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Addressing Surgical Uncertainty Surrounding Fat Grafting for Breast Cancer Reconstruction by Creating Novel *In Vitro* Migration Assays

Matthias Alexander Sauter

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Vorsitz: apl. Prof. Dr. Klaus-Peter Janssen

Prüfer*innen der Dissertation:

1. Prof. Dr. Hans-Günther Machens
2. apl. Prof. Dr. Marc Martignoni

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Abstract

The transfer of autologous fat tissue to the breast has emerged as a novel reconstruction method for breast cancer patients. The translocated fat tissue contains Adipose Derived Stem Cells (ASCs); pluripotent stem cells which play a substantial role in the incorporation of the graft through the paracrine promotion of angiogenesis and cell survival. These specific properties of ASCs are potentially of oncogenic risk, by triggering residual breast cancer cells (BC cells) and therefore promoting recurrence. An extensive body of preclinical research postulates that ASCs are inherently oncogenic by supporting BC cells, which is in stark contrast to clinical studies finding no increase of recurrence risk. This drove us to challenge existing *in vitro* models, specifically researching the migratory patterns of ASCs towards BC cells by the creation of a novel assay.

In an *in vitro* assay, silicon chambers were used to seed isolated populations of ASCs and HS-27 fibroblasts together with either benign MCF-7 or malignant MDA-MB-231 BC cell lines in a petri dish. After adherence of the populations, chambers were lifted, the dish filled with media, and cells observed for migratory capacities. Populations were seeded in varying distances from each other, to emphasize trophic tendencies. Migration of ASCs alone towards varying concentrations of BC cells was tested. Cytokine arrays were performed on the supernatant of our novel assay and transwell migration assays for comparison.

We clearly observed a dynamic where ASCs were mobilized by a static population of BC cells, especially towards BC cells of higher malignancy. When the distance between the populations was increased, only migration of ASCs towards MDA-MB-231 cells, the most malignant population used in this study, was traceable. Increase in BC cells numbers seeded enhanced the migratory properties of ASCs, especially towards MDA-MB-231 cells. Cytokine profiling showed higher expression levels of Interleukin 6 (IL-6) and C-C-Cytokine Ligand 2 (CCL-2) in the transwell assay compared to our novel assay.

With this study we have created a new *in vitro* model for the observation of ASC and BC cell migration. We were able to confirm the migratory properties of ASCs towards BC cells, especially of higher malignancy. This was done in the presence of HS-27 cells and over varying distances; confirming a selective migratory impact of BC cells on ASCs, rather than local fibroblasts. We further saw that migration of ASCs is directly

proportional to the number of BCs in the locality. IL-6 and CCL-2, which are both cytokines linked to cancer progression and cell recruitment to the tumor, were more expressed in the transwell assay than our novel one. This shows that the expression of factors linked to oncogenicity is dependent of the modality of the assay used. These data might help in understanding the disparity of preclinical and clinical research on the question of ASC oncogenicity. Compendial assays used to test migratory properties might not be fit to accurately depict ASC BC cell interactions.

Introduction

The modern day perception that plastic surgery is based on aesthetic enhancements is highly misleading, since its roots lie in treating complex traumata and disfigurations. Plastic surgery is a surgical discipline which can significantly enhance and often restore life quality in patients. A historic, groundbreaking example is Gaspare Tagliacozzi's invention of nose reconstruction, by temporarily anastomosing an arm flap to the face, published as early as 1597 (Tomba et al., 2014). Throughout the centuries the treatment of disfigurations still presents a key role in the plastic surgical field of expertise.

One aspect of this endeavor is the surgical reconstruction of the female breast. The most common reason for breast reconstruction being the primary loss of the natural breast through a mastectomy (Dieterich et al., 2017). Mastectomies are a standard procedure performed to treat breast cancer disease (Homsy et al., 2018). Before further analyzing means and ways of reconstruction one must first study and comprehend the breast cancer disease more closely.

Breast cancer

By a share of 31% breast cancer is the most common type of cancerous disease within the female population in Germany. Throughout her lifespan a woman has an 1 in 8 chance of developing breast cancer. This accounts to almost 70.000 newly diagnosed cases in Germany alone in 2016 (Koch-Institut & e.V., 2019). Patient care as envisaged by the latest 2020 German S3 guidelines on the treatment of mamma carcinoma usually involve neoadjuvant chemo- and anti-hormonal therapy. This is always to be followed by a mandatory surgical excision of the tumor. Depending on the tumor biology, hormonal status and the overall stage of disease, surgery is connected to an adjuvant therapy plan involving chemotherapy, radiotherapy and hormonal therapy. The surgical procedure selected may be a local excision, a partial or complete mastectomy (Wöckel & Stüber, 2019). After successfully completing an initial treatment, including full mastectomy, locoregional recurrence events of breast cancer are observed between 4 and 20% of the patients within 12 years (Voduc et al., 2010).

Breast reconstruction

27% percent of breast cancer patients in Germany in 2015 had to undergo a full mastectomy and about a third of these women aimed to get a breast reconstruction procedure (Gerber et al., 2015). The practice of reconstruction has repeatedly shown to improve body image, sexuality, mental health, and the overall quality of life for women qualifying for it (Dauplat et al., 2017; Fanakidou et al., 2018; Santosa et al., 2018).

Reconstruction options are most commonly divided between the insertion of silicone implants or an autologous reconstruction using various options of skin and muscle flaps (DellaCroce & Wolfe, 2013).

Autologous Fat grafting

A novel approach targeted at becoming a mainstay for patients seeking reconstruction is the application of autologous fat grafting. The idea of transferring fat within the body is not a new one. In 1889 the Dutch physician Van der Meulen used autologous fat to treat a diaphragmatic hernia by placing a free omental flap between the liver and the diaphragm (Bellini et al., 2017). Only 4 years later, Gustav Neuber performed the first ever free fat tissue transfer, harvesting fat from the upper arm to a depressed and adherent facial scar sequelae caused by osteomyelitis (Mazzola & Mazzola, 2013). The multitude of qualities fat tissue possesses was already understood by surgeons then. Through its availability, simple harvest procedure, non-immunogenicity, and its biomechanical suitability, autologous fat tissue is the ideal filler material for structural and soft tissue defects (Coleman et al., 2018).
In 2006 Coleman published a report on “structural fat grafting” and hereby founded the “Coleman technique” which led to the rediscovery and new application of fat transfer by plastic surgeons (Coleman, 2006). Coleman describes three vital phases to cut through for successful grafting; the harvest, the processing and the injection. The initial step is the harvest of the fat from an appropriate donor site, most commonly the gluteal region, the abdominal fat depots or the upper thighs. Harvesting is performed via liposuction, whereby a small cannula is placed in the desired depot and the fat is disconnected from the surrounding tissue through the aid of mechanical force (proposed by Coleman), water jets (Taufig, 2006), ultrasound (Duscher et al., 2016) or

laser-assisted liposuction (Goldman & Gotkin, 2009). However acquired, the lipoaspirate is usually afterwards processed, which means the actual fat tissue is separated from other unnecessary fractions; the oily and liquid portion of the aspirate (Girard et al., 2015). The then isolated fat tissue is injected in the desired recipient site. The semi liquid properties of the fat by then allow to administer the transplant via syringes and small cannulas (Coleman, 2006).
This in summary presents further advantages of autologous fat grafting. Fat is, as above mentioned, readily available, the procedure as described only requires minimal incisions for the cannulas in the donor and recipient site, and consequently only slightly scars the patient. The liposuction itself can be performed under local anesthesia and is to be considered as a relatively complication and side effect free procedure (Coleman & Saboeiro, 2007; Raj et al., 2020).

Before discussing the applicability of fat grafting in a post breast cancer setting the limitations of the method must also be addressed.
When Neuber initiated the use of autologous fat transfer, the method over time did not find widespread implementation until the mid 20th century. After the second world war, fat grafts gained popularity amongst others by treating post-traumatic deformities acquired during the first and second world war. (Mazzola & Mazzola, 2013; Raj et al., 2020). More widespread use however highlighted a major limitation of autologous fat grafting. Initially satisfying aesthetic results were compromised by the inability of the grafts to maintain volume long term. This was caused due to high rates of reabsorption of the transplanted tissue. The first favorable long-term results only occurred when in the 1990s Coleman and Chajchir facilitated liposuction for the harvest and Coleman introduced standard operating procedures described above (Chajchir & Benzaquen, 1989; Coleman, 2020). This practice led to more satisfying results in term of graft retention but mainly for small volume grafts (<150ml) as used for procedures such as facial contouring and rejuvenation (Gal et al., 2019). Even so, the issue of graft reabsorption remained. A meta-analysis about the efficacy of fat grafting for breast reconstruction conducted by Herly et al. found that the average number of performed procedures before reaching satisfactory aesthetical results is 2,84 - 4,66 for mastectomy patients and 1,72 for patients after breast conserving therapies (Herly et al., 2018).

To recapitulate, fat grafting has become a valid option to correct structural defects by facilitating natural filler. The main issue though is its highly variable reabsorption rate, which often calls for multiple procedures. Trying to solve this issue has been a topic of research in the last 15 years. But to understand the methods applied to maximize fat graft retention, it is important to explore the cellular structure of fat tissue and the underlying pathogenesis of graft reabsorption.

Fat reabsorption

Observing the process of fat reabsorption reveals the following, as described by Eto et al. Adipose tissue components formerly kept in situ by connective tissue and sustained by blood vessels delivering oxygen and nutrients are disrupted and disintegrated by the process of liposuction. When the harvested and processed fat is transferred into the recipient site, the first few days the graft undergoes an initial phase of hypoxia in which macrophages, histocytes and polynucleated cells are recruited, and phagocytose. They thereby effectively remove the hypoxically damaged fraction of the graft. Right after the transplantation, neovascularization occurs, with vessels sprouting from the healthy host tissue which incorporate the graft. The current literature describes three zones, a slim “surviving zone” on the outside, supplied by vessel-independent oxygen diffusion, a “regenerating zone”, and the necrotic zone (Eto et al., 2012). The bigger the graft, the bigger the necrotic zone, which explains the favorable retention results for smaller volume transplants.
Mature adipocytes, which make up most of the volume of the graft, are known to be extremely sensitive to trauma like sheer stress and moreover, ischemia (Wolter et al., 2005). Preadipocytes, fatty progenitor cells, are much less susceptible to those stressors. Their state as precursor cells allows for a lower metabolic activity and therefore oxygen consumption. These non-adipocytes (which will be more thoroughly explained later on), are possibly the chief surviving cell population in the hostile recipient site and therefore make up for the retained transplant portion finally incorporated (Bellini et al., 2017).

Components of fat tissue

Microscopically, fat consists abundantly of adipocytes containing vacuoles of fatty acids. More precise observation reveals that even though mature adipocytes occupy

most space, they are not the most abundant cells making up the tissue. The largest cell proportion is made of “stromal vascular fraction” (SVF), which consists of an abundance of different cell types such as preadipocytes, fibroblasts, macrophages, pericytes, endothelial cells, endothelial progenitor cells (EPU), neutrophils, lymphocytes and stem cells (Ràfols, 2014). A critical part of the SVF are the adipose derived stem cells (ASC); a fraction of cells defined by their multipotency. They possess the ability to undergo transformation into myogenic, chondrogenic, adipogenic and osteogenic lineages *in vitro* (Zuk et al., 2002). Furthermore, the ASCs are reported to not only serve as a source for adipocyte homeostasis through adipogenic differentiation. ASCs also secrete an abundance of paracrine signaling molecules when cultured *in vitro*. This secretome broadly consists of pro-angiogenic factors like VEGF, PDGF, FGF and HGF, immunomodulatory and proinflammatory cytokines and growth factors, neurotrophic factors and adipokines (Dubey et al., 2018). This distinguishes the ASC with the mesenchymal derived stem cell (MSC), given the ready availability compared to bone marrow derived MSCs (Strioga et al., 2012). What drives the decision of using adipose derived SVF in a regenerative setting are a number of clinically desirable features associated with it. Possibly through the paracrine functions of the ASCs, the SVF is reported to possess pro-angiogenic, antiapoptotic, antifibrotic, immune regulatory and anti-inflammatory properties *in vitro* (Andia et al., 2019).

Cell assisted lipotransfer (CAL)

Fat grafting stands to be a valuable therapeutic option for breast augmentation in various settings. As stated previously, the benefits of fat grafting are compromised mainly by the issue of high and unpredictable fat reabsorption rate (Herly et al., 2018). This reabsorption occurs after necrosis of the transplanted fat due to insufficient nutrition. The SVF, and the ASCs within the fat tissue cell population possess properties which are known to enhance angiogenesis and neovascularization (Andia et al., 2019). In 2007, Yoshimura and colleagues tried to utilize the SVF for better fat grafting outcomes. They hypothesized that the SVF regenerative features could come into effect by increasing the quantity of the SVF within the fat graft. For that purpose, a liposuction was performed, and half of the obtained fat was processed to extract the SVF. The extracted cells were then mixed with the other half of the obtained fat tissue, and this so called “ASC enriched fat” was then injected into the receiving site. Results of this new approach in a limited case study showed favorable cosmetic results within

3 months but more conclusive clinical data was necessary (Yoshimura et al., 2007). More clinical trials have been performed since. Kølle et al. in their double-blind placebo-controlled trial reported of residual volumes of around 80% in CAL compared to their placebo group with conventional fat grafting and retention rates of 16% at 121 days after injection (Kølle et al., 2013). A conducted meta-analysis and systematic review of CAL demonstrated improved efficiency over conventional fat grafting, although both call for bigger and more conclusive clinical trials before implementing it into regular clinical practice (Toyserkani et al., 2016; Zhou et al., 2016).

They also emphasize on the yet to be fully understood underlying mechanism of how exactly the ASCs or SVF promote their regenerative potential. Different explanations have been proposed. An early idea was the differentiation of the preadipocytes into adipocytes, which might help sustain volume of the graft with newly created adipocytes which could then replace the old adipocytes that are lost due to the hypoxic conditions in the graft site. This mechanism has been observed *in vitro* but not confirmed *in vivo* (Bora & Majumdar, 2017).

A more plausible option would be the stabilization of the graft through the direct function of the SVF/ASCs. This could be through differentiation of fractions of the SVF itself into endothelial cells that would be capable to form neovasculature to support nutrition. Freiman and collegues for example observed human derived ASCs co-cultured with “human adipose microvascular endothelial cells” in a 3D scaffold to form neo vessel networks (Freiman et al., 2016). This again highlights the importance of the SVF secreting paracrine signals that promote that neovascularization. Garza et al recorded gene expression in human ASCs which were added into human fat grafts transplanted into immunocompromised rodents. They found the expression of genes for BMPR2, CD90, CD105, FGF2, CD248, TGFβ1, and VEGF-A, all associated with neovascularization (Garza et al., 2015). Worthy of mention in this context are also the hypoxic conditions in the graft site, which augment the aforementioned properties of the SVF. SVF cultured *in vitro* under hypoxic conditions showed an increase in their ability to proliferate. Furthermore, the levels of expressed mRNA of VEGF and FGF2 were increased (Kakudo et al., 2015), a finding confirmed *in vivo* by Mytsyk et al. The authors seeded SVF cells on collagen sponges, expanded them under severe hypoxia (<1% oxygen concentration) and subsequently implanted the sponges into

immunocompromised nude rats. They later measured cell survival and gene expression. Data after *in vitro* expansion showed no alteration in SVF survival and increased expression of VEGF and the number of proliferating cells. The collagen sponges with hypoxic SVF showed vascular networks after 28 days post implantation which were longer and denser then the normoxic control group (Mytsyk et al., 2021).

