timepoints (Fig. 15a).

Furthermore there was no CCL5 detectable by exposing the MDA MB 231 cells to direct WI-38 contact (Fig. 15b). Interestingly CCL5 could be measured in the cell culture of HMEC and in the Co-Culture of HMEC together with MDA MB 231 (data not shown).

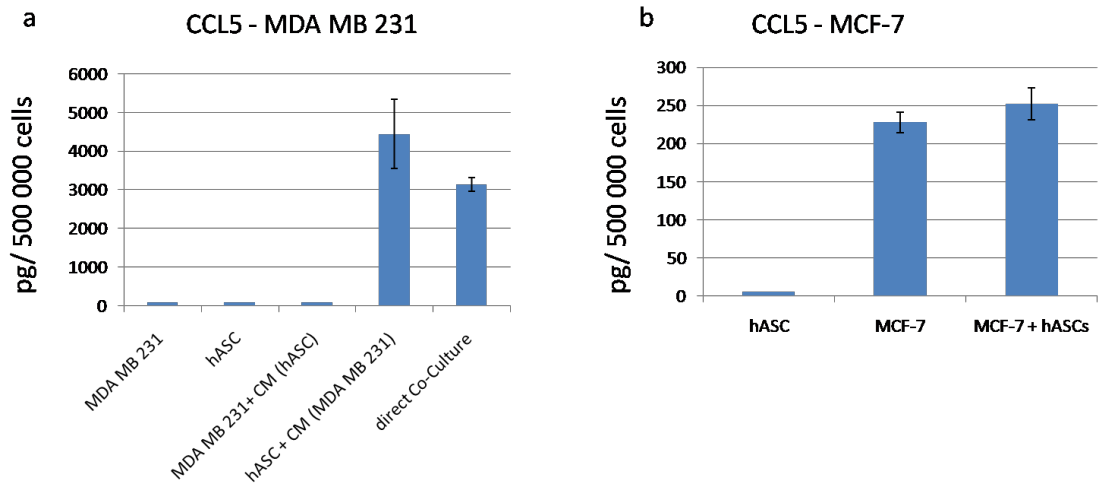

Fig.14 a) CCL5 is produced by hASCs when exposed to tumor conditioned medium (MDA MB 231) or in direct Co-Culture with MDA MB 231. All conditions were analyzed with ELISA after 48h incubation. b) CCL5 is produced by MCF-7 cells and not significantly more in direct Co-Culture with hASCs. All columns without error bars indicate that no CCL5 was detectable with ELISA.

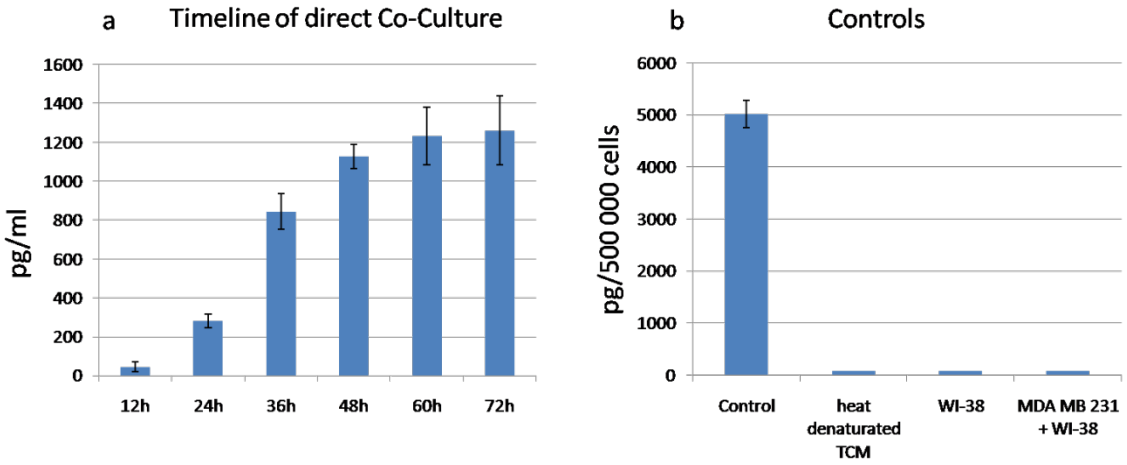

Fig.15 a) Secretion kinetics of CCL5 in direct Co-Culture of MDA MB 231 and hASCs. b) hASCs exposed to tumor conditioned medium (MDA MB 231) was used as a control for this ELISA. Heat denaturation of TCM (MDA MB 231) eliminated the humoral factors secreted by MDA MB 231 cells to stimulate hASCs. CCL5 was not detectable in supernatants of WI-38 cell culture and direct Co-Culture of MDA MB 231 and WI-38 cells.

When measuring the supernatants of MCF-7 cultures a baseline level of CCL5 was secreted by MCF-7 cells. In contrast to the direct Co-Culture of MDA MB 231 with hASCs no induction of CCL5 secretion could be observed in the MCF-7/hASCs – direct co-cultures (Fig. 14b).

From this point on we focused on the interaction of MDA MB 231 cells and hASCs since only in this context CCL5 was induced in hASCs in such a dramatic manner.

6.3.2 Humoral MDA MB 231 derived factors induce CCL5 secretion in hASCs

To answer the question which cell line would be responsible for the CCL5 secretion the conditioned media of both hASCs and MDA MB 231 cells were used to stimulate the counterpart respectively (Fig. 16).

