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Understanding crosstalk between mesenchymal stem cells and cancer cells metastasizing to bone – a whole secretome approach

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Summary

The effect of mesenchymal stem cells (MSC) on cancer progression is still a matter of debate. It is increasingly appreciated that MSC can migrate towards the site of the primary tumor and participate in tumor stroma formation, supporting tumor growth and priming cancer cells for dissemination. Less is known about MSC or other stromal cells at the site of metastasis. Some cancers show a strong tendency to metastasize to bone, a tissue of mesenchymal origin and a prominent site of MSC. Recent reports have suggested that bone-metastasizing cancers may mimic the process of homing of hematopoietic stem cells to their bone niche, in which MSC play a crucial role. With regard to the fact that MSC play an important role in cancer progression, I aimed to dissect the interactions and the dynamics between tumor cells and MSC in metastasis formation.

In order to understand the role of MSC in metastasis formation, I investigated primary human bone marrow MSC with established cancer cell lines able to metastasize to bone in a transwell migration assay. Combining this experimental set up with impedance measurements allowed quantitative analysis of cancer cell migration towards MSC in a time-resolved fashion and high-throughput format. This enabled an unbiased approach taking the complete secretome of MSC into consideration.

The results showed that MSC induced a rapid migration response of prostate and breast cancer cell lines. In contrast to this finding, fibroblast cell lines were not able to induce a comparable migration response indicating specific MSC – cancer cell crosstalk. In order to identify the factors stimulating cancer cell migration, MSC cell culture supernatant was then purified by size exclusion and ion exchange chromatography. This was followed by mass spectrometry as well as antibody array analysis of the chromatographic fractions inducing migration. With this approach I identified extracellular matrix proteins to be the main drivers of rapid cancer cell migration requiring as little as two hours for a full migration response. These factors included type I and III collagen, fibronectin and Laminin 421, which were confirmed using recombinant proteins. RNAi experiments showed that the response to these molecules required the extracellular matrix receptor β_1 integrin in the migrating cancer cells.

This study shows that MSC are very potent mediators of cancer cell migration, based on diffusible gradients of extracellular matrix proteins acting independently of chemokines.

Zusammenfassung

Welche Rolle mesenchymale Stammzellen (MSC) in Krebserkrankungen spielen, ist weitgehend ungeklärt. Es wird jedoch zunehmend davon ausgegangen, dass MSC zu Tumoren wandern können, dort an der Bildung des Tumorstromas beteiligt sind und dadurch Tumorwachstum und die Streuung der Tumorzellen unterstützen. Wie MSC konkret am Ort der Metastasen wirken, ist dagegen weniger bekannt. Manche Krebserkrankungen bilden besonders häufig Metastasen in Knochen, einem Gewebe mesenchymalen Ursprungs und wichtiger Sitz zahlreicher MSC. Zudem mehren sich die Vermutungen, dass metastasierende Krebszellen den physiologischen Einnistungsprozess hämatopoetischer Stammzellen in das Knochenmark nachahmen könnten. Da dieser Einnistungsprozess von MSC beeinflusst wird und auf Grund ihrer Rolle in Tumor-Stroma Interaktionen, habe ich die Wechselwirkungen zwischen MSC und metastasierenden Krebszellen untersucht.

Um zu verstehen, welche Rolle MSC bei der Entstehung von Metastasen spielen, wurden primäre MSC aus dem Knochenmark isoliert und in einem Migrationsassay analysiert, wie sie Migration von Krebszelllinien, von denen bekannt ist, dass sie Knochenmetastasen bilden, auslösen. Die Verwendung eines impedanzbasierten Assay erlaubte quantitative und zeitaufgelöste Messungen in einem Hochdurchsatzformat zur funktionalen Analyse des gesamten MSC Sekretoms.

Meine Ergebnisse zeigen, dass MSC eine schnelle Migration von Brust- und Prostatakrebszellen auslösen können. Im Gegensatz hierzu waren Fibroblastenzelllinien dazu nicht in der Lage, was darauf schließen lässt, dass die beobachteten Wechselwirkungen MSC spezifisch sind.

Um diejenigen Faktoren zu identifizieren, die für die Induzierung der Krebszellmigration verantwortlich waren, wurde der MSC Zellkulturüberstand mittels Größen- sowie Ionenaustauschchromatographie aufgereinigt. Chromatographiefraktionen, die Krebszellmigration auslösten, wurden anschließend mittels Massenspektrometrie und Antikörper-Array analysiert. Dieses Verfahren erlaubte es mir, extrazelluläre Matrixproteine als diejenigen Faktoren zu identifizieren, welche die beobachtete Krebszellmigration auslösten. Zu diesen Matrixproteinen zählten Typ I Kollagen und Typ III Kollagen, Fibronektin und Laminin 421, deren migrationsinduzierende Wirkung mittels rekombinanter Proteine bestätigt werden konnte. RNAi Experimente konnten zudem aufzeigen, dass die beobachtete Migration der Krebszelllinien abhängig war vom Zellrezeptor β_1 Integrin.

Meine Studien konnten somit belegen, dass MSC mittels extrazellulärer Matrixproteine und unabhängig von klassischen Chemokinen eine starke Migration von Krebszellen auslösen können.