Application of AFG in oncoplastic surgery

In conclusion then, the transfer of autologous fat is by now well studied treatment option for soft tissue defects of various origins. Beyond its many advantages the main disadvantage remains the unpredictable reabsorption rate of the graft after the transplantation. This is the issue that CAL aims to diminish, if not eliminate. The addition of isolated SVF to the fat graft improves the incorporation of the tissue into its new site and improves retention. This is very likely to be possible due to the paracrine functions of subpopulations within the SVF. They appear to possess a secretome that enhances neo-vascularization and therefore ensures an improved nutrition that allows the graft to incorporate into the recipient site. This effect is probably boosted by the hypoxic conditions in which SVF show enhanced proliferation and increased pro-angiogenic gene expression.

Fat grafting therefore seems to be a perfect surgical procedure to be incorporated in the treatment plans of post mastectomy patients. Yet there remains opposition within the ranks of reconstructive breast surgeons as well as scientists about using fat grafting for this oncological setting.

Prooncogenic effect of ASCs

Since first being described in 2001 by Zuk (Zuk et al., 2002), ASCs have been studied and grown in relevance for their pro regenerative potential, using them for various therapies (Zuk et al., 2001). This went hand in hand with questioning and researching whether this regenerative potential also proves to be a beneficial factor for malignant cells. ASCs have been studied *in vitro* and *in vivo* with all variants of cancer types such as melanoma, lung cancer, ovarian cancer, hepatocellular carcinoma, glioma and glioblastoma, amongst others (Scioli et al., 2019). This work will be focusing on the

research investigating the effect of ASCs and breast cancer cell populations on each other.

In these *in vitro* studies various cell types are used, a few of which are to be explained here for a brief overview. In general, breast cancer is not a single disease but rather a heterogenous group of different distinct tumors that can be categorized through various classification systems. Means to distinguish are through histology (Sinn & Kreipe, 2013), tumor progression (Giuliano et al., 2018) or the hormonal receptor status of breast cancer cells. Here there are three receptors that have been identified to play a role. The estrogen receptor (ER) , progesterone receptor (PR), and HER2/neu receptor (human epidermal growth receptor 2) are prognostic as well as predictive factors (Allred, 2010). A negative receptor status for ER and PR was found to be linked to an increased mortality risk for patients (Dunnwald et al., 2007). This distinctive feature of breast cancer is accounted for in two of the most abundantly used breast cancer cell lines, MCF-7 and MDA-MB-231. MCF-7 is ER and PR positive cell line which was isolated from a primary breast adenocarcinoma of a 69-year-old patient. The cell line is poorly aggressive and non-invasive with a low metastatic potential (Gest et al., 2013).
MDA-MB-231 in contrast, is a cell line isolated from a metastasis of a ER, PR and HER2/neu negative adenocarcinoma of a 51 year old patient (Lacroix & Leclercq, 2004). With this lack of all three clinically important receptors this cell line represents “triple negative” breast cancers. This type of cancer is known to be a highly invasive, aggressive, and poorly differentiated. Its invasiveness *in vitro* translates to a high capability to form metastasis *in vivo* (Chavez et al., 2010). These cell lines exist amongst various others but have been widely used in basic breast cancer research since they were established. MCF-7 and MDA-MB-231 together with T-47D account for more than two-thirds of all breast cancer cell mentions in abstracts scanned in a Medline survey (Lacroix & Leclercq, 2004).
The following section will be dedicated to describing different features of ASCs that have been deemed prooncogenic.

Number 1 - ASCs (SVF) promote Angiogenesis

It is known that in order to properly sustain themselves, expand and metastasize, tumor cells are dependent on a vasculature network. These networks provide nutrition for the

tumor mass to grow and expand and provide tracks for segregated tumor cells to relocate within the body and metastasize (Nyberg et al., 2008). This process of vessel formation has been described to be accelerated in the presence of ASCs (Kakudo et al., 2015; Mytsyk et al., 2021). Clues strengthening this hypothesis are delivered by various studies. Razmkhah et al. compared the expression of VEGF in ASCs of fat tissue taken from excised breast tumors and from fat tissue of matched controls. They found a 2-fold increase of VEGF in the tumor derived fat tissue (Razmkhah et al., 2010). Lalala et al. isolated a c-Kit positive subpopulation of ASCs from Balb/c mice and co-injected them with 4T1 breast cancer cells. They found an increase in tumor mass compared to the tumors grown from 4T1 injected cells alone. ELISA test assays *in vitro* and *in vivo* showed enhanced concentrations of IL-1 (Interleukin-1), CXCL-12 (Stromal-Cell-Derived-Factor-1) and VEGF-A, all cytokines linked to angiogenesis (Li et al., 2017). Other findings like the ones by Orecchioni and her colleagues suggest that not only ASCs but other subpopulations of the SVF like endothelial progenitor cells (EPC) play into this prooncogenic effect. They found that in murine breast tumor models EPCs migrate into lymph nodes in the proximity of the tumor mass and locally stimulate the formation of neo-vasculature (Orecchioni et al., 2013).

Number 2 - ASCs promote Epithelial to Mesenchymal Transition (EMT)

Another aspect of tumor biology that seems to be affected by the ASCs is the EMT of the tumor cells. This describes the shift of carcinoma cells from their epithelial origin towards more mesenchymal traits, such as an enhanced ability for cell mobility. This is known to contribute to a change of the tumor to a more invasive and malignant subset (Campbell, 2018). Ritter et al. conducted a study where they cultured MCF-7 breast cancer cells with ASCs in a transwell system with no direct cell to cell contact but both cell types sharing the same medium. Over a course of 14 days they observed a changing of morphology of the MCF-7 cells. Cells lost their epithelial polarity and changed into a more mesenchymal appearance. Through immunofluorescence staining of the cancer cells a molecular change from the epithelial marker E-cadherin to the mesenchymal markers N-cadherin and vimentin was shown. Inhibition of the PI3K pathway inhibited this process hinting on an involvement of this pathway (Ritter et al., 2015). Findings like these have been made for other breast cancer cell lines like SKBR3 which also showed a mesenchymal like change of morphology when co-cultured with ASCs which was inhibited by pharmacologically blocking the PIK3 and

MAP pathways (Kucerova et al., 2013). Another study observed ASCs co-cultured with estrogen sensitive breast cancer cells to an EMT and metastasis linked secretome of SERPINE1, MMP2 and Interleukin 6 (IL-6). This was inhibited by knockdown of leptin, postulating the involvement of this hunger hormone into breast cancer EMT (Strong et al., 2015).

Number 3 - ASCs change the microenvironment

Another prooncogenic change ASCs have been linked to is the alteration of the tumor microenvironment (TME). Ramskah and colleagues observed that ASCs isolated from breast cancer patients produce a secretome which alters the T-cell regulation and therefore boosts local inflammation which serves to protect breast cancer cells from an immune response (Razmkhah et al., 2011). Other studies highlight that observed secretomes of the ASCs seem to have a beneficial effect on the proliferation of breast cancer cells. Trivanonović et al. found that ASCs obtained from healthy fat tissue and from fat tissue adjacent to breast cancer tumors both improved the proliferation of co-cultured hormone sensitive breast cancer cell lines. This effect was not observable using only the conditioned medium of these ASCs, driving the authors to link direct cell to cell contact to this pro-proliferative effect (Trivanović et al., 2014). Others found that proliferation was increased for different breast cancer cell lines when they were brought into contact with the conditioned medium rather than direct co-culture (Kucerova et al., 2011).
These studies are but an example of the current literature which most endorses this idea of an altered tumor microenvironment through ASCs. While this cross talk between cancer and stem cells is not yet fully understood it is acknowledged that different chemokines, cytokines and hormones contribute dynamically into a microenvironment favorable for tumor progression (Cammarota & Laukkanen, 2016). There are factors like TGF-ß or IL-6 which have been repeatedly described to be present in BC cell/ASC assays and are clearly linked to tumor progression and a change into more malignant cancer types (Schweizer et al., 2015).

TGF-ß is very well described and a neat example of the theory that ASCs are sometimes titled as a “double edged sword”. TGF-ß is known to play a crucial role in wound healing, mediating collagen deposition and the remodeling of extracellular matrix in tissue defects (Gadelkarim et al., 2018). This effect, favorable in healthy

tissue yet also seems to affect breast cancer cells. For example, TGF-ß secreted by cancer associated fibroblasts (CAFs) is described to promote EMT (Yu et al., 2014). Other reports tell of tumor-derived extracellular vesicles (TEVs) which are secreted by a triple negative highly malignant breast cancer cell line. When these were mixed with ASC cultures, the cells showed higher secretion of VEGF, and a switch to a myofibroblastic phenotype. This process was found to be controlled by TGF-ß related signaling (Song et al., 2017).

Number 4 - ASCs change into cancer associated fibroblasts

Several studies investigate a change of phenotype the ASCs exhibit when they are met with a breast cancer environment. Specifically, they report of the switch of the ASCs to so called cancer associated- or tumor associated fibroblasts (TAF) (Cho et al., 2012; Jotzu et al., 2011). This phenotype seems to then contribute to an increase tumor proliferation. The alteration might be induced by the Wnt-pathway which was proven through inhibition of the same (Visweswaran, Keane, et al., 2018).

Number 5 - ASC homing

A well described ability of ASCs is their homing towards tumor sites. ASCs are able to accumulate at the tumor from even distant body areas through blood circulation. This has been described for MSCs and ASCs respectively *in vivo* in rodent models (Karnoub et al., 2007; Zhang et al., 2009). The reason for this is explained by the similarity of the tumor microenvironment with an inflammatory site, like a tissue defect (Chulpanova et al., 2018). A multitude of inflammatory cytokines have been described to play role in this. For example, IL-6 is apparently responsible for the recruitment of ASCs into the hypoxic tumor environment (Rattigan et al., 2010). Furthermore, there are reports that breast cancers are even able to remotely mobilize MSCs and ASCs from the bone marrow and fat tissue and home them to the cancer site. Recruited MSCs differentiated into tumor associated fibroblasts (TAFs) while the ASCs that were incorporated into the tumor stroma differentiated into vascular and fibroblast-like structures (Kidd et al., 2012). In the last years, scientists have aimed on facilitating this homing effect for therapeutic purposes. The idea was that ASCs could be used as carriers for agents that have a noxious effect on the tumors. For example, ASCs can be loaded with Paclitaxel (PTX). This anti-tumoral drug inhibits the construction of

tubulin structures therefore stalling mitosis and having an anti-proliferative and anti-angiogenic effect on the tumor (Howat et al., 2014). When PTX loaded cells were cultured and their conditioned medium was tested against cell lines of osteosarcoma, leukemia and prostatic carcinoma they found it to negatively affect cell proliferation of these cancers (Bonomi et al., 2013). A more elaborate approach is the crafting of nanoparticles which were loaded with PTX and then injected into MSCs. These were then used in a rodent model. They showed that these loaded ASCs were able to significantly suppress the growth of MCF-7 tumors (Wu et al., 2016). This drug loading is far from the only approach that has been studied. Nanotechnologies, drug loaded exosomes or the secretion of micro RNA all have been reported to negatively affect tumor progression in various cancer models (Gentile & Garcovich, 2019).

Number 6- ASC migration/tropism

The chemoattraction breast cancer cells create for ASCs and the SVF has been described various times *in vitro*. A study from 2010 uses a two-layer migration assay to determine ASC migration towards tumor conditioned medium containing platelet derived growth factor (PDGF). A well was filled with the conditioned medium and an insert with 3µm pores was placed into it with ASCs seeded on top. After 8 hours cells were fixed on the bottom site of the porous insert and stained. The authors saw that cell migration of the ASCs was significantly enhanced by the conditioned medium and even more so for more malignant cancer cell lines (Gehmert et al., 2010). Based on these findings, further research was conducted by the same group. They found the Phosphoinositide 3-kinase (PI3K) inhibitor to mediate the secretion of PDGF by breast cancer cells. This significantly enhanced the migration of the ASCs when they were brought in contact with conditioned medium containing the isoform PDGF-BB in the same two-layer migration assay described above (Salha et al., 2018). Other groups like Koellensperger et al. have confirmed these findings using the same transwell system with slight alterations in pore size of 8µm and a co-culture time period of 24 hours (Koellensperger et al., 2017). This attraction of ASCs towards breast cancer cells has been described in other assays as well. For example, some groups have used wound healing assays. Here two cell populations are seeded in one well and are distanced from each other with a normalized spacer. Once cells have been allowed to grow, the spacer is removed, and the growth and closure of the gap are observed. When running the wound healing assay, Ritter et al. saw the ASCs showing an

increased number of protrusions when seeded opposite of breast cancer cell line MCF-7 (less malignant) and MDA-MB-231 (highly malignant). This directional growth of the ASCs towards the breast cancer cells is called tropism, rather than a free movement of singular cells which would be labeled migration (Ritter et al., 2015).
This attraction of ASCs towards breast cancer cells doesn't necessarily translate into an inverse attraction. Teufelsbauer et al. seeded breast cancer cell line MDA-MB-231. After they reached confluence, a scratch was put into the wells with a plastic tip and the medium was changed with either regular fresh medium or SVF-conditioned medium and the gap was monitored until it closed. The authors did not observe a significant difference in closure time between media (Teufelsbauer et al., 2019). Another example would be the above-mentioned study of Koellensperger. Here the migration assay that was performed using the transwell approach saw an increased migration of some cancer cell lines. MCF-7 and MDA-MB-231 showed a slightly increased migration when seeded on top of ASCs (Koellensperger et al., 2017). Notably, the literature tells of a much more pronounced migration of ASCs towards BC cells than vice versa.

Inhibitory effect of ASCs on breast cancer

The reports on the interaction between ASC/SVF and breast cancer cells *in vitro* are conflicting. Apart from the many reports on pro-oncogenic properties there are several studies as well proclaiming an inhibitory effect of the stromal fraction of the adipose tissue on breast cancer cells. One study used conditioned medium of ASCs and combined it with MCF-7. They performed simple cell viability assays on those cells after a period of culturing with this conditioned medium. What they saw was a decrease in viability for BC cells cultured with ASC conditioned medium rather than unconditioned medium (Visweswaran, Arfuso, et al., 2018). Another study conducted in 2014 similarly found that MCF-7 cells showed a decrease in viability when cultured in ASC pre-conditioned medium. The effect was proportionate to the number of ASCs that were used to condition the medium. When they used transwell assays to investigate if viable ASCs in the top-well would have an effect on MDA-MB-231 and MCF-7 cells in the bottom-well, they found a higher rate of apoptosis in both breast cancer cell lines measured by flow-cytometry (Ryu et al., 2014). Similar results using conditioned medium was obtained by other groups. Wu and colleagues exposed epithelial cell lines derived from healthy female mammary gland breast tissue to the

conditioned medium. The decrease in viability was not observed for the healthy cell lines as it was for the cancer cells (Wu et al., 2019). There are also papers describing both prooncogenic and tumor suppressive effects of ASCs. Kucerova observed the effect of ASCs on the BC cell line SKBR3. On the one hand they found that co-culture with ASCs led to an epithelial to mesenchymal transition with is linked to tumor progression. On the other hand, they saw that direct co-culture or culture of the tumor cells with ASC conditioned medium both led to a decrease in proliferation of the tumor cells (Kucerova et al., 2013).