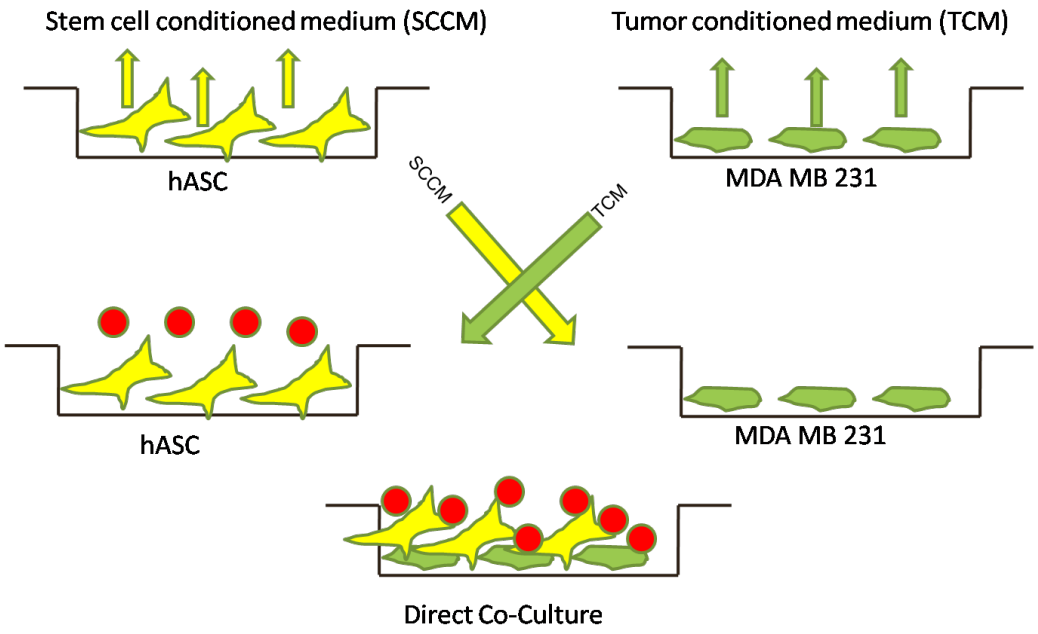

Fig.16 Scheme of cross conditioning. Green (TCM) and yellow (SCCM) arrows indicate transfer of tumor conditioned medium (TCM) and stem cell conditioned medium (SCCM) to the according cell line as indicated. Red circles mark secretion of CCL5.

CL5 was markedly high produced only by hASCs (Fig. 14a) after exposing them to tumor conditioned medium (TCM) while the MDA MB 231 cells did not secrete any CCL5 after being incubated with stem cell conditioned medium (SCCM).

In order to see whether the humoral factors secreted by MDA MB 231 cells were proteins by nature, the TCM had been heat denatured before being used for hASCs stimulation. In this case there was no CCL5 measurable any more (Fig 15b).

6.4 The CCL5-receptor CCR5 is expressed by both hASCs and MDA MB 231

The main receptor for CCL5 is CCR5 and has been shown to play a pivotal role in the interaction of breast cancer cells and bone-marrow derived mesenchymal stem cells.

Therefore the expression of CCR5 (hASCs, MDA MB 231) as well as the exposure of the receptor on the cell surface has been investigated with RT-PCR and Immunohistochemistry (Fig. 17 and Fig. 18) respectively.

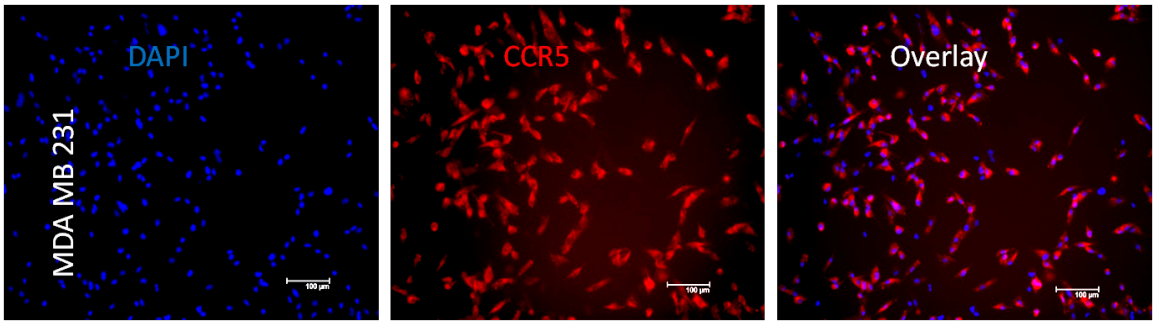

Fig.17 Immunostaining (MDA MB 231) for CCR5.

RT-PCR for CCR5 was performed by using GAPDH as reference gene. The mRNA expression levels of the two cell lines relative to GAPDH for the CCL5 (RANTES) receptor CCR5 were calculated as shown below. The mRNA expression of CCR5 in MDA MB 231 was 40.09 fold higher as compared to hASCs in Passage 4. The relative expression was calculated according to the formula below.