1. Introduction

1.1. Cancer progression and the tumor microenvironment

Cancer describes a pathology in which normal cells acquire the characteristics of uncontrolled and chronic proliferation. During the progression of cancer, the cancerous cells undergo an evolution from proliferative to tumorigenic and finally malignant state. Malignant cells then grow beyond the regular tissue boundaries, invade surrounding tissue and finally spread throughout the body.

Although there are a large variety of different types of tumors, most forms of cancers seem to undergo similar changes in cellular characteristics. These have been termed the hallmarks of cancer[1,2]. They include self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death, sustained angiogenesis, limitless replicative potential, tumor promoting inflammation, reprogrammed energy metabolism and, lastly, tissue invasion and metastasis. The reason why cancer pathologies do differ and form the complexity observed when comparing different cancer types or cancer patients is that although most cancers undergo these hallmarks of cancer, they do so based on different genetic or environmental reasons and in varying chronological order.

Additional complexity of the disease arises from the fact that a tumor is not a homogenous tissue but comprises a heterogeneous cell population with differing plasticity, including cancer stem cells. Furthermore, tumors are surrounded by a complex tumor microenvironment, which is build up during the course of the multistep tumorigenesis described above[3]. This tumor microenvironment, also called tumor stroma, is made up of numerous cells that are recruited by and interact with the tumor cells. These cellular interactions are reciprocal, allowing both the tumor cells and the surrounding tissue to transform from normal tissue to high-grade malignancies[4]. The cells of the tumor stroma include endothelial cells and mesenchymal pericytes aiding in angiogenesis, immune inflammatory cells facilitating tumor promoting inflammation and various stromal cells of mesenchymal origin, such as cancer associated fibroblasts (CAF). These stromal cells supply growth factors and cytokines to the cancer cells as well as extracellular matrix proteins to facilitate the framework of the tumor microenvironment.

The final step of cancer progression is the spread of tumor cells from the primary tumor to distant metastatic sites. This step is normally responsible for most of cancer lethality with metastatic tumor growth disrupting the function of vital organs such as lungs, liver and bone marrow.

In order for cancer cells to successfully metastasize to distant organs they need to invade the surrounding tissue of the primary tumor and then intravasate into lymphatic or blood vessels for dissemination throughout the body. Once the circulating cancer cells have reached the potential

site of metastasis they need to interact successfully with the microenvironment at the metastatic site. Paracrine factors from the microenvironment facilitate adherence to the vessel wall and extravasation into the surrounding tissue. The microenvironment also helps facilitate proliferation, evasion of apoptosis and angiogenesis; all contributing to the formation of a new tumor[5]. This essential requirement for cancer cells to closely interact with their metastatic surrounding is the reason why metastasis formation is a non-random event and why cancers differ in their propensity to metastasize to certain organs. This phenomenon has been termed the 'seed and soil' hypothesis, describing that metastasizing cancer cells and the site of metastasis need to match and contradicts the opposing view that metastatic dissemination occurs solely through mechanical factors caused by the anatomical structure of the vasculature[6-8].

1.2. Breast and prostate cancer metastasize to bone

Breast and prostate cancer are both the second most common cancer in females and males respectively. For both cancers the skeleton is the preferred site of metastasis in over 70% of breast and prostate cancer patients. The majority of bone metastases are found in the highly vascularized metaphyseal bone at the end of long bones, ribs and vertebrae[9]. Once the tumors have metastasized to the bone they cause severe morbidity and are considered incurable, because the homeostasis of bone formation and bone degradation becomes out of balance leading to a loss of bone integrity resulting in skeletal complications[10].

The healthy bone microenvironment is made up of mineralized extracellular matrix and the two key cell types, osteoclasts and osteoblasts (Figure 2). Osteoclasts, which are of hematopoietic origin, are able to secrete acid and a number of proteases demineralizing and degrading the bone matrix. Osteoblasts on the other hand are of mesenchymal origin and are responsible for the secretion of the organic matrix, which is then calcified to form the mineralized bone tissue. Not only do osteoblasts and osteoclasts counteract each other functionally, but they also regulate each other with osteoblasts enhancing osteoclast maturation and activation. This occurs via the secretion of a number of factors including macrophage colony stimulating factor (M-CSF) aiding in maturation of progenitor cells as well as receptor activator of nuclear factor (NF)-κB ligand (RANKL) leading to activation of osteoclasts[11]. In contrast, osteoclast maturation can also be inhibited by osteoblasts via osteoprodegrin (OPG)[12]. Osteoblast maturation is also tightly regulated by transforming growth factor β (TGF-β) or bone morphogenetic proteins (BMP), which are stored in the mineralized bone matrix and can be released from it upon osteoclast activity.

This finely tuned cycle of osteoblast as well as osteoclast maturation and function is disrupted upon metastasis formation. In general, bone metastasis can be classified as predominantly osteolytic or osteoblastic[13]. Breast cancer induces mainly osteolytic lesions in which bone tissue is lost and

patients experience severe pain, pathological fractures and spinal cord compression. Prostate cancer on the other hand induces mainly osteoblastic lesions with excessive bone formation of poor quality, causing pathological fractures[14].

The progression of tumor growth in bone metastasis depends on the reciprocal interaction between the metastasizing cancer cells with osteoblasts and osteoclasts in the bone microenvironment. Cancer cells can secrete stimulating factors such as parathyroid hormone related peptide