Summary of in *vitro*/*in vivo* studies

To summarize here, the research that has been conducted *in vitro* and *in vivo* on the role on SVF/ASCs on breast cancer cells is conflicted. Many authors, considering the pro-tumorigenic results they obtained, call for caution in facilitating fat grafting and especially cell assisted lipotransfer. The authors also quote the lack of comprehensive clinical studies (Koellensperger et al., 2017; Ritter et al., 2015).

Clinical data on oncological safety of AFG/CAL

Ever since fat grafting has been reintroduced into the surgical world of reconstruction, physicians investigated its safety. In 2009 the American Society of Plastic Surgeons (ASPS) already published a joint statement in which they attested the autologous lipotransfer to be a safe method; not increasing the risk of tumor recurrence (Gutowski, 2009). The current German guideline published by the Deutsche Gesellschaft der Plastischen, Rekonstruktiven und Ästhetischen Chirurgie (DGPRÄC) in 2015, states that there is no evidence to be found of fat grafting in healthy participants causing breast cancer. However it does infer that autologous fat grafting creates an increased risk for locoregional recurrence of breast cancer in a post-cancer setting (Broelsch et al., 2017). A new guideline is currently being written and expected to be released in 2023.

Nevertheless, in the last years a multitude of clinical studies have been conducted further investigating this oncological safety. Initially, patient cohorts were looked at from a retrospective point of view. For example, Petit et al. in 2012 published data where they examined 321 patients which received either a breast conserving therapy (BCS) or a mastectomy (MST) and afterwards a reconstruction via fat transfer.

Together with a matched control group they followed up on these patients for 28 months and could not find significant differences in the prevalence of either local recurrence (LR) or distant metastasis (DM) between groups. A more thorough analysis found a significantly increased risk for LR within a subgroup of *in situ* cancers (Petit et al., 2012). A following retrospective study by Petit and his group focused on this subset of patients with intra-epithelial neoplasia and could indeed confirm a higher risk of LR (18% case group vs. 3% control group) (Petit et al., 2013). Although limitations were the small number of enrolled patients and the retrospective design these were the first clinical studies to report on an oncological safety issue with autologous fat grafting. In 2017 however, the group around Petit stated, that when they analyzed the same subset of patients for recurrence rates with a longer follow up, the statistically increased risk of cancer recurrence after AFG was no longer observable (Petit et al., 2017). Many of these retrospective analyses were conducted which led to more brought observations done through meta-analyses.

In 2018 Krastev et al. included retrospective studies into their analysis and specifically questioned the occurrence of locoregional recurrences (LRR) after AFG. Having investigated studies with collectively over 4000 patients, by then the meta-analysis with the highest number of included patients, they found no increase in LRR after AFG in these studies (Krastev et al., 2018). A second meta-analysis was performed by Li et al. in 2021 on the question of BC recurrence after AFG, specifically LRR and DM. The studies included into the analysis were retrospective matched cohort studies. Patients were matched for various prognostic factors such as age, size of the tumor, histological properties, date and method of surgery, tumor staging, lymph node affliction and importantly receptor status of estrogen, progesterone and HER-2/neu expression. AFG didn't increase the probability of LRR or DM in the meta-analysis of all 17 studies with 7494 included patients. Apart from the limitation of the inferior retrospective design of these studies the authors also point to the time-period that was observed as a major drawback. The mean follow-up time after surgical tumor removal was 74.9 months and after fat grafting 43.1 months (Li et al., 2022). A retrospective analysis carried out in 2016 investigated the time dynamic of breast cancer recurrence of 3000 women. They found that 40% of cancers recurred after more than 5 years post-diagnosis (Wangchinda & Ithimakin, 2016). Krastev et al. discuss, that in theory, regenerative and therefore possibly oncogenic effects of the ASCs within the fat graft should take place in the first few months or a year after grafting. This line of thought makes it

unclear whether recurrence multiple years after grafting could be attributed to the fat grafting procedure in the first place. Still, the lack of data prohibits any informed clinical assertion on the safety of AFG (Krastev et al., 2018). The same issues present themselves with retrospective studies that performed the AFG procedure immediately after the MST or BCS. Two studies performed in 2017 and 2018 reported a follow up of 36 months and 40.8 months respectively (Biazus et al., 2018; Khan et al., 2017), both not crossing the 5-year mark.

It is important to distinguish that these studies focus on the question on the oncogenic risk factors of regular autologous fat transfer. The question remains about the safety of cell assisted lipotransfer. In Yoshimura's original paper the concentration of ASCs in CAL-fat is twice as high as the concentration of ASCs in regular, non-enriched fat (Yoshimura et al., 2007). In more recent protocols for CAL, autologous ASCs are expanded *in vitro* before being mixed with the fat graft. ASC concentrations of these grafts are 2000 times higher than the physiological level of ASCs found in regular fat tissue (Kølle et al., 2013).

The only noteworthy clinical study to determine the oncological safety of CAL was the RESTORE-2 trial. In this multicentric prospective single arm trial CAL was tested on 71 patients. They were enrolled with a median time period of 51.6 months after their primary BC diagnosis. CAL was used to correct tissue defects created through either breast conserving therapy or radiation therapy. Half of the harvested volume of fatty tissue was used for enzymatic processing and collection of SVF, such that the SVF concentration in the final graft was roughly doubled. After a follow up period of 12 months MRI scanning was performed in which 54 out of 66 patients showed an improved result in breast contour. Oncological safety was determined by MRI scans 6 and 12 months after treatment. There were no signs of local recurrences found (Pérez-Cano et al., 2012). This study remains the only clinical approach to evaluate the oncological safety of CAL to date. Although no cancer recurrences were detected this study presents not merely enough evidence to determine the question at hand. Limitations are the lack of a control group, the relatively low number of participants and not least of all the short time of oncological follow up. Moreover, the sole focus on local recurrence might be concealing any systemic recurrence in the form of metastasis. A systematic review on the state of clinical CAL trials already conducted in 2016 found 11 studies that were performed with only three of them incorporating a control group. And only one of the trials (RESTORE2) raises the question on oncological safety. An

overview of larger clinical trials that wanted to test the safety and efficiency of CAL was presented, many still in the early stages of recruitment but some already ongoing. Up to date there still is a lack of conclusive high-quality data assessing oncological safety of the procedure. Kølle et al. published a randomized controlled trial researching the efficiency of CAL using *ex vivo* expanded ASCs and compared it to regular AFG. While they saw promising higher retention rates in the CAL group compared to the AFG group 4 months after the procedure (80,4% to 45,1%), oncological safety was not evaluated. They nevertheless debate on the issue and argue that with the clinical safety of regular AFG the assumption could be made that this also applies for CAL (Kølle et al., 2013).

Surgical approach of fat grafting for breast reconstruction

Where does that now leave the issue? On the one hand there are countless examples of *in vitro* (Koellensperger et al., 2017) and *in vivo* (Zhang et al., 2009) studies showing in their study setups a definitive prooncogenic effect of SVF/ASCs on breast cancer tumors. These papers are calling for rigorous clinical research to be performed to rule out the likelihood of cancer recurrence promotion. Furthermore, some call for a pre-operation discussion to be had with the patient to advise on potential oncological risks of AFG (Koellensperger et al., 2017). Other papers showing preclinical findings, are not able to show an effect of SVF/ASCs on BC cells they deem of clinical importance. Teufelsbauer et al. claiming ASCs used for AFG are not expected to alter tumor progression or recurrence (Teufelsbauer et al., 2019). Clinical data that has been provided, especially on conventional AFG does not achieve the gold standard of double-blind placebo controlled trials. The large retrospective meta-analyses that were performed on the issue attest oncological safety of the procedure (Krastev et al., 2018; Li et al., 2022; Tukiama et al., 2021). Large reviews that discuss the matter acknowledge the lack of clinical evidence of AFG causing recurrence, but also warn of the strong implications that preclinical papers make on the prooncogenic influence of ASCs, namely the promotion of EMT and invasiveness (Scioli et al., 2019). In 2013, a survey was sent out to the American plastic surgeon community aiming to gain insight into how often physicians apply AFG, and their thoughts and concerns about it. While 62% of the participants claimed they used AFG for breast reconstruction, 50% strongly agreed that the non-resolved question of oncological safety remains an issue to implement AFG further into their clinical practice (Kling et al., 2013).

A novel approach on researching the issue of AFG oncological safety

If the preclinical findings are not translatable in clinical trials, one logical deduction would be that either the preclinical or the clinical trials are flawed in their setup, giving results which are not entirely accurate. The point has been made that for the clinical side, even huge retrospective cohort analysis might not be fit to determine the safety of AFG for patients (Krastev et al., 2018). Li et al. in their meta-analysis claim that an inherent limitation of the retrospective studies they investigated is that despite extensive matching, confounders like different surgical modalities, or varying histological characterization of the tumors cannot be entirely be corrected, and therefore might invalidate the results (Li et al., 2022). A definitive conclusion could probably only be drawn through high quality double-blinded placebo controlled clinical trials, but these are immensely difficult to initiate. It is argued that one of the main issues regarding the acquisition of clinical data is the difficulty in designing high quality clinical trials in the first place. Since AFG fundamentally differs from other reconstruction methods like the free or rotating flap surgery, or the implantation of silicon, one cannot really name an adequate control group and therefore a randomized controlled prospective trial is hard to establish (Krastev et al., 2018).

In vitro assays measuring migration

All this considered, there is value in reviewing the preclinical studies more carefully and critically. As described earlier, one key finding in preclinical *in vitro* and *in vivo* studies is the migration of ASCs towards breast cancer cells. Cell migration is the metric which many studies aim to analyze, and have used to infer oncogenicity. These investigations typically set up and observe migration *in vitro* using a transwell assay. These assays are comprised of a plastic well in which cells are seeded. Into that well, a plastic insert is put which provides a membranous surface for another type of cell to be seeded. The surface of the insert contains small pores of variable diameter. The assay is performed by letting well and insert sit for a certain amount of time together whilst medium is shared. During that time, the cells seeded in the insert can move through the pores to the bottom surface of the insert. Eventually the insert is put out of the well and the cells that migrated through the pores are counted via microscopic imaging (Senger et al., 2002). This assay was used by several authors to depict migration of ASCs towards different breast cancer cell lines (Gehmert et al., 2010;

Koellensperger et al., 2017; Salha et al., 2018). The migration that is perceived is unidirectional and the migratory properties of only one cell type at a time can be studied while the other cell type in the bottom well remains static.

Another approach of measuring cell migration *in vitro* are scratch assays. Here a cell type is cultured to high confluence. Afterwards a pipette tip or other sterile object with a defined diameter tip is used to "scratch" over the well in a straight line. This line disturbs the confluence of the cells and the closure time of the newly created gap can then be measured microscopically (Liang et al., 2007). An evolution of this method was the engineering of silicon chamber implants. These small structures could be adhered in a watertight manner onto the base of a dry cell culture surface. The implant consisted of silicon walls that created two chambers, the two chambers were separated by a straight wall with an exact predefined thickness. The same, or two distinct cell types could be seeded in the two chambers and after reaching confluence the chambers were removed from the well. The wall between the two chambers left a cell-free imprint on the well with a known length and closure of that imprint can be measured through imaging. The silicon chambers therefore were created to provide a more standardized way of conducting scratch assays (Huang et al., 2019). With regard of our method, Ritter et al. used the chambers to seed ASCs opposite of BC cell lines with a cell free gap of 500µm. After lifting the chambers the investigators found protrusions of the ASCs directing towards the BCC and interpretated that for ASC homing towards breast cancer (Ritter et al., 2015).

Transwell assays and scratch/silicon chamber assays are well studied and established ways to investigate migration. Consequently, they are the setups utilized to observe the patterns of movement of ASCs and BCC *in vitro*. Results of these studies confirm this attraction of ASCs towards BCC or BCC conditioned medium (Koellensperger et al., 2017). Our goal in designing this study was to question the status quo on this specific field or research. We hypothesized that the reliance on these limited assays to depict migration presents an issue. It might be that focusing on unidirectional migration through transwell assays poorly illustrates the real cell-cell relationship. After grafting, the populations of residual breast cancer cells, *and* ASCs introduced to the site by grafting, theoretically have the option to migrate. It is an important consideration in *in vitro* experimental design to facilitate unlimited, free cell migration, while still maintaining assay integrity and quantifiability. Finally, an assay that only takes into account two different cell types is a stark simplification of the *in vivo* situation. It is

known that fat tissue consists of a multitude of different cell types (Ràfols, 2014) as described above. These cell types also have a potential to influence ASC and BCC migration.

Creation of a novel assay more “organically” depicting patterns of tropism

We designed a set of experiments to take these theoretical considerations into account. The assays we created are meant to depict multidirectional migratory patterns between ASCs, fibroblasts and BCC lines of varying malignancy. The setups were chosen to provide a setting for the different cell types that more closely mimics the clinical conditions found in the oncological application of AFG. By creating those assays we aimed to increase the knowledge of how ASCs interact with BCC and therefore shed some more light into the question of their true oncogenic potential.

Materials and Methods

Cell procurement, culturing, and passaging

The following breast cancer cell lines were purchased from ATCC: MDA-MB-231 (estrogen receptor negative, progesterone receptor negative and HER2 receptor negative, ATCC® HTB-26™), MCF-7 (estrogen receptor positive, progesterone receptor positive, HTB-22™). Fibroblast cell line HS-27 was purchased from Sigma Aldrich (cat# 94041901-1VL). Human adipose derived stem cells were acquired from Poietics™ (donors 29635, 31363, cat# LO PT-5006). MDA-MB-231 MCF-7 and HS-27 cell lines were set into culturing flasks with the addition of Dulbecco's modified Eagle's medium (DMEM, Biochrom), 10% Fetal Bovine Serum (FBS, Biochrom) and 1% of antibiotic and antimycotic supplement (Penicillin G, Streptomycin and Amphotericin C), (Capricorn Scientific). For culturing of ASCs, Stem MACS™ MSC Expansion Media (Miltenyi Biotec) was used with the addition of 1% of antibiotic and antimycotic supplement (Penicillin G, Streptomycin and Amphotericin C), (Capricorn Scientific). Medium changes were performed every 2-3 days and cell cultures were monitored until reaching 80 to 90% confluence and then passaged. Passaging was performed with 0.25% trypsin and EDTA (Biochrom). For culturing, cells were kept in incubators set at 37°C in a humified atmosphere that containing 5% CO_2 (Sauter et al., 2019).

Cell staining

Before experimental set up cells were fluorescently stained. MDA-MB-231 cells were labelled with Mitotracker CMXRos (Thermo Fischer). ASCs and HS-27 cells were labelled with Mitrotracker Red Green FM (Thermo Fischer). Stains were performed according to manufacturers protocol. In brief, the stock was dissolved in DMSO with a 1nM concentration. The stock solution and culturing medium of the different cell types were mixed in a ratio of 1 to 2000 (CMXRos) and 1 to 10.000 (Red Green FM). The final concentration achieved was 500nM. The cells were cultured in the stained medium for 4 hours. Before experiments, cells were washed and then placed for respective *in vitro* co culture setups (Sauter et al., 2019).

In vitro cell tropism models preparations

The four different cell types (ASCs, HS-27, MDA-MB-231 and MCF-7) were arranged in various formations in individual colonies, in one well of a 6 well plate. The various setups are schematically depicted in **Figure 1**.

Joined experimental preparations

Before culturing, silicon spacing chambers (Ibidi) were attached to the bottom of the plastic well. Cells were lifted from their culturing flasks using 0.25% trypsin and EDTA (Biochrom). Cells were then manually counted using Neubauer counting chambers. Cells were put into their respective silicon chamber in a defined quantity dissolved in 70µl of DMEM. They were given 12 hours in a 37°C humidified incubator with 5% CO_2 for attachment and growth before the chambers were gently lifted using forceps (Sauter et al., 2019).