1) Expression (ΔCT) = C_T(target gene) - C_T(reference gene)

2) relative Expression = $2^{-\Delta\Delta CT[\Delta CT(\text{MDA MB 231}) - \Delta CT(\text{hASCs})]}$

Figure 17 and 18 show DAPI staining for the nuclei, the red signal shows CCR5 on the cell surface and the overlay shows the association of receptor and nuclei staining for MDA MB 231 and hASCs as indicated.

Interestingly both the cancer cell line MDA MB 231 and the hASCs showed a regular pattern of CCR5 on the cell surface. This shows that CCL5 has the possibility to bind to its main receptor on MDA MB 231 cells and hASCs. Following this insight the role of CCL5 in the context of enhanced invasion in the interaction of MDA MB 231 cells and hASCs needed to be clarified.

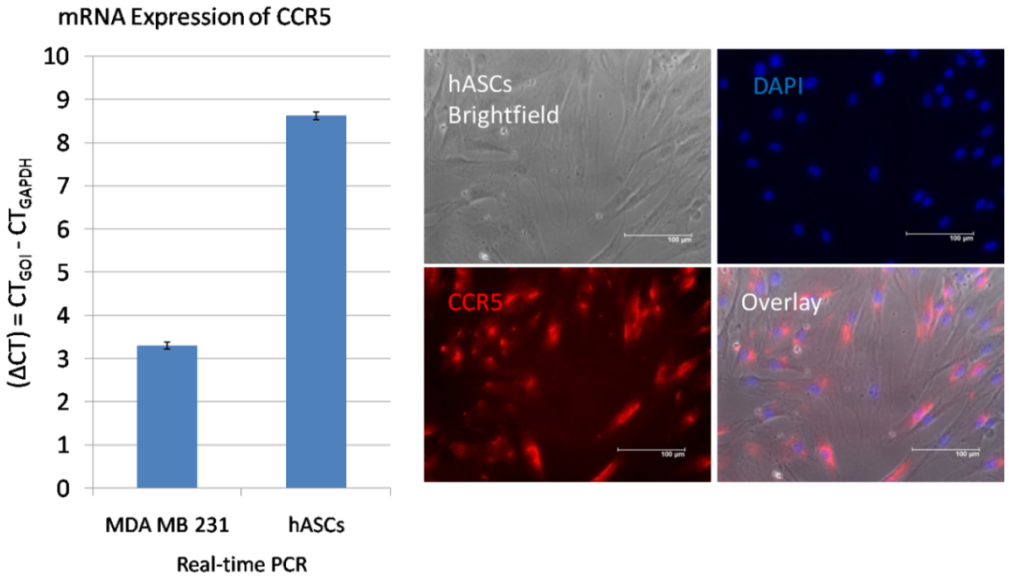

Fig.18 mRNA expression of CCR5 in MDA MB 231 and hASCs and Immunostaining (hASCs) for CCR5.

6.5 CCL5 is necessary to promote invasion of MDA MB 231

With CCL5 being secreted by hASCs only after being stimulated by humoral tumor derived factors the question arose whether CCL5 is involved in the promotion of MDA MB 231 invasion. Therefore the invasion assays have been repeated with additionally incubating the cells in the coseeding with a neutralizing CCL5 antibody.

These invasion assays showed that adding the CCL5 antibody blocked the invasion enhancing effect of hASCs on MDA MB 231 (Fig. 10f). To exclude the possibility that random binding of the antibody was responsible for reducing MDA MB 231 invasion the control IgG antibody has been incubated during the invasion assays as well and did not show any alteration in MDA MB 231 invasive behavior.

6.6 MMP-9 is a candidate protease contributing to higher invasion of MDA MB 231

To further characterize the consequences of hASCs interacting with MDA MB 231 cells several zymographies have been carried out. Zymography is a valuable method to evaluate the production of certain enzymes and at the same time to see their enzymatic activity. In this case two candidate proteases have been looked at, Matrixmetalloproteinase-2 (MMP-2/Gelatinase A) and Matrixmetalloproteinase-9 (MMP-9/Gelatinase B).

6.6.1 MMP-9 activity is upregulated in direct co-culture of hASCs and MDA MB 231

The settings for measuring the MMP-9/MMP-2 activity were the same as described above. MDA MB 231 (5 x 10^4 cells/well) and hASCs (10^5 cells/well) were seeded alone or together in direct co-culture (ratio of MDA MB 231 to hASCs was as before 1:2). The supernatants containing active MMP-2/MMP-9 were harvested as mentioned before and either directly used directly for zymography or stored at -20 ^{o}C until use.

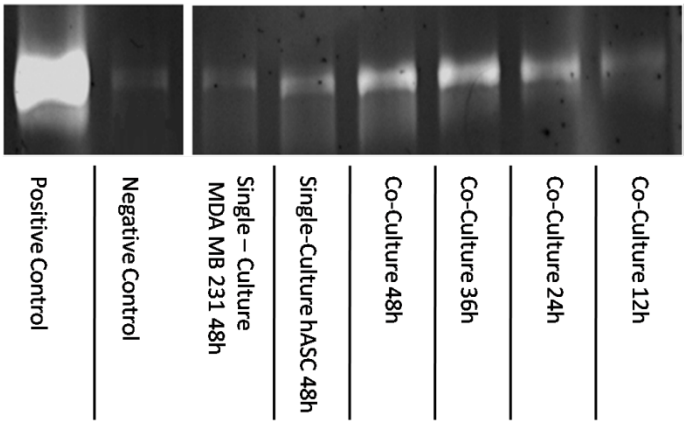

Fig.19 MMP-9 activity in MDA MB 231 and hASCs Co-Culture. MMP-9 activity was analyzed with a pre-cast (Gelatin) Zymogram gel. Lanes show clear bands of MMP-9 activity.