Assay 1

Three silicon chambers were placed on the bottom of 1 well of a 6 well plate. The middle chamber contained 10.000 cells of either MCF-7 or MDA-MB-231. In both cases, ASCs were seeded perpendicular to the right of the central chamber, HS-27 cells were seeded perpendicular to the left, both in a concentration of 12.000 cells, as can be seen in **Figure 1a**. Breast cancer cells were stained in advance with CMXRos and ASC and HS-27 cells were stained using Red Green FM. After incubation time of 12 hours silicon chambers were lifted. That left a gap of 1000µm to either side of the central BC cell colony. The well was filled with 2ml of DMEM and Fluorescence-Imaging of both gaps was performed in 24 hour time distances until gap closure was achieved. Fluorescent images were analyzed through pixel densiometry (Image J) (Sauter et al., 2019).

Assay 2

Three silicon chambers were placed on the bottom of 1 well of a 6 well plate. They were arranged in a triangular shape as can be seen in **Figure 1b**. Breast cancer cells (MCF-7 and MDA-MB-231) were placed in the silicon chamber on top in a concentration of 10.000 cells. The silicon chamber containing the HS-27 population was placed in an 70° angle below the BC population with a distance of 15mm to it, in a concentration of 12.000 cells. The silicon chamber containing the ASC population

was placed in an 120° angle below the BC population with a distance of 15mm to it, in a concentration of 12.000 cells. Breast cancer cells were stained in advance with CMXRos and ASCs and HS-27 cells were stained using Red Green FM.
After a 12 hour attachment period chambers were lifted for all three populations. The well was then filled with 2ml of DMEM. Fluorescence images were taken of the top right corner of the HS-27 population and the top left corner of the ASC population at 0, 12 and 24 hour timepoints (Sauter et al., 2019).

Assay 3

Two silicon chambers were placed on the bottom of 1 well of a 6 well plate. Each chamber was placed on either side of the well. The top chamber contained either MCF-7 or MDA-MB-231 cells with the following concentrations: 1.000, 10.000 and 100.000 cells. Each time the bottom well was seeded with 12.000 ASCs. As can be seen in **Figure 1c**. Cells were given a 12h period to attach to the bottom of the well and afterwards the chamber containing the ASCs was lifted while the chamber containing the BC cells remained in place. The well was then flooded with 2ml of DMEM such as to overflow the brim of the silicon chamber containing the BC cells. Brightfield images were taken of the edge of the ASC population facing the BC cells at timepoints of 0 and 24 hours (Sauter et al., 2019).

Assay 4

For probing of paracrine factors influencing the cells tropism a human cytokine array (C5, RayBiotech) was performed. Two different experiments were set up for direct comparison.
For the first experiment, two silicon chambers were placed on the bottom of 1 well of a 12 well plate. Each chamber was placed on either side of the well. One chamber contained MDA-MB-231 cells in a concentration of 15.000cells. The other chamber contained ASCs in a concentration of 20.000 cells. Cells were given a 12h period to attach to the surface. Afterwards the silicon chamber containing the ASCs was lifted while the chamber containing the MDA-MB-231 cells stayed in place. The well was filled with 1,5ml of DMEM containing 1% FBS and was incubated for 48 hours.
For the second experiment, Boyden Chamber (Sigma Aldrich) culturing inserts were purchased with a pore size of 8µm. On the bottom of the well MDA-MB-231 cells were

seeded in a concentration of 15.000 cells. In the chamber on top ASCs were seeded in a concentration of 20.000 cells. The well was filled with 1,5ml of DMEM with 1% FBS. As such the cells were incubated for 48 hours. A group of 15.000 MDA-MB-231 cells incubated for the same time with 1,5ml of DMEM, with 1% FBS serving as a control.

All samples within each experiment had three replicates. After 48 hours 330ul of each sample of each respective experiment was taken and pooled to make 1ml of test solution per experiment. The solution was used to perform the blot according to manufacturer's instruction. Blot measurement was done using BioRad ChemiDoc™ MP Imaging System. Exposure time lasted for 15 seconds. Images were analysed using pixel densiometry (ImageJ) and values were used to create heatmaps (Sauter et al., 2019).

Statistical Analysis

The acquired data was presented with a mean +/- standard deviation. Two-tailed student's t-tests were facilitated to compare two groups with corrections for multiple comparisons. The cut off for statistical significance was chosen at a p value of < 0.05. Statistical analysis was done through GraphPad Prism software. (GraphPad Software Inc., La Jolla, CA, USA) (Sauter et al., 2019).

Results

ASCs and fibroblast display similar attraction to MCF-7 cells, MCF-7 population remains in place

The migration assay that was established as shown in **Figure 2a**, had MCF-7 cells in the center while ASCs were seeded to its right and HS-27 to its left. After removal of the silicon chambers, gap closure was observed microscopically (**Figure 2b**). The gap between MCF-7 cells and HS-27 closed after 96 hours while the gap between MCF-7 and ASCs was closed after 120 hours. The differences in closure time were quantified and testing revealed the difference as not statistically significant ($p = 0.102$) (**Figure 2c**). Microscopic observation of the gaps indicated that gap closure was largely driven by HS-27 and ASC population migration. The MCF-7 cell population primarily remained static within the initial boundaries of the silicon chambers (Sauter et al., 2019).

ASCs show significant attraction towards MDA-MB-231 cells while MDA-MB-231 cells translocate to stagnant fibroblast population

The migration assay shown in **Figure 1a** placed MDA-MB-231 cells in the middle with populations of ASCs to the right and HS-27 to the left respectively. After removal of the silicon chambers gap closure was observed microscopically (**Figure 2b**). After 48 hours fluorescent microscopy showed a clear migratory growth of ASCs towards the MDA-MB-231 population, with ASCs developing micro-protrusions towards the BC population. The BC cells facing the ASCs remained relatively static. When observing the HS-27/MDA-MB-231 front over 48 hours, fluorescent imaging revealed a migratory redistribution of the BC population towards the HS-27 population, HS-27 on the other hand remained stagnant (**Figure 3a**). To confirm this observed loss of cell density in the MDA-MB-231 population facing the HS-27, images were analyzed via pixel densiometry. Results showed a significant depletion of BC cells facing the HS-27 compared to the BC cells facing the ASCs after 36 hours ($p = 0{,}037$) (**Figure 3b**). Gap closure progressed faster on the HS-27/MDA-MB-231 front than it did on the ASC/MDA-MB-231 front with the HS-27/MDA-MB-231 gap closing significantly faster at 16, 24 and 26 hours ($p = 0.027$ (**Figure 3c**). When comparing both assays, gaps seemed to close faster when MDA-MB-231 cells were present (48 hours) compared to when MCF-7 cells were used (128 hours) (Sauter et al., 2019).

Immobile MDA-MB-231 cells affect tropism of ASCs over longer distances

To challenge the above described migratory effects, a set up was created as depicted in **Figure 1b**, with a population distance of 15mm. When the breast cancer population that was seeded on top was MCF-7, ASC and HS-27 populations remained rather dormant. Over a course of 24 hours fluorescent microscopy showed the original boundaries of the silicon chambers (indicated by white dotted lines) were not strongly altered by either the HS-27 or the ASC population (**Figure 4a**). However, culturing MDA-MB-231 revealed a clear response of targeted tropism of the ASC population towards the MDA-MB-231 population seeded above. The ASCs population migrated beyond the boundaries of the silicon chambers. The HS-27 population however appeared to remain dormant within the silicon chamber boundaries (indicated by the white dotted line) (**Figure 4b**). This experiment shows that the attraction dynamic between ASCs and MDA-MB-231 seems to be ASCs homing to MDA-MB-231 cells (Sauter et al., 2019).

ASC migration is increased as BC cell number is raised, especially with malignant cancer type

Microscopic observation showed that ASCs migration was enhanced in the presence of 100.000 rather than 1000 MCF-7 cells, seen in **Figure 5a**. For 1000 MCF-7 cells the ASC population stayed roughly within their former silicon boundaries after 24 hours (indicated by blue dotted line). Trophic growth was enhanced for 10.000 MCF-7 cells and even more so for 100.000 MCF-7 cells.

The same experimental set up with the exchange of MCF-7 cells with the more malignant MDA-MB-231 cells showed that trophic growth was observable with all 3 chosen BC cell concentrations as seen in **Figure 5b**. The effect got stronger, and the ASCs presented more trophic growth, when the dosage of MDA-MB-231 cells was increased. Especially when comparing migration patterns of the ASCs of this group with the ASCs of the MCF-7 group, the MDA-MB-231 cells showed to have a much higher influence on ASC migration (Sauter et al., 2019).

Transwell chamber conditioned media reveals higher concentrations of 2 signaling molecules linked to oncogenic pathways

Samples of the conditioned media of the transwell chamber set up and our novel set up were taken and analyzed. ASC migration over the boundaries of their former silicon

chambers was confirmed via brightfield microscopy after an incubation time of 24 hours, as shown in **Figure 6a**. Pixel densitometry read out of the array revealed that in the samples with the transwell chamber conditioned media, the molecules Interleukin-6 (IL-6) and C-C chemokine ligand 2 (CCL-2) were overexpressed. This expression pattern was slightly apparent for the novel assay conditioned media as well, compared to MDA-MB-231 cells conditioned media alone. A heatmap was created comparing the pixel densiometry values of the transwell chamber and the novel assay conditioned media (**Figure 6b**). Screening revealed that IL-6 and CCL-2 were both distinctly more present in the transwell chamber conditioned media than in the novel assay conditioned media. Images of the CCL-2 and IL-6 of the direct bioluminescent array readout for the transwell chamber and novel assay were cropped out for analysis (**Figure 6c**) (Sauter et al., 2019).

Discussion

Our aim in this current study was to create a setup *in vitro* that more organically mirrors the actual *in vivo* breast cancer microenvironment. A key factor of the experimental set up was the cell freedom of migration while in a 2D environment. As discussed earlier, research focusing on the question of the potential oncogenity of ASCs relies heavily on the facilitation of unidirectional transmembrane systems (Gehmert et al., 2010; Jotzu et al., 2011; Koellensperger et al., 2017; Salha et al., 2018). This transmembrane tool has been applied to an abundance of ASC research. Scientists studied the role of ASC migration in the repair of cardiomyocytes after myocardial infarction (Ji et al., 2020), the attraction of ASCs towards the epidermal growth factor and therefore to sites of injury (Baer et al., 2009), the possibility of treating inflammatory bowel disease with ASCs (Stavely et al., 2015) or ASC homing abilities towards prostate cancer cells (Mamchur et al., 2018). This, one the one hand, highlights the importance of the transwell system as a method for *in vitro* migration research. On the other hand, it raises the question if this monopoly of the transwell system leads to research with a unilateral perspective. Our unique setup of experiments serves not as a challenger to the commonplace transwell systems, but tries to investigate if there are other ways to show the same cell migration patterns. Worth noting is that the readout of both methods is different. The transwell inserts can be stained to reveal the number of cells that have migrated through the pores after a fixed time; providing a quantitative readout (Wang et al., 2012). Our novel method differs in that the actual process of migration can be observed much more closely. While numerical quantitation of migration is difficult, it is possible to keep track of migration speed (surface area covered) or changes of cell morphology at any chosen number of timepoints during the experiment. Similarly, our novel assay allows for a much clearer view of which cell population is mobilized by the other, by offering an unguided and uninfluenced *in vitro* arena.

Another aspect to consider, that was implemented in our assays, is the utilization of more than 2 distinct cell types at once. In the last years it has become increasingly evident, that breast cancer consists not only of neoplastic tissue but a heterogeneous mix of cell types, extracellular matrix and cytokines, all significantly contributing to cancer development, progression and metastasis (Soysal et al., 2015). The different cell types that can be found in the local stroma surrounding the tumor body are

adipocytes, immune cells like leukocytes, myoepithelial and epithelial cells, endothelial cells and fibroblasts (Coleman et al., 2013) A critical part within this environment is played by these cancer associated fibroblasts (CAF). They are the most abundant cell type in the microenvironment and have been found to secrete various soluble factors such as chemo- and cytokines. These are believed to change the tumors into more aggressive phenotypes stimulating tumor growth and tumor invasion (Folgueira et al., 2013). Considering this special role of the CAFs we aimed to account for that in the set-up of our assay. Although ASCs were the main focus of our investigation, we wanted to challenge them in a situation that mimics the actual state of breast tumors *in vivo*. This then consequently led to the creation of an assay implementing not only breast cancer cells but ASCs and HS-27 fibroblasts. The cell line HS-27 does not directly represent CAF since they are not of malignant origin but rather are fibroblasts originally harvested from a healthy donor (Sigma Aldrich cat# 94041901-1VL). Recent studies have been conducted on a single cell level closely comparing CAF and healthy fibroblast populations (Hosein et al., 2019), emphasizing distinct differences between the two but also the heterogeneity within their populations. Another aspect we considered is the unclear and possibly mixed origin of CAFs. These cells could originate from formerly healthy fibroblasts around the progressing tumor (Kalluri, 2016) or might be recruited by the tumor from distal locations (Direkze et al., 2006). Our choice to use healthy fibroblasts was therefore mainly motivated by the fact that in a scenario of an autologous fat transplantation healthy fibroblasts would also be relocated within the fat tissue, and given the current state of knowledge, they could also very well be the origin of fat transfer-associated CAFs.

The tropism patterns that were observed in our first assay where ASCs and fibroblasts were cultured together with cancer cells spanned two distinct breast cancer cell lines. The cell migration we observed in assays involving MCF-7 population agrees with earlier reports describing a slower migration rate. Scratch assays conducted with MCF-7 cells were monitoring them for 24 hours and saw a closure percentage of around half of the gap (Ahmadiankia et al., 2016; Anaya-Eugenio et al., 2021). The MCF-7 population boundary line in our Assay 1 remained static with no clear cell migration towards both the fibroblast or ASC population. Previous findings indicate that co-cultures of ASCs and MCF-7 cells suppress MCF-7 proliferation (Ryu et al., 2014) which might account in part for the adynamic state of the MCF-7 population frontier.

Other studies investigating the effect of ASCs on MCF-7 proliferation describe an enhancement of proliferation but that effect is much less pronounced for MCF-7 than for other, more malignant, BC cell lines (Teufelsbauer et al., 2019). This is likely explained by the benign properties of this BC cell line. MCF-7 are a cell line which is ER and PR positive. This specific subtype generally does not metastasize compared to hormone receptor negative BC subsets (van Uden et al., 2019). The fact that the HS-27/MCF-7 interface of the assay closed faster than the MCF-7/ASC interface is also supported by the literature. Chen et al. used a transwell assay to determine the migratory capacity of MCF-7 cells towards healthy fibroblasts and CAFs. Migration was highest towards CAFs but also significantly increased towards healthy fibroblasts compared to a blank control (Chen et al., 2012). Explaining the slower gap closure of the MCF-7/ASC interface likely involves several reasons. Other groups have already shown that the migratory capacity of ASCs towards MCF-7 cells is not very pronounced. When Koellensperger et al. seeded ASCs in a transwell system there was no significant difference in migration towards the MCF-7 population seeded below compared to ASCs cultured in the absence of MCF-7 (Koellensperger et al., 2017). Taken together these data suggest there is not a powerful chemokinetic role of MCF-7 cells on ASCs.