Notably after 36 and 48 hours there was a significant increase of active MMP-9 in the supernatants of the direct co-culture (Fig. 19). MMP-2 did not show any change in activity when MDA MB 231 cells were interacting with hASCs. The differences between bands of active Enzyme activity were confirmed by measuring the average density with the Software Image J.

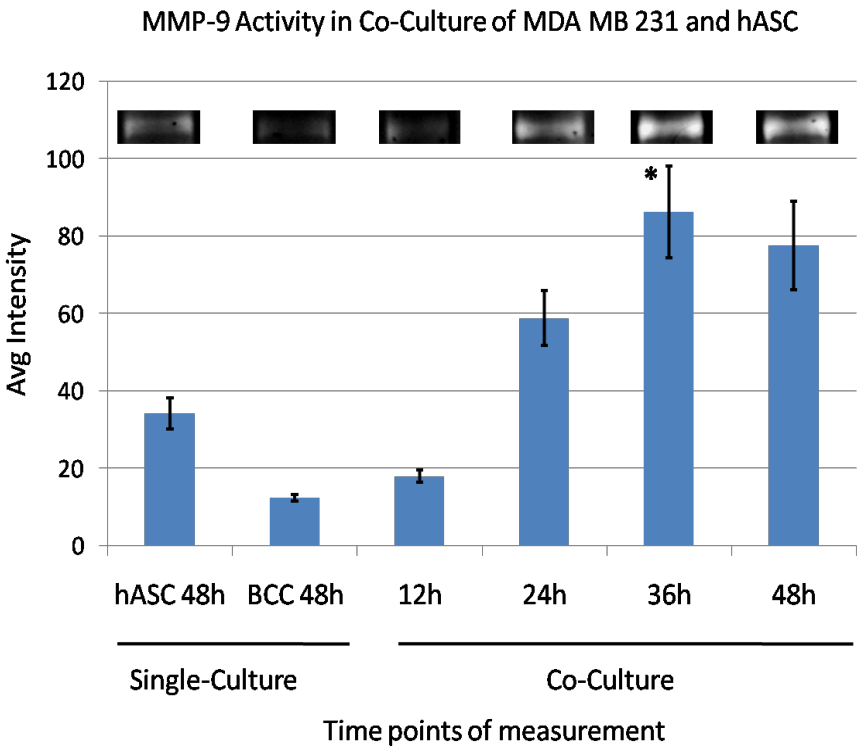

Fig. 20 Quantitative data of MMP-9 activity. The correlating bands of the zymography gel are displayed above each column. *P< 0.05 compared to hASCs cultured alone at 48 hours.

6.6.2 CCL5 does not increase MMP-9 Activity in MDA MB 231 but in hASCs

With CCL5 and MMP-9 being upregulated in the supernatants of MDA MB 231 and hASCs co-cultures we wanted to answer the question whether MMP-9 might be a downstream effector molecule of CCL5. Since we already found that MMP-9 and MMP-2 are higher expressed on the RNA level of MDA MB 231 after being stimulated for 2h with human recombinant CCL5 in a pilot experiment (data not shown) we wanted to confirm this observation on the level of enzyme activity by zymography (Fig. 21).

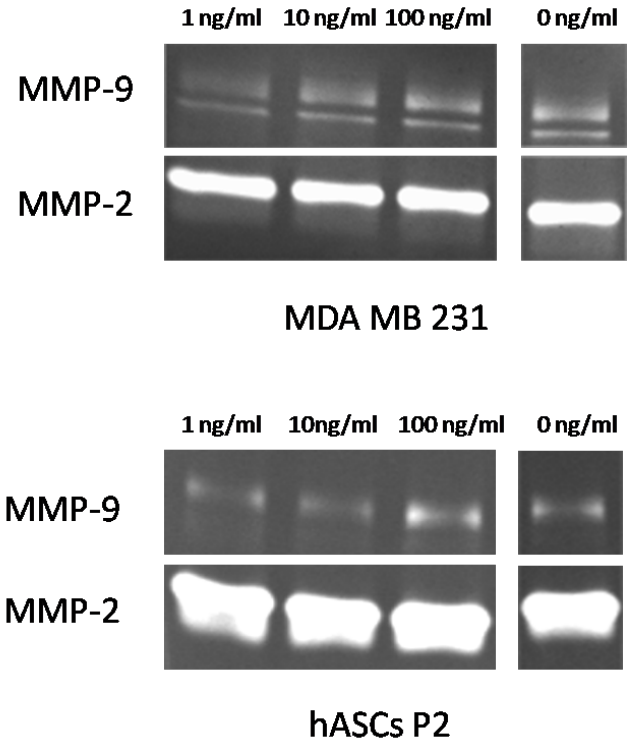

Fig.21 MMP-9 and MMP-2 activity of MDA MB 231 and hASCs after stimulation with recombinant human CCL5. Concentrations of CCL5 were applied as indicated above each condition.

In the direct co-culture of MDA MB 231 and ASCs the highest activity of MMP-9 was detected between 36h and 48h. To determine the role of CCL5 in this context MDA MB 231 were stimulated with CCL5 for 48h and the activity of MMP-9 and MMP-2 in the supernatants was analyzed by zymography.

The activity of neither MMP-2 nor MMP-9 was markedly altered in the treated MDA M 231 cells as compared to the control group. However the activity of MMP-9 seemed to be lower in the 1ng/ml group (average density 96.37 ± 6.384 as compared to 144.809 ± 24.603 in the control group).

Interestingly the zymography of hASCs stimulated with CCL5 also showed a lower average density in the 1ng/ml group (107.941 ± 16.224 as compared to 120.972 ± 24.814 in the control group) but a higher average density in the 100 ng/ml group (146.147 ± 36.882 as compared to 120.972 ± 24.814 in the control group).

6.7 Summary of Results

Taken together the results can be summarized as the following:

1) Adipose tissue derived stem cells (ASCs) promote breast cancer cell invasion in vitro

2) Humoral tumor derived factors stimulate ASCs to produce CCL5 (RANTES)

3) CCL5 plays a pivotal role in promoting breast cancer cell invasion in vitro

4) MMP-9 is up regulated in the direct co-culture of breast cancer cells and ASCs

5) CCL5 enhances MMP-9 activity in ASCs but not in MDA MB 231 breast cancer cells

7. Discussion

The aim of this study was to clarify how adipose tissue derived stem cells (ASCs) as part of the breast cancer microenvironment might be involved in breast cancer metastasis. Furthermore, we sought to identify potential molecular targets in this interaction that could lead to the development of tumor microenvironment specific therapies in breast cancer.

Evidence has been growing over the last decades that the tumor microenvironment is significantly involved in tumor progression on all stages, from primary tumor growth to development of distant metastases (Albini and Sporn, 2007; Elenbaas and Weinberg, 2001; Witz, 2008). In this context, cancer associated fibroblasts (CAF), myofibroblasts, tumor associated macrophages (TAM), monocytes, as well as bone marrow derived progenitor and stem cells have been studied extensively and described in various aspects (Olumi et al., 1999; Jeon et al., 2008; Allavena et al., 2008b; Kaplan et al., 2006).

Furthermore, a variety of bone marrow derived cells have been shown to be involved in tumor growth through providing cellular components for neoangiogenesis, supporting the vascularization that is necessary for a tumor to grow beyond a certain size (Du et al., 2008). The group of Karnoub has investigated specifically the interaction of bone marrow derived stem cells and breast cancer cells in primary tumor growth as well as the rate of lung metastases in a murine breast cancer model (Karnoub et al., 2007).

Interestingly the role of adipose tissue derived stem cells (ASCs) has not been addressed in the context of breast cancer microenvironment and metastasis. With the growing evidence for alteration of the tumor microenvironment resembling inflammatory processes, we hypothesized that ASCs might represent an important source of early multipotent response cells being manipulated by breast cancer cells, and thus contribute to creating a reactive environment, for example, by either

producing chemoattractant factors that subsequently recruit inflammatory cells or by secreting factors that act in a paracrine fashion on breast cancer cells or the surrounding microenvironment.

In order to investigate the interaction of ASCs and breast cancer cells, we started to evaluate the effect of stem cell conditioned medium on breast cancer cell proliferation by exposing human breast cancer MDA MB 231 cells to stem cell conditioned medium (SCCM) and compared their growth rate to MDA MB 231 cells in regular growth medium.

Similar experiments with various cancer types have been done by other groups (Zhu et al., 2009; Ohlsson et al., 2003; Ramasamy et al., 2007) and show an inhibition of cancer cell proliferation. However, in these cases, irradiated stem cells, virally transfected stem cells, or much higher tumor cell-stem cell ratios had been used for the experiments, which might have altered the outcome of these studies.

In contrast to these findings, we (Muehlberg et al., 2009) and other groups (Karnoub et al., 2007; Mishra et al., 2008) showed that co-injection of breast cancer cells and mesenchymal stem cells from the bone marrow or tissue resident stem cells derived from the adipose tissue enhanced tumor growth tremendously in nude mice models. The proliferation experiment of the present study confirmed these in vivo observations in such a way that we could detect a higher number of MDA MB 231 cells after being exposed to SCCM over four days. The first significant difference in cell number was observed on day four.
Furthermore, this experiment was important towards ruling out the possibility that proliferation differences within the first 48 hours would affect the final results of the invasion assays.

To study the effect of a direct co-culture of ASCs and breast cancer cells on the invasive capacity of the tumor cells, several invasion assays have been carried out. We chose the murine breast cancer cell line 4T1 and the two human breast cancer cell lines MCF-7 and MDA MB 231 that have been characterized for different invasive

capacities. In both the 4T1 and the MDA MB 231 co-culture, we could observe an increase in invasive cancer cells of 137% and 126% respectively.
Since the MCF-7 cell line did grow into a colony, we were not able to quantify the visual impression of an increased number of invasive tumor cells. However the increase of invasive MCF-7 cells when co-seeded with ASCs seemed not to be as dramatic as compared to 4T1 and MDA MB 231 cells.

This might imply that the invasion-promoting effect of ASCs becomes more important according to the initial invasiveness of the breast cancer cells, although we could not confirm this hypothesis due to difficulties in quantification of invasive MCF-7 cells. These results are in line with the in vivo studies that have been done by the laboratory of Karnoub (Karnoub et al., 2007).