In comparison to the setup with MCF-7, the MDA-MB-231 assay reveals much more dynamic cell migration. Not only is complete gap closure achieved faster (after 48 hours) compared to the MCF-7 setup (120 hours) but movement patterns are also different. There are studies suggesting that the secretome of CAF upregulates MDA-MB-231 growth and progression (Suh et al., 2020), and that CAFs are attributed to accelerate wound closure time of MDA-MB-231 cells *in vitro*. The effect is attributed to the soluble factor CXCL12 secreted by the CAFs, which is thought to alter the extracellular matrix of the breast cancer cells to enhance motility (Dvorak et al., 2018). Others confirm the enhanced migratory capacity *in vitro* again through the use of scratch and transwell assays. They link this effect to G Protein coupled factor, an estrogen sensitive receptor promoted by CAF which then leads to an enhanced cancer cell migratory capacity (Yang & Yao, 2019). Some studies propose a mechanism in which triple negative breast cancer cells firstly activate fibroblasts which then promote their metastatic potential (Chen et al., 2021). These studies are all unusually unified in suggesting that a strong migratory impact is exerted on triple negative breast cancer cells by fibroblasts/CAFs (Yu et al., 2018). In our setup we were able to reproduce

similar results in that the MDA-MB-231 were much more mobile on the HS-27/MDA-MB-231 frontier than the BC cells facing the ASCs. While there are a plethora of studies investigating the effects of fibroblasts/CAF on breast cancers *in vitro* and *in vivo* (Katanov et al., 2015) there is little literature describing migratory patterns of healthy fibroblasts and triple negative breast cancer cells. This might be attributed to what we saw in our particular setup. The fibroblasts we observed stayed mostly dormant and showed no real shift towards the MDA-MB-231 cells. One group claims that cancer progression like endothelial to mesenchymal transition (EMT) in triple negative breast cancers might be due to soluble factors the cancer cells secrete. They found that secretion of prooncogenic factors such as CCL-2 or Interleukin 1ß was enhanced in cultures of BC cells with stromal cell conditioned media. Some other factors like CCL-5 require direct cell-to-cell contact of stromal cells and BC cells (Liubomirski et al., 2019). One possible explanation of the strong attraction of the MDA-MB-231 cells towards the fibroblast population in our setup is the strategic relocation of MDA-MB-231 cells to co-opt local fibroblasts into CAFs via membrane-membrane (juxtacrine) cell contact. Fibroblasts have been described in the literature as a predecessor for CAFs (Shen et al., 2020). Conversion of fibroblasts towards CAF could promote MDA-MB-231 cells further towards metastatic spread (Dvorak et al., 2018). Statistical analysis of the comparison of gap closure time did not significantly differ between the HS-27/MDA-MB-231 and the ASC/MDA-MB-231 gap; both gaps were closed after 48 hours. While the HS-27/MDA-MB-231 gap was chiefly closed by MDA-MB-231 migration, ASC/MDA-MB-231 gap closure was mostly achieved through ASC migration. As discussed before, this relationship has been observed and reported multiple times *in vitro* (Senst et al., 2013) and *in vivo* (Zhang et al., 2009). To summarize, the novelty of our *in vitro* assay lies in the shared media between three cell types, while still being able to discern cell migration patterns across replicates and experiments. One key finding was that over a distance of 1000µm, and in the presence of locally seeded fibroblasts, ASCs showed a clear trophic attraction towards BC cells (especially MDA-MB-231) cells.

To further understand the kinetics between fibroblasts, ASCs and BC cells, we decided to alter the assay by amplifying the distance between the cell types from 1mm to 15mm. The question was to observe if these migratory properties remain similar even when the populations are located 15 times further away. Part of this challenge stemmed

from surgical rationale. The elongated distance accounts for the distance grafted ASCs might travel to any residual tumor cells which would probably be in the order of millimeters from ASCs. Another factor influencing this experiment design was to add an extra angle to the conventional scratch assay, which usually uses scratch or gap widths of around 500 µm (Huang et al., 2019; Ritter et al., 2015).

What appeared to us as most striking in this particular setup was the fact that in the assay with MCF-7 cells almost no migratory activity was visible for both stromal cell populations. While we saw slow migration of the HS-27 and ASC cells in assay 1 we were not able to reproduce this in assay 2. It is important to consider that data on the properties of ASCs as they promote/inhibit MCF-7 are conflicting. While some report that ASCs have an inhibitory effect on BC growth (Ryu et al., 2014), other studies claim that ASCs actually decrease MCF-7 affinity for chemotherapeutic drugs and promote cancer progression (Lu et al., 2017). Other papers found that MCF-7 influence ASCs. ASCs cultured in MCF-7 conditioned medium showed an upregulated proliferation, and upregulation of stemness regulating genes and autophagy (Garroni et al., 2021). What unites these studies is that they use MCF-7 conditioned medium, which globally affects the cultured cells immediately from use. From these experiments, the researchers suggest *in vivo* paracrine signaling and a potential crosstalk between the cell types. Our study shows that migration over a larger distance (15mm) in non-preconditioned media does not take place. The follow up logic from this is whether use of conditioned media skews the assay to show migration that is not representative of the *in vivo* situation. If the cells are unable to communicate across 15mm *in vitro,* there is a chance that the paracrine communication across 15mm *in vivo* is also hindered.
As expected, cell migration becomes more dynamic when the MCF-7 cells are replaced with the more malignant MDA-MB-231 cells. The effect of using a metastatic BS cell line compared to a benign one is striking in that with the elongated distance the paracrine impact still appreciably exists. The ASCs show a clear guided growth towards the MDA-MB-231 population. Considering this result, it makes sense to more closely take into account the tumor microenvironment (TME). Many studies acknowledge that ASCs influence the TME. They have shown to create inflammatory conditions favoring tumor progression (Eterno et al., 2014). The contribution could also be more indirect. As explained earlier, there is also a body of literature claiming a transition of ASCs into CAF through the influence of BC (Cho et al., 2012; Jotzu et al.,

2011). These studies create an artificial TME by bringing ASCs into close proximity with BC cells through *in vitro* assay design, or murine co-injections *in vivo* (Eterno et al., 2014). Analysis on the actual cell composition of the TME shows no real evidence of substantial contribution of ASCs as a cellular component (Mao et al., 2013; Soysal et al., 2015). These reviewers acknowledge that stromal cells however are a key component and this is where the knowledge of ASC to CAF transition could explain the adoption of local ASCs into tumor tissue. ASCs might simply not be observable because they transition into stromal cells supporting the TME. A key question that arises is how the composition of the TME is achieved. Kidd and colleagues researching the contributions towards BC TME found that the stromal cells of murine breast and ovarian cancer models originate from two sources. One fraction of the tumor associated fibroblasts were recruited from MSCs originating from bone marrow sources while another distinct subtype was mostly recruited from neighboring adipose tissue and therein adipose derived stromal cells (SVF) (Kidd et al., 2012). The conclusion from this is two-fold; firstly, fibroblasts not undergoing chemoattraction towards cancer cells *in vivo* is a finding which is mirrored in our *in vitro* assays, corroborated by negligible fibroblast migration observed when seeded 15mm away from BCs. Secondly, co-opted stromal cells from adipose tissue make up a substantial fraction of the TME. Our results showing very clear and directional movement of ASCs towards the MDA-MB-231 population captures this relationship clearly. Comparing mesenchymal stem cells derived from bone and fat shows strong similarities in morphology, differentiation potential or immunophenotype (Strioga et al., 2012). Per the literature, this similarity seems to extend also to tumor attraction. There have been studies of systemically injected ASCs and MSCs which were able to home to breast cancer tumors in rodent models (Karnoub et al., 2007; Zhang et al., 2009). Although our *in vitro* model is clearly not fit to align ASC directional migration *in vitro* with the systemic homing of ASCs *in vivo*, we still found it interesting that we could in some form reproduce this strong attraction that BC cells exert upon ASCs, even over a distance much longer than typical assays.

In our following assays we wanted to investigate if the migration of ASCs is proportional to the number of BC cells they interact with. The rationale for this approach was again inspired by clinical reflections. We not only wanted to know if ASCs are drawn to a bigger tumor mass, but also if this attraction is observable for even a small number of

BC cells. In a breast reconstruction (BR) scenario after cancer treatment, detection of tumor remnants or cancer cells is very difficult/impossible through histological and radiological analysis. The British Association of Plastic Reconstructive and Aesthetic Surgeons (BAPRAS) and the Association of Breast Surgery (ABS) together released a guideline in 2013 on the topic of BR. There they emphasize that oncological considerations should be applied, specifically, pre-operative staging needs to be considered as part of planning BR surgery (Cutress et al., 2013). This would imply that BR would only be performed when the smallest amount of tumor cells is likely to remain in the residual breast tissue, theoretically minimizing local migration of ASCs newly relocated by the BR. To mimic the migration of ASCs towards an immobilized population of cancer cells, an adjustment that was made for assay 3 compared to the prior two, which was that the silicon chamber holding the BC cell population in place was not removed during the experiment. Instead, the well of the six well plate was filled with media so that it would pour over the rim and flood the silicon chamber containing the cancer cells. We wanted to take into account the existing literature reporting deviating paracrine effects the CM of BC cells has on ASCs, in contrast to the juxtacrine effect these same BC cells have with direct cell to cell contact (Kucerova et al., 2013). Our goal was to restrict the ability of the BC cells to migrate or relocate directly to the ASCs. Instead, we wanted to investigate if migratory behavior of ASCs is triggered through paracrine signaling, as this is the most acknowledged cell contact mechanism by which BC influences ASCs (Lyes et al., 2019). Going a step further, we decided to restrict the number of cell types used down to only BC cells and ASCs for this assay. The insights we collected in assay 1 and 2 showed little to no migratory capacities of the HS-27 fibroblasts and the focus of this investigation was to be about the migratory potential of ASCs towards breast cancer cells, as they are discussed as the critical driver of a potential oncogenicity of AFG and CAL. In contrast to that extensive body of literature there are, to our best knowledge, no publications studying attraction of ASCs to various concentrations of BC *cells in vitro*. Studies that do exist only rely on one set concentration of either ASC and BC cells for their migration experiments and are generally either transwell or scratch assays (Koellensperger et al., 2017; Ritter et al., 2015; Teufelsbauer et al., 2019).

The results we saw when we cultured freely migrating ASCs with different concentrations of restricted BC cells agreed largely with the findings of our previous

experiments. For one, the MCF-7 setup again failed to initiate major change in the ASC population kinetics. That being said, there was a clearly visible increase in ASC migration when the dosage of MCF-7 cells was increased. This minor, but apparent migratory potential for large numbers of cells (100,000) gets more interesting when comparing it to other migration studies of MCF-7 and ASCs. For example, Koellensperger and colleagues, using a transwell system where they seeded only 6000 cells over 24 hours in the bottom on the well, found no enhancement in ASC migration through the transwell membrane (Koellensperger et al., 2017). This does align well with our results from assay 1 and 2 in which we used MCF-7 cell numbers of 10.000 cells and found no real stimulus of ASC migration. It might be then, that only larger bodies of ER/PR positive BC cells are able to stimulate migratory effects. The situation gets more complex observing clinical data. Tumor size is used as a prognostic factor for time of survival, even before other factors like lymph node involvement; a finding that also applied for ER positive breast cancers, represented here by MCF-7 (Liu et al., 2021). Zhang and colleagues postulate that MSCs drive tumor progression of MCF-7 cells (Zhang et al., 2013). There is a potential that only large numbers of tumor cells are actually attracting ASCs which then progress tumor development. This is in conflict to the conclusion drawn from data that show no effect of ASCs on MCF-7 cell proliferation (Koellensperger et al., 2017). Again, this presents a vivid example on how the current literature is not unified about the role of ASCs. The data we acquired might help to explain some of these inconsistent findings.

While there is a plethora of studies measuring the effect of ASCs on the migratory capacities of MDA-MB-231 cells (Orbay et al., 2018; Park et al., 2016; Rowan et al., 2014; Teufelsbauer et al., 2019), not as many focus on the inverted attraction of ASCs towards MDA-MB-231 cells, and those that do, neglect to take into account the dose dependent effect of cancer cells on ASCs (Koellensperger et al., 2017; Ritter et al., 2015). It is commonly acknowledged in current research that the crosstalk between ASCs and BC cells is mediated through soluble factors (Guillaume et al., 2022) (de Miranda et al., 2021). While there is no clear hypothesis of how the secretome of BC cells influences ASC migration, what is made abundantly clear by the data from Assay 3 is the linear relationship between ASC migration and BC cell number.

After performing assay 1 to 3 and finding a highly reproducible, strong attraction of ASCs, especially towards MDA-MB-231 cells, we decided to compare our newly

established silicon chamber migration assay with the customary transwell migration assay. A pore size of 8µm was selected since this was found in most studies utilizing the method (Bahman Soufiani et al., 2021; Chen et al., 2012; Koellensperger et al., 2017). Direct comparison of both assays via migration would not have been possible, given the quantitative readout for the transwell assay and more qualitative readout for our novel assay. To level these assays against each other, and since it is established that BC ASC interaction is mediated through paracrine signaling (Guillaume et al., 2022) we decided to measure and compare cytokine expression of both assays. The Human Cytokine Array (C5, RayBiotech) was selected since it allowed to detect various cytokines that are related to migratory properties and have already been described by other papers to be found in BC ASC interactions, such as Interleukin-6 (IL-6) (Wei et al., 2015), Interleukin-8 (IL-8) (Razmkhah et al., 2010) or CC-chemokine ligand 5 (CCL-5) (Brett et al., 2020).

MSC migration or homing is not an effect that is only observed in cancers. There exists well documented research studying the role of MSCs in wound healing (Piccolo et al., 2015). Mesenchymal stem cells have been detected in a plethora of human organs (Vizoso et al., 2017), among them, ASCs have been found to reside within the subcutaneous tissue (Marfia et al., 2015). ASCs have been suggested to possess the ability to migrate towards injured tissue. In a murine model of dermal wound healing, ASCs were either applied topically, or through intravenous or intramuscular injection, and in each study arm, they accelerated wound closure compared to controls (Kim et al., 2019). This homing was proven through ASC staining prior to intravenous injection, and surviving cells were confirmed within the defect accelerating vessel formation (Zhou et al., 2019). Mechanistically, the first step of MSC tissue homing is the adhesion of the systemically-injected MSC to the endothelial wall (Sackstein et al., 2008). Once attached, the MSCs are activated by CXCL-12. CXCL-12 was found to be significantly increased in acute wound fluid compared to chronic wound fluid. ASCs in turn express the CXCL-12 C-X-C chemokine receptor 7 (CXCR7) (Stuermer et al., 2015), which facilitates the adhesion further. Following this activation, the MSCs enter arrest and then are stimulated to transmigrate through the endothelial cell layer and base membrane with the help of Matrix Metalloproteases (MMPs) (Steingen et al., 2008). This process is called extravasation and is the hallmark of rapid cell migration around the body. After the adhesion, the activation, the arrest and the transmigration, the

MSCs are then in close proximity to the wounded site. The final step of homing is migration through the interstitium, the extracellular matrix, to reach the tissue defect. This MSC migration is triggered through various factors liberated by the defect. Described are platelet-derived growth factor (PDGF), the insulin-like growth factor 1 (IGF-1), CCL 5, macrophage-derived chemokine (MDF) or CXCL-12 (Ponte et al., 2007). An important role is also attributed to inflammatory cytokines like Interleukin 8 (IL-8) to stimulate MSC migration to injured sites (Bayo et al., 2017). Once arrived, they are known to secrete VEGF, promoting vessel formation and consecutive wound healing (Hou et al., 2014). This capacity to especially exhibit homing to inflammatory sites is especially striking for MSCs. François et al. observed MSC homing after systemic infusion in a murine model. They compared healthy rodents to some that underwent systemic or local irradiation, creating local or systemic inflammation. MSC homing was significantly increased for both those groups compared to control (François et al., 2006).