According to the observations described above we focused on MDA MB 231 cells for further investigation. Our question was how the ASCs could possibly enhance the invasive behavior of breast cancer cells.

In a candidate approach we measured the chemokine CCL5 (RANTES) in the supernatants of ASCs, MDA MB 231, and direct co-cultures in a ratio of stem cells to tumor cells of 1:2.

Remarkably, a high concentration of CCL5 could only be detected in the direct co-culture of MDA MB 231 cells with hASCs and was not detectable at all in the supernatants of the corresponding single cultures.
Through further experiments, we concluded by ELISA that humoral tumor derived factors secreted by MDA MB 231 cells induce a de novo secretion of CCL5 by ASCs. According to different studies (Altman et al., 1997; Azenshtein et al., 2002), epithelial cells, immunocompetent cells and tumor cells either constitutionally express CCL5 or are stimulated by tumor necrosis factor α (TNFα) and consequently express CCL5 (Robinson et al., 2002; Balkwill, 2004).
However, the TNFα level that we measured in the supernatants of MDA MB 231 tissue cultures was very low, and experiments where we incubated the supernatants of MDA

MB 231 cells with neutralizing antibodies against TNFα before exposing ASCs to the tumor conditioned medium did not result in a decrease in CCL5 secretion by ASCs.
We concluded that either other factors are responsible in this context for the induction of CCL5, or the complex interplay between several factors is causing the secretion of CCL5.
Possible ways to identify these mediators are to use shRNA treated MDA MB 231, thus knocking down different genes encoding possible mediators, or by screening the supernatants of MDA MB 231 cells for active proteins and subsequently characterizing those. The group of Glod (Lin et al., 2008b) used an interesting experimental approach including mass spectrometry to identify factors secreted by MDA MB 231 cells responsible for the chemoattraction of mesenchymal stem cells from the bone marrow (MSCs).
Cyclophilin B and hepatoma-derived growth factor were the two proteins characterized for their promigratory effect on MSCs. It would be interesting to see whether these factors are also involved in the stimulation of ASCs.

After having identified CCL5 as a de novo secreted chemokine in the interaction of ASCs and breast cancer cells, we asked whether CCL5 might be involved in promoting the invasion of MDA MB 231 cells. We therefore incubated the direct co-cultures of ASCs and MDA MB 231 cells with a neutralizing antibody against CCL5.
We found that blocking CCL5 completely abolished the invasion-promoting effect of ASCs but did not inhibit the intrinsic invasion capacities of MDA MB 231. These in vitro findings support the importance of CCL5 for MDA MB 231 metastasis as shown by the group of Karnoub (Karnoub et al., 2007). We confirmed with a control IgG antibody that only specific blocking of CCL5 was responsible for this observation.
Interestingly the study of Karnoub et al. (Karnoub et al., 2007) showed in a mouse model that systemic application of a neutralizing CCL5 antibody significantly inhibited the metastatic spread from the primary tumor site.

Trastuzumab and bevacizumab, two monoclonal antibodies targeting HER-2 and VEGF respectively, are already in use in the context of managing metastatic disease in breast cancer patients. Women with widespread disease involving multiple

symptomatic sites especially seem to be better served with an initial systemic treatment. However, only five to ten percent of patients with MBC survive five or more years. Clinical trials evaluating the benefit of early breast cancer stage patients treated with trastuzumab did not show a clear benefit (Mackey et al., 2009).
Therefore, new molecular targets such as chemokines might help in finding optimal combination therapies that finally lead to better control of metastatic dissemination in breast cancer patients.

We asked whether MMP-9 might be involved in the interaction of ASCs and MDA MB 231 cells, and subsequently conducted experiments to measure MMP-9 activity in the supernatants of direct ASC - MDA MB 231 co-cultures at different time points and found that MMP-9 is upregulated in a time-dependent manner (Fig. 18).

However, it was still not clear which of the two cells or if maybe both were secreting MMP-9. It has been shown that MMP-9 is regulated by various factors such as IGF, TNFα, TGFβ (Mira et al., 1999; Hagemann et al., 2004) and other factors like the chemokine CCL5 (Okita et al., 2005; Chabot et al., 2006), in some cases dependent on the Smad-, Ras-, and PI3-kinase-signaling pathway (Stuelten et al., 2005).
We first wanted to see whether CCL5 is possibly involved in upregulating MMP-9 or MMP-2 in either the MDA MB 231 or the ASCs, and therefore checked for the expression of the major CCL5 receptor CCR5.

In order to see whether MDA MB 231 cells or ASCs express the main CCL5 receptor CCR5, we used RT-PCR and. Interestingly, we found that the expression of CCR5 in MDA MB 231 cells was 40.9-fold higher than normal, according to our RT-PCR results. The immunostaining revealed that CCR5 was detectable in a more homogenous pattern on the cell surfaces of the MDA MB 231 cells as compared to the ASCs, but a clear signal was stably detectable in both cell lines, and the no-primary controls were negative in all cases.

It has been well established that breast cancer cells express CCR5 (Manes et al., 2003; Zlotnik, 2006), but regarding ASCs we found controversial statements about the expression of CCR5 in mesenchymal stem cells.