The similarities between tissue defects and cancers have been apparent for years. An article wrote more than 30 years ago by Dvorak et al. initially made this connection (Dvorak, 1986). Research that followed has found some key paracrine factors common to wounds and cancer, most prominently the inflammatory cytokine IL-6, which we were also able to detect in our cytokine array. IL-6 is a cytokine produced in response to infections or tissue defects and is a powerful stimulant of the acute immune response (Tanaka et al., 2014), and plays a pivotal role in wound healing (Mateo et al., 1994; McFarland-Mancini et al., 2010). IL-6 stimulates immune cell chemotaxis (Weissenbach et al., 2004), and regulates chemokine secretion of stromal cells, keratinocytes, endothelial cells and macrophages and therefore acting as a regulator creating a physiological wound healing environment (Wright et al., 2014). In cancer, this regulatory effect is compromised. IL-6 has been found to be highly elevated in the serum of breast cancer patients also, correlating with disease stage (Kozłowski et al., 2003). IL-6 signaling pathways promote cancer cell proliferation (Hirano et al., 2000), lead to the expression of anti-apoptotic proteins - enhancing cancer cell survival (Gritsko et al., 2006) - and enhance tumor invasiveness and metastasis (Kim et al., 2009). IL-6 was found in the conditioned media of MDA-MB-231 and MCF-7 breast cancer cells, with higher malignancy cell lines expressing more IL-6 (Senst et al., 2013). This fits in with studies linking higher IL-6 blood levels to worse prognosis in breast cancer patients (Lippitz, 2013). ASCs have also been described to secrete IL-

6, thereby promoting migration and invasion of breast cancer cells, notably shown only by transwell assays (Walter et al., 2009). Others describe that IL-6 and VEGF combined are driving BC cell migration (De Luca et al., 2012).

The detection of IL-6 in our cytokine panel is not surprising at all, since it has been described before to be a driver of MSC recruitment to the tumor site (Rattigan et al., 2010). Others have confirmed this attraction of MSCs towards IL-6 independently. In a 2D chemotaxis assay, MSCs were found to exhibit significant directional attraction towards an IL-6 gradient. In a follow up experiment, they created 3D spheroids of MSCs which they cultured with IL-6 supplemented media. In comparison to the control spheroid only cultured with DMEM, the MSCs from the IL-6 spheroid detached and migrated out of the main cell body (Casson et al., 2018).

The other cytokine we found to be upregulated in our array was CCL-2. CCl-2 is yet another important cytokine linked to breast cancer prognosis (Ghoneim et al., 2009). The physiological role of CCL-2 is that of a pro-inflammatory chemokine that acts as a chemoattractant to immune cells like monocytes, dendritic cells or T-cells to the site of inflammation (Carr et al., 1994). Further properties include angiogenesis (Gangadaran et al., 2021). It was illustrated that BC cell conditioned media lead to the release of CCL-2 and CXCL-8 from MSCs, which led to the conversion of MSCs to CAFs; clearly highlighting the involvement of CCl-2 to TME modelling in an *in vitro* platform (Katanov et al., 2015). Others report of co-cultures containing MSCs and BC cells that led to robust upregulation of CCL-2 secretion. CCL-2 rich CM of these co-cultures was able to induce BC cell sprouting and enhance migration in MDA-MB-231 cells (Liubomirski et al., 2019). Other groups have found this induction of CCL-2 expression also holds true for co-cultures of ASCs and BC cells. This effect was especially pronounced for the co-culture of ASCs with MDA-MB-231 (Koellensperger et al., 2017). Importantly, this induction in CCL-2 secretion was also reported for co-cultures with other cancers, such as malignant melanoma (Preisner et al., 2018). Apart from these reports on CCL-2 inducing breast cancer progression, there is also some evidence for CCL-2 to influence ASC migration. Wang et al. wanted to improve the homing abilities of systemically administered ASCs onto dystrophic muscle tissue in a rodent model. They used lentiviral vectors to induce the expression of CCL-2 receptor (CCR-2) in ASCs which led to a significantly increased homing abilities towards dystrophic tissue in these mice (Wang et al., 2021).

Assay 4 reveals the upregulation of two cytokines, IL-6 and CCL-2, that both repeatedly have been named as drivers of breast cancer progression. The fact that less IL-6 and CCL-2 was detected in our assay compared to the transwell assay, could lead one to believe that our assay possibly shows a less oncogenic profile. The question of whether or not high expression of IL-6 or CCL-2 is directly translatable to higher oncogenicity is not a definitive one. The inconclusiveness of the current research can be observed in the example of IL-6. There are reports that IL-6 is a mediator of the binding of cytotoxic T-cells on the tumor vascular endothelium and therefore a driver of an anticancer immune response (Fisher et al., 2011). Other groups saw that through acute inflammatory responses, like fever, the recruitment of lymphocytes to lymph nodes in the tumor proximity was improved. This process aids the immune system to fight the tumor and is mediated by IL-6 (Carrière et al., 2005; Chen et al., 2006). This research sheds a more nuanced light on the role of IL-6 in cancer progression and the recruitment of various cell types to the tumor site, if, as our research suggests, IL-6 indeed is a driver of ASC tumor migration. There still remains the question if ASCs do in fact possess tumor driving properties. An interesting paper highlighting this issue was written by Mohd Ali and colleagues. They isolated exosomes harvested from indirect co-cultures of either MCF-7 or MDA-MB-231 cells with ASCs and analyzed the expression profiles and cancer morphology. What they saw was that under the influence of ASCs, both BC cell lines switched to a phenotype more associated with dormancy, especially suppressing EMT. Interestingly though, this state of dormancy improved the chemoresistance of these cells when faced with chemotoxic agents (Mohd Ali et al., 2020). Papers like this highlight how difficult it can be to find a definitive conclusion on the question of ASC oncogenity. Our present paper underlines this. We created a novel assay with which we were able to reliably show migratory properties of ASCs towards malignant BC cells, even with the implementation of a third cell type. In our comparative assay 4, involving the comparison of our novel assay and the commonly performed transwell assay, we cultured the same cell types for the same amount of time in the same volume of shared media and found an altered cytokine profile. These findings lead us to propose that especially *in vitro* research on this topic has to be expanded. The current knowledge does not possess the weight to support a definitive recommendation on the support of AFG or especially CAL on BC patients, especially considering the lack of clinical

evidence on AFG oncogenicity. Our paper delivers some insight to the question, in that it shows that there are multiple ways to show the same effects with various readouts. This study has limitations in that the research was conducted only *in vitro* and the hypothesis was not challenged *in vivo*. Furthermore, we acknowledge the limitations of qualitative results compared to quantitative results. We understand that the data of these experiments were designed to allow us to observe a more real and unbiased view of cell kinetics, on which more quantitative work can be performed. Since these are newly designed assays, there is no direct comparison available in the literature, which is both a limitation and a novelty.

Conclusion

In this paper we were able to create a series of novel assays showing the migration of ASCs towards breast cancer cells of different malignancies. Migratory properties of ASCs towards breast cancers, especially of higher malignancies were confirmed in the presence of fibroblasts, thereby creating an environment more accurately depicting the cancer microenvironment *in vivo*. We confirmed that migratory capabilities of ASCs are elevated in a dose dependent manner. Finally, by comparing the cytokine profile of our novel migration assay with the established transwell migration assay, we saw altered cytokine expression. This data taken together highlight that the current state of research on this topic remains inconclusive. Confounding data in this field is the result of realistic *in vivo* studies showing the capacity of ASCs in migration, immunomodulation and cancer progression, and unrepresentative *in vitro* studies which fail to encompass the complicated tumor microenvironment. This body of work within this thesis highlights what a custom-designed, non-biased *in vitro* assay can illustrate in an otherwise nebulous field of cancer biology.

Appendix I – Figures

Figure 1

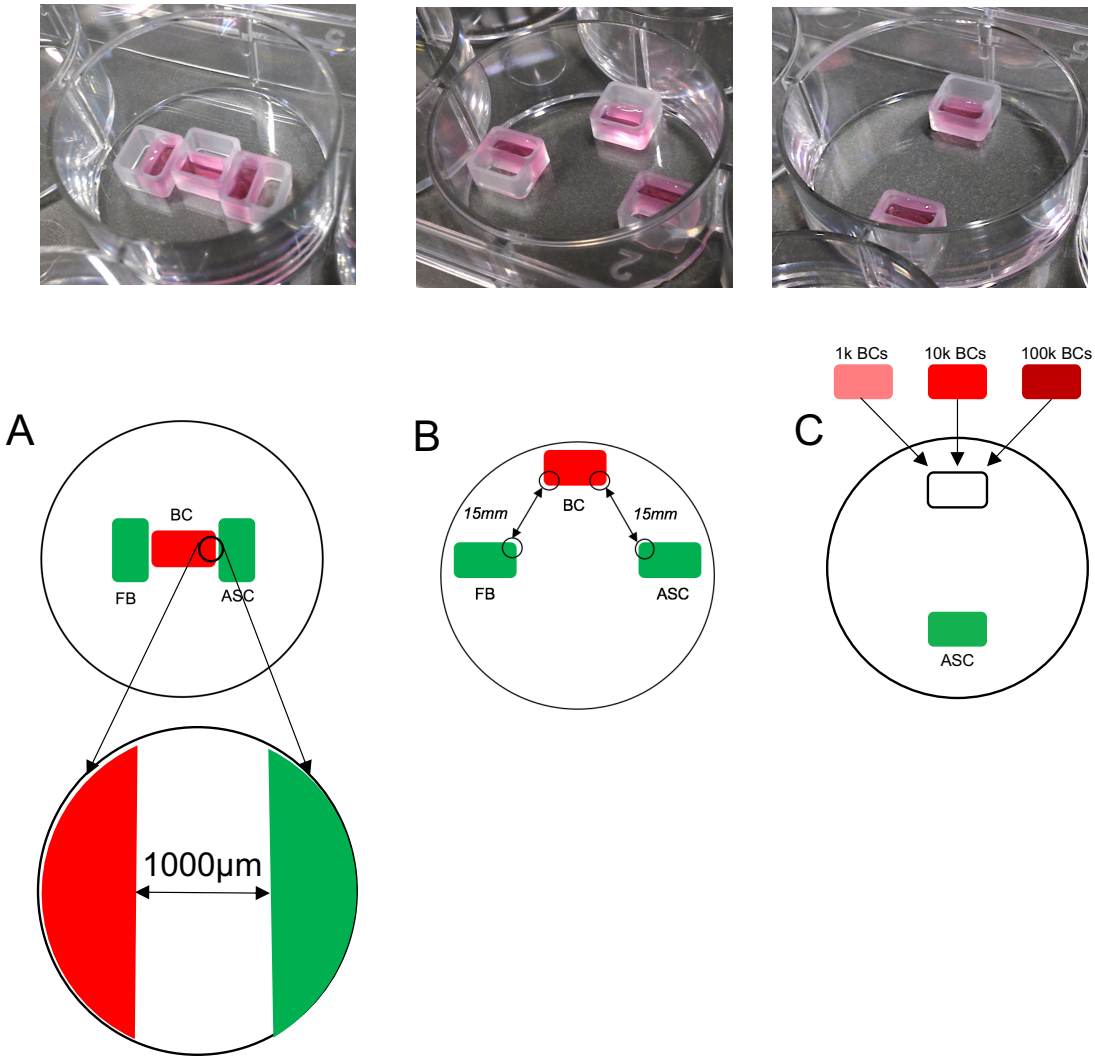


Figure 1 – description

A. Setup of 3 silicon chambers. Chamber placed in the center horizontally, containing BC cells (either MCF-7 or MDA-MB-231). Chamber seeded perpendicularly to the right and left of the central chamber containing either fibroblasts (FB) or Adipose derived stem cells (ASC).

B. Setup of 3 silicon chambers. 3 chambers placed in a triangular fashion. Topmost chamber containing BC cells (either MCF-7 or MDA-MB-231). Silicon chambers on the

bottom corners of the isosceles triangle with a distance of 15mm to the topmost chamber. Left chamber seeded with FB, right chamber with ASC.

C.Setup of 2 silicon chambers. The top chamber was seeded with BC cells (either MCF-7 or MDA-MB-231). Concentration of seeded cells as 1000, 10.000 or 100.000 cells. The bottom chamber was seeded with ASCs.

(Sauter et al., 2019)

Figure 2

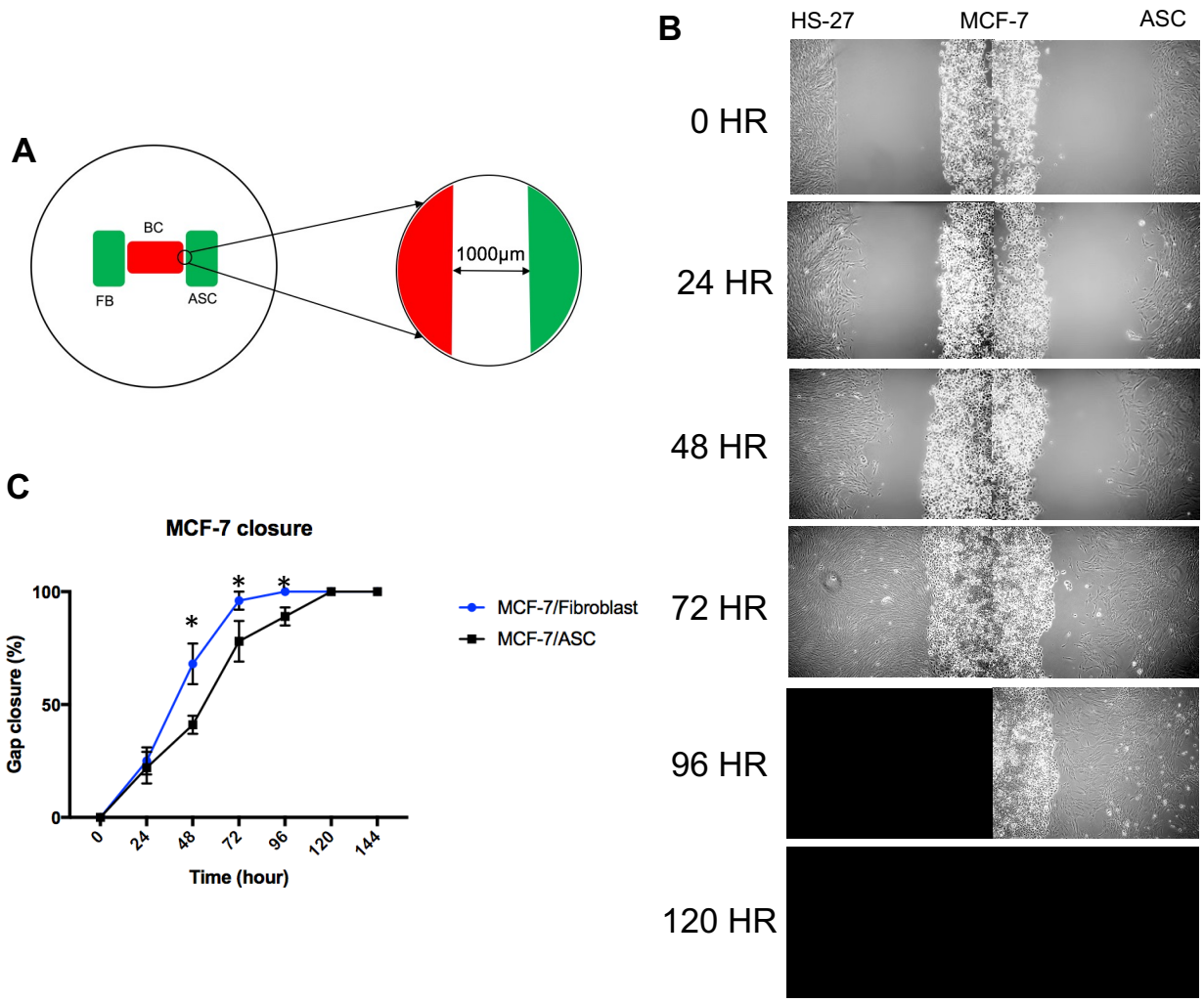


Figure 2 – description

Presentation of data obtained from running in vitro setup shown in Figure 1A.