The group of Kroeze (Kroeze et al., 2009) found by flow cytometry that mesenchymal stem cells from adipose tissue do not express CCR5, whereas the group of Ji (Ji et al., 2004) showed by PCR, flow cytometry, and immunostaining that CCR5 was expressed on rat mesenchymal stem cells derived from the bone marrow, which is in direct contradiction to the work of the group of Karnoub (Karnoub et al., 2007) claiming that bone marrow derived stem cells did not express CCR5.
Hence we concluded that surface expression of certain receptors might differ according to the origin, passage, and culture conditions of the cell type or applied methodology.

Since the expression of CCR5 on human adipose tissue derived stem cells showed in our experiments to be consistently expressed in both RT-PCR and immunostaining, we hypothesized that CCL5 might also act in an autocrine fashion on ASCs.

Therefore, we conducted experiments where we stimulated MDA MB 231 cells and ASCs for over 48 hours with human recombinant CCL5 at different concentrations (Fig.20) and measured the activity of MMP-9 and MMP-2 in the supernatants with zymography.
We found that both cell lines showed slight MMP-9 and strong MMP-2 activity. Interestingly, we could only detect an elevated level of MMP-9 activity in the supernatants of ASCs when stimulated with CCL5 at a concentration of 100ng/ml, but not in MDA MB 231 cells.
Accordingly we hypothesized that CCL5 is possibly involved in the upregulation of MMP-9 in mesenchymal stem cells derived from the adipose tissue. These results support the idea that CCL5 plays a role in upregulating MMP-9 in immature dendritic cells and T-cells (Chabot et al., 2006; Okita et al., 2005).

Further investigation is necessary to clarify the relative importance of CCL-5 induced MMP-9 secretion as compared to migration enhancing effects of CCL5. A schematic overview of the results of this study is shown in Fig. 22.

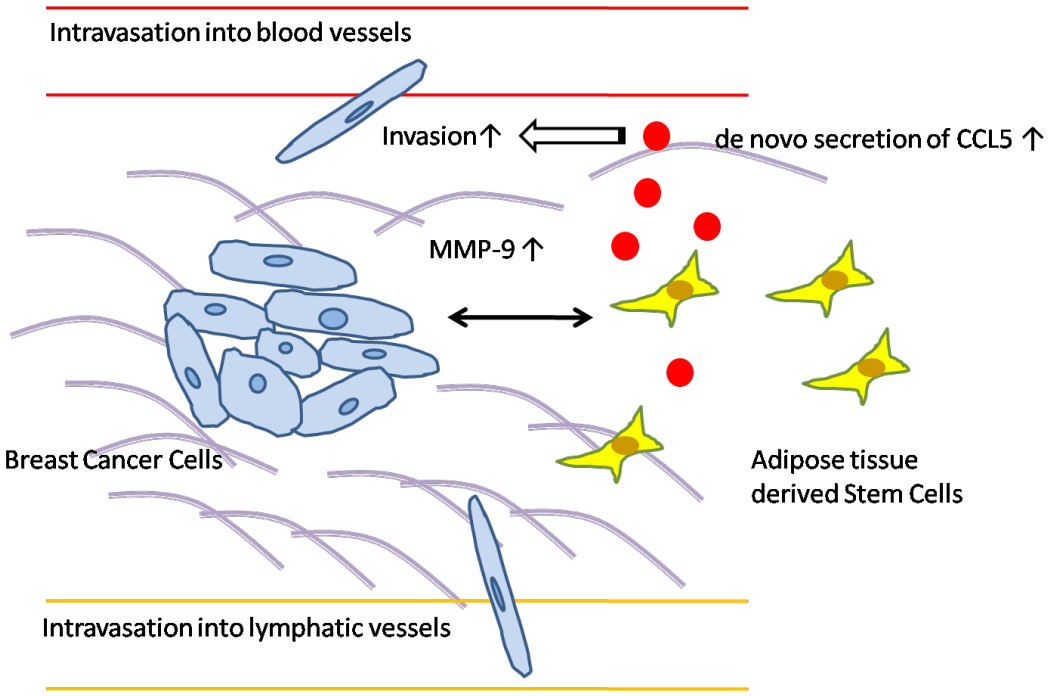

Fig. 22 Schematic overview of aspects in the context of MDA MB 231 breast cancer cells (blue) interacting with human adipose tissue derived stem cells (yellow). Red circles represent secreted CCL5 (RANTES).

It would be interesting now to see whether MDA MB 231 cells also secrete factors that attract and mobilize adipose tissue derived stem cells. This seems to be very likely according to the findings of the group of Lin (Lin et al., 2008a) and Dwyer (Dwyer et al., 2007), which showed that bone marrow derived stem cells (BMSC) home to tumor sites and described scenarios of BMSC as carriers of therapeutic drugs, astested in some in vivo tumor models (Picinich et al., 2007). Nevertheless BMSCs are not as easy accessible as ASCs. and the tumor homing capacity of ASCs has been investigated by the group of Lamfers (Lamfers et al., 2009; Kucerova et al., 2007), who

showed that ASCs might be an alternative for bone marrow derived stem cells as anti-cancer drug delivery vehicles.

Taken together, the results of this study suggest that CCL5 might represent an interesting target for breast cancer treatment, especially for highly metastatic breast cancer subtypes.

Although bone marrow derived stem cells have been characterized in several studies according to their interaction with tumor cells, the study of (Tomiyama et al., 2008) indicates that despite a common origin from the mesenchymal germinal sheet, this does not necessarily mean that stem cells from the adipose tissue share complete functional identity with bone marrow-derived stem cells.

Interestingly ASCs also seem to differ in their ability to promote angiogenic recovery and were subsequently found to differ in their tube formation and matrixmetalloproteinase expressing profile (Kim et al., 2007) in such a way that blood flow recovery in a hindlimb ischemia model of nude mice was stronger when ASCs were applied.

Furthermore, we hypothesize that the role of tissue resident stem cells needs to be reconsidered for developing a more complete concept of breast cancer pathogenesis that includes the role of the huge variety of cells composing the tumor stroma, which crucially contributes to reorganizing the tumor microenvironment, creating the inflammatory or desmoplastic stroma which permits tumor growth over small sizes and finally enables tumor cells to metastasize.

Despite the growing evidence for a metastasis promoting function of CCL5, the exact role of CCL5 in breast cancer still remains elusive since the group of Jayasinghe (Jayasinghe et al., 2008) has shown that tumor derived CCL5 does not affect tumor growth and metastasis by using interference RNA to inhibit CCL5 translation in the highly metastatic murine breast cancer cell line 4T1 and by overexpressing CCL5 in a less metastatic murine breast cancer cell line 168.

Despite the methodological problems that come along with this approach and the intrinsic difference between metastasis and tumorgrowth subclones, this study supports the significance of host derived CCL5 in the context of breast cancer

progression. To prove this conclusion, CCL5 knockout mouse might provide important insights.

To summarize the present study, we identified CCL5 (RANTES) as a potential mediator in the interaction of human adipose tissue derived stem cells and breast cancer cells in tumor cell invasion and characterized its effect on Matrixmetalloproteinase 9 activity, and conclude that CCL5 or its receptor CCR5 might represent potential targets for mammary cancer progression, especially in highly invasive cases.

Cancer remains one of the most complex and challenging diseases in human beings, not only because of the myriad of potential mechanisms that lead to the malignant transformation on the cellular level, but also because of the enormous involvement of local and systemic factors that differ in every human individual. Finding therapies for cancer requires the investigation of each single parameter in this multistep process in order to define targets that can be focused on and thus promise a cure, longer lifetime, or at least an increase in quality of life for patients suffering from cancer

7.1 Summary of discussion

Breast cancer is the cancer with the highest incidence among women. Although tumor evolution and progression has been well studied in the past decades on the cellular level of cancer cells, the interaction of breast cancer cells with surrounding non-tumorigenic tissue still leaves many questions unanswered.
Immunomodulatory cells like macrophages, monocytes, and T-cells, as well as other bone marrow derived cells, have been characterized in many aspects of their involvement in breast cancer development and metastasis. Nevertheless, the specific role of tissue resident stem cells has not been investigated yet.
It has been shown that the tumor microenvironment not only provides the cellular and extracellular material for primary tumor growth, but also plays a pivotal role in tumor metastasis.

Therefore, we sought to elucidate whether and how adipose tissue derived stem cells might contribute to breast cancer cell invasion, and which mediators could be involved.
After observing that adipose tissue derived stem cells enhance tumor invasion in vitro by roughly doubling the amount of invasive cells, we could identify CCL5 (RANTES) as an important mediator in this process.
Interestingly we found that human breast cancer cells MDA MB 231 induce a de novo secretion of CCL5 by secreted factors, which are still to be identified.
Furthermore, we found that Matrixmetalloproteinase 9 is upregulated in the context of breast cancer cells, interacting directly with adipose tissue derived stem cells. We also showed that hASCs produce more active MMP-9 when being stimulated with CCL5.
We conclude that adipose tissue derived stem cells might represent an important factor in breast cancer metastasis for microenvironment-targeted therapies.

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9. Declaration

Eidesstattliche Erklaerung

Hiermit bestätige ich, Severin Pinilla, bei der Anfertigung der vorliegenden Dissertation keine anderen Quellen als die angegeben verwendet zu haben.
München, den 14.06.2012

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(Severin Pinilla)

10. Acknowledgments

My cordial thanks to Prof. Eckhard Alt, MD for the opportunity to work in his laboratory at the University of Texas, MD Anderson Cancer Center in Houston and Priv. - Doz. Dr. med. Ralf Schmidmaier, MME, for his extraordinary support at the Ludwig-Maximilians-University in Munich.

I gratefully acknowledge Dr. Yao-Hua Song, Ph.D. who guided and supported me throughout my project and critically reviewed all my manuscripts.

Special thanks to all my dear lab colleagues, who shared bench and beer with me and made the year as a graduate research student in Texas an unforgettable experience.

I would like to express my highest gratitude to Xiaohong Leng, Ph.D. who inspired me scientifically and personally during my whole year in Houston.

My sincere and cordial thanks to Mirijam Fries, Till Wagner and Vani Rajendran as critical reviewers of my thesis.

I duly appreciate the administrative co-workers, especially Linda Jackson for making my stay in Houston possible.

My deepest gratitude and warmest feelings for my family and friends, who gave me their kind support and encouragement.

11. Publications

Jotzu, C., Alt, E., Welte, G., Li, J., Hennessy, B.T., Devarajan, E., Krishnappa, S., **Pinilla, S.**, Droll, L., Song, Y.H. (2010). Adipose tissue-derived stem cells differentiate into carcinoma-associated fibroblast-like cells under the influence of tumor-derived factors. Cell Oncol (Dordr). *34*, 55-67.

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12. Addendum

12.1. Figures

12.2. Tables