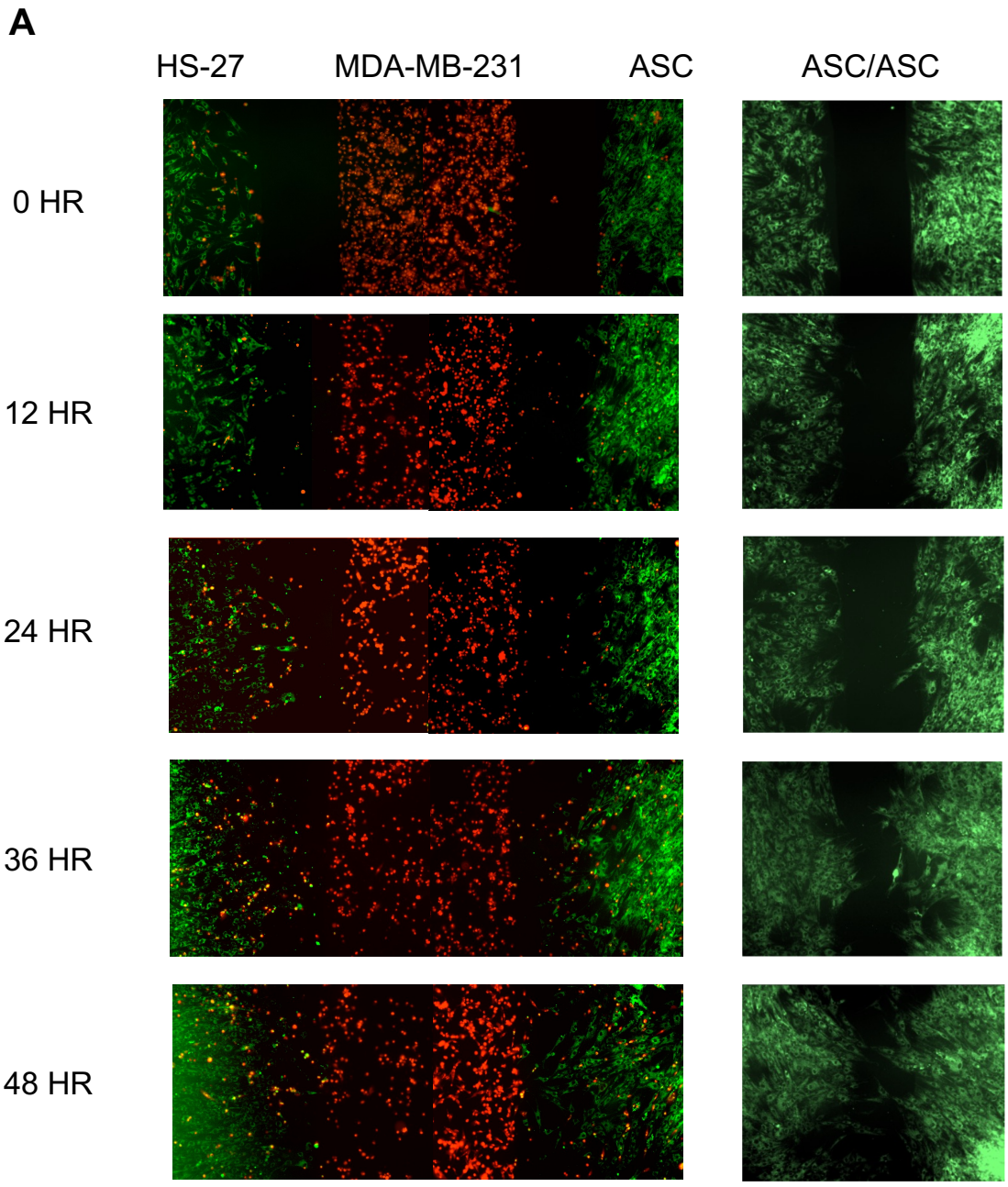
A. Schematic of the set up of Assay 1.

B. Brightfield microscopy images showing left and right edges of MCF-7 population seeded centrally and edges of HS-27 population (left) and ASC population (right). Six timepoints show the timeline of 1000µm gap closure.

C. Gap closure quantification. Significant differences were detected at time point 28, 72, and 96 hours. After 96 hours closure of the HS-27/MCF-7 gap. After 120 hours closure of the. Results were considered significant at $p < 0.05$.

(Sauter et al., 2019)

Figure 3



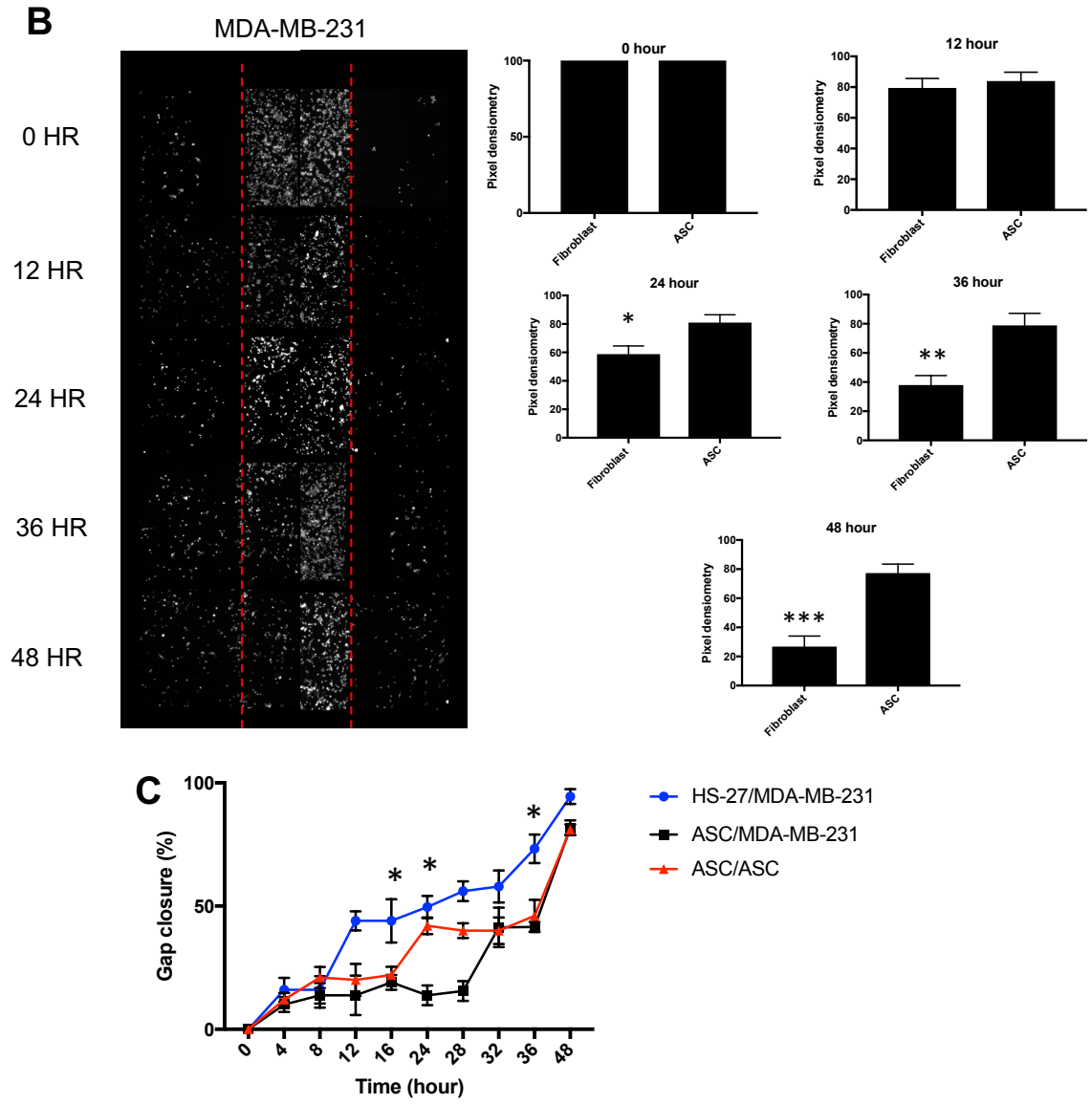


Figure 3 – description

Presentation of results obtained from running in vitro setup shown in Figure 1A.

A. Fluorescent microscopy images showing MDA-MB-231 in the center (red colored) HS-27 population (left) and ASC population (right), colored green. During 48 hours MDA-MB-231 cells are detaching and moving towards HS-27 population. On the right gap, green ASCs are migrating towards static BC population.

B. Pixel densiometry of red florescent staining and results of respective quantification via ImageJ. At timepoint 24, 36 and 48 hours, significantly less fluorescence is detectable on the side facing the HS-27 population compared to the side facing the ASC population, *p < 0.05.

C. Gap closure quantification. Changes in gap closure were non-significant. *p < 0.05. (Sauter et al., 2019)

Figure 4

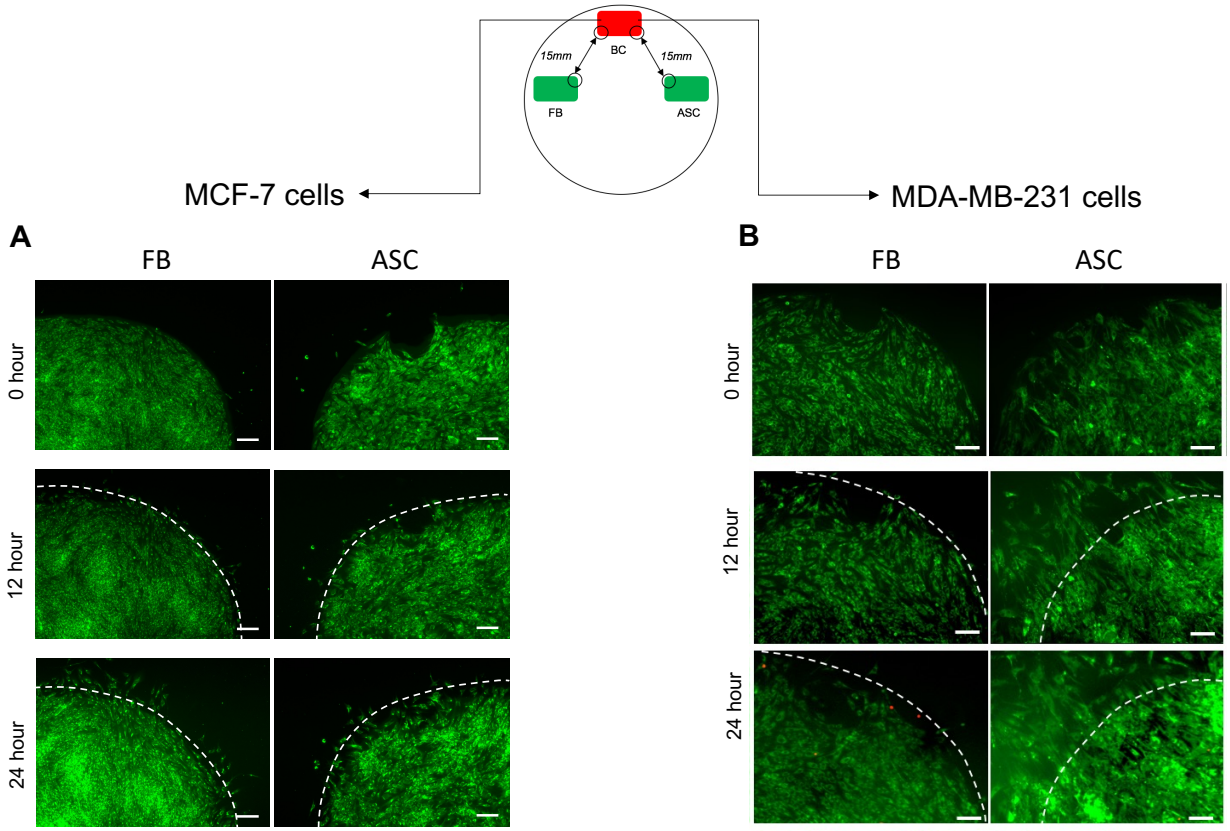


Figure 4 – description

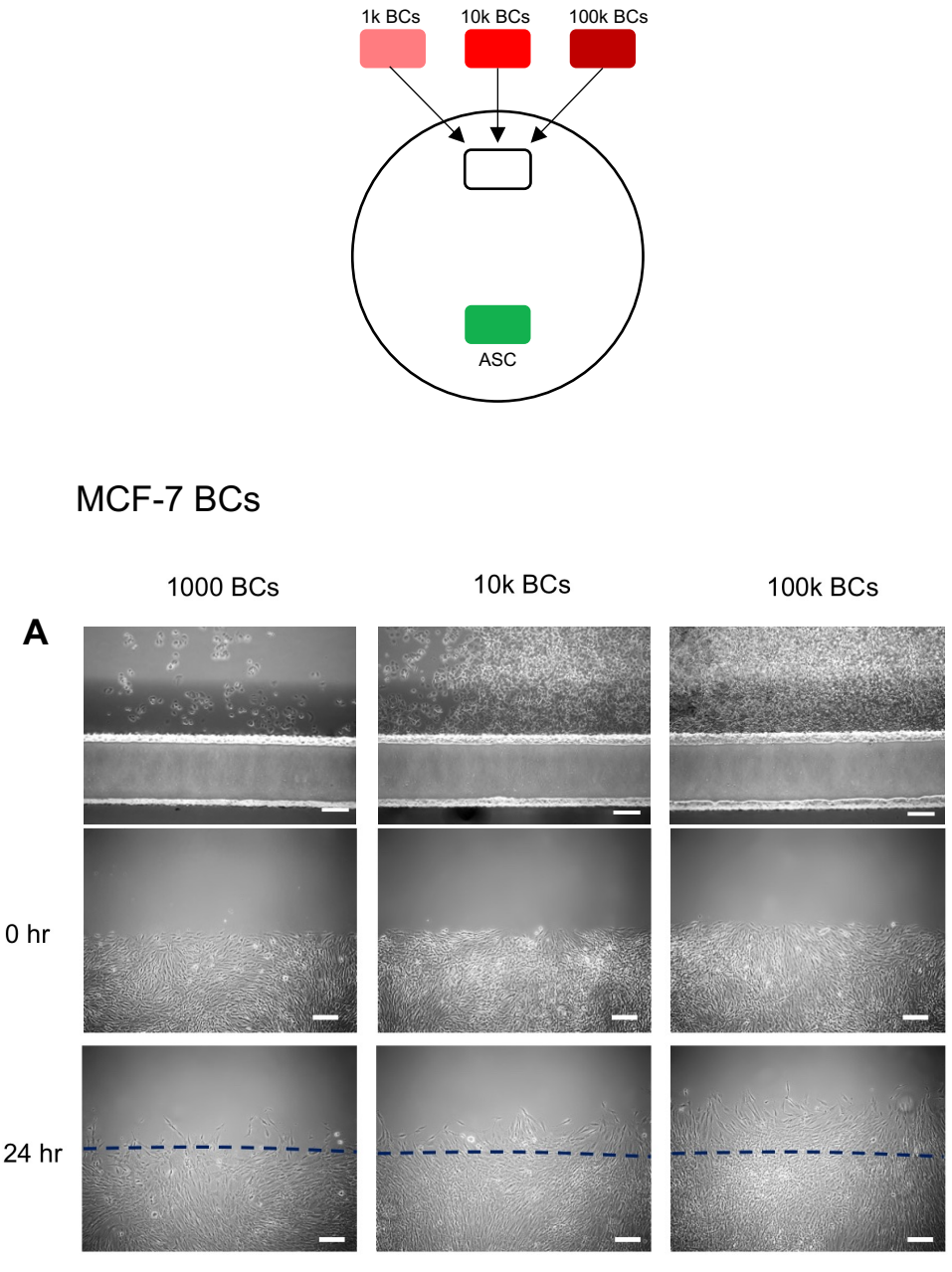
Presentation of results obtained from running in vitro setup shown in Figure 1B. 3 Chambers were arranged in a triangular shape. Either MCF-7 or MDA-MB-231 (top), HS-27 (left), ASC (right). White dotted line indicate the original boundaries of the silicon chambers. White bars in all images scale for 100 µm.

A. Fluorescent imaging reveals little migration of HS-27 or ASCs over their original boundaries after 12 or 24 hours.

B. Fluorescent imaging reveals little migration of HS-27 over the original boundaries after 12 or 24 hours. ASCs show clear, directional migration towards MDA-MB-231 population after 12 and 24 hours.

(Sauter et al., 2019)

Figure 5


1k BCs
10k BCs
100k BCs
ASC
MCF-7 BCs
1000 BCs
10k BCs
100k BCs
A
0 hr
24 hr

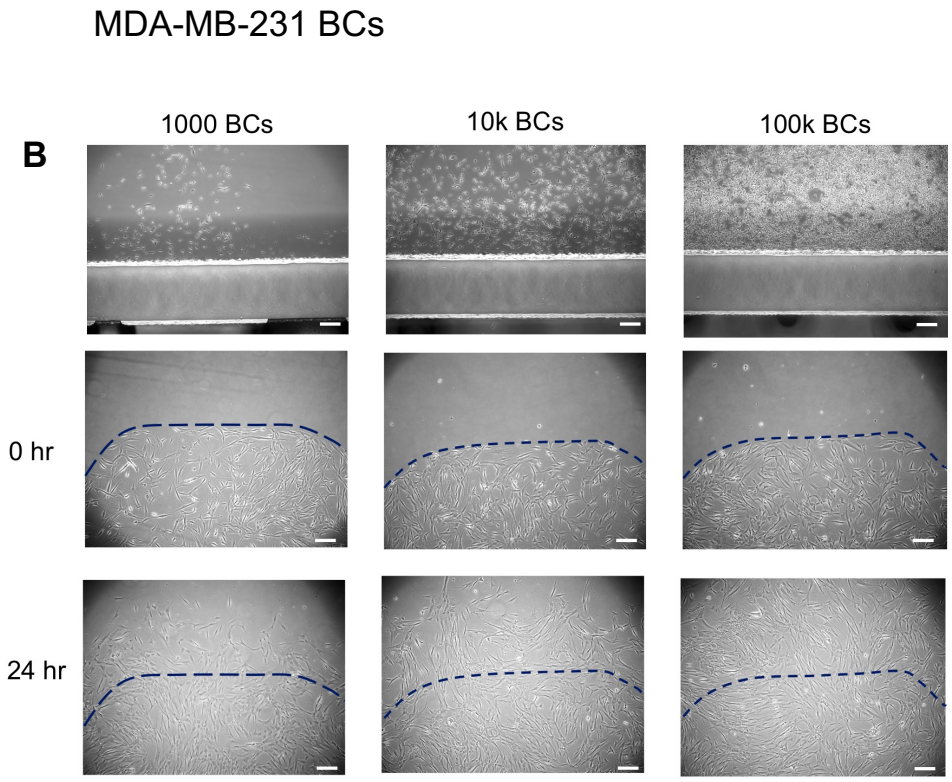


Figure 5 – description

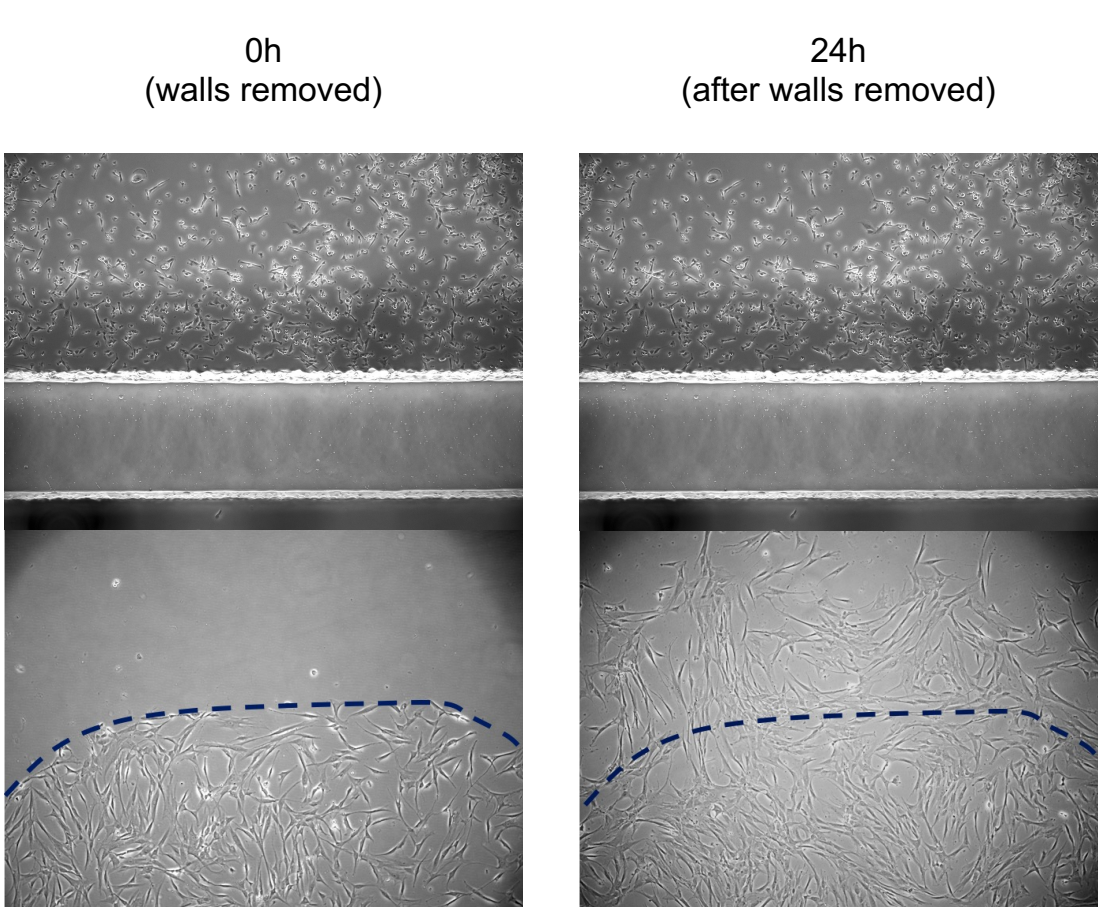
Presentation of results obtained from running in vitro setup shown in Figure 1C. 2 Chambers were arranged in the well with either MCF-7 or MDA-MB-231 cells on the top in concentrations of 1000, 10.000, or 100.000 cells. ASCs were seeded on the bottom chamber. Blue dotted lines indicate original boundaries of the silicon chamber holding the ASCs. The chamber holding MCF-7 was kept in place.

A. Scant migration of ASCs towards various MCF-7 cell concentrations is enhanced over 24 hours when number of cells is increased

B. Distinct migration of ASCs towards various MDA-MB-231 cells over 24 hours. Migratory capacities are clearly enhanced when concentration of BC cells in increased. (Sauter et al., 2019)

Figure 6

A



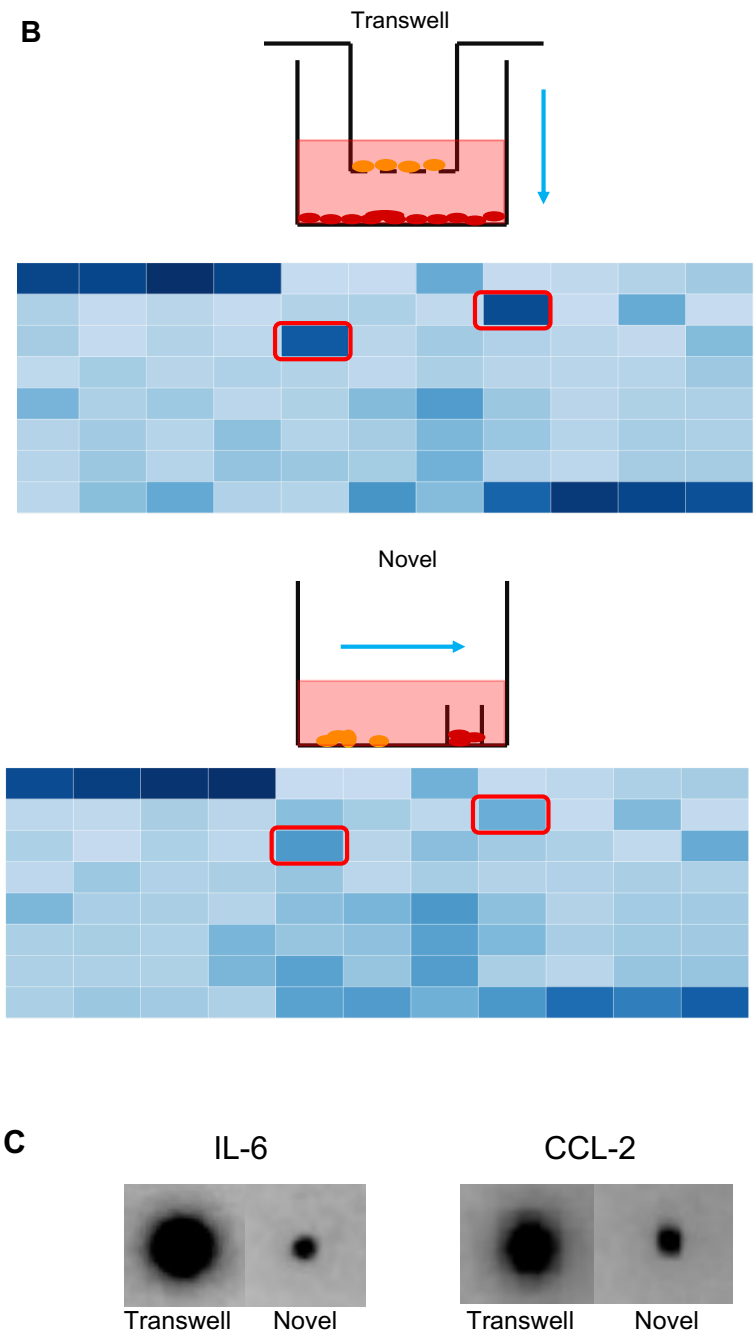


Figure 6 – description

Presentation of results obtained from running a cytokine array on the supernatant of our novel assay to a transwell migration assay.

A. Brightfiled images showing migration of ASCs towards stagnant MDA-MB-231 population after 24 hours. Original boundaries of chambers are indicated by blue dotted lines.

B. Schematic depiction of the transwell chamber (top) and our novel assay (bottom). ASCs (yellow) migrate towards MDA-MB-231 cells (red), blue arrows show the

migratory track. Heatmaps show the cytokine profiles obtained from supernatants after 48 hours. IL-6 and CCL-2 and circled red for both maps.
C. Images of the cytokine array. Comparison of the expression of IL-6 and CCl-2 for both assays. Transwell left, novel assay right.
(Sauter et al., 2019)

Appendix II - Abbreviations

4T1 – rodent breast cancer cell line

ABS – Association of Breast Surgery

AFG - Autologous Fat Grafting

ASPS – American Society of Plastic Surgeons

Balb/c mice – Inbred mouse strain commonly used for animal research

BAPRAS – British Association of Plastic Reconstructive and Aesthetic Surgeons

BC – Breast Cancer

BCS – Breast Conserving Therapy

BMPR2 – Bone Morphogenetic Protein Receptor Type II

c- kit – Receptor Tyrosine Kinase

CAF – Cancer Associated Fibroblasts

CAL - Cell Assisted Lipotransfer

CCL-2 – CC-chemocine ligand 2

CCR-2 – CC-chemocine ligand receptor 2

CD105 – Cluster of Differentiation 105

CD248 – Cluster of Differentiation 248

CD90 – Cluster of Differentiation 90

CXCL-12 – C-X-C motif chemokine 12

CXCL-8– C-X-C motif chemokine 8

CXCR-7 – C-X-C chemokine receptor 7

DGPRÄC – Deutsche Gesellschaft der Plastischen, Rekonstruktiven und Ästhetischen Chirurgie

DM – Distant Metastasis

E-cadherin – Epethelial Cadherin

EMT – Endothelial to Mesenchymal Transition

EPC – Endothelial Progenitor Cells

ER - Estrogen Receptor

FGF – Fibroblast Growth Factor

FGF2 – Fibroblast Growth Factor II

HER2/neu – Human Epidermal Growth Receptor 2

HERS-2 - Human Epidermal Growth Factor Receptor 2

HGF – Human Growth Factor

IL-1 – Interleukin-1

IL-6 – Interleukin-6

LR – Local Recurrence

LRR – Locoregional Recurrence

MAP pathway – Mitogen-Activated Protein Kinase Pathway

MCF-7 – Michigan Cancer Foundation-7 (ER/PR positive BC cell line)

MDA-MB-231 - M.D. Anderson - Metastatic Breast 231 (ER/PR negative BC cell line)

Micro RNA – Micro Ribonucleic Acid

MMP – Matrix-Metalloproteinase

MMP-6 – Matrix-Metalloproteinase-6

MST – Mastectomy

N-cadherin – Neuronal Cadherin

PDGF – Platelet Derived Growth Factor

PI3K – Phosphoinositide 3-Kinase

PI3K pathway – Phosphatidylinositol 3-Kinase Pathway

PR - Progesterone Receptor

PTX – Paclitaxel

SERPINE1 – Serine Protease Inhibitor I

SKBR3 – Human adenocarcinoma breast cancer cell line

SVF - Stromal Vascular Fraction

T-47D – Human Epithelial Breast Cancer Cell Line

TAF – Tumor Associated Fibroblasts

TGFβ1 – Transforming Growth Factor ß 1

TME – Tumor Microenvironment

TVE – Tumor-Derived Extracellular Vesicles

VEGF – Vascular Endothelial Growth Factor

VEGFA – Vascular Endothelial Growth Factor A

Appendix III - Bibliography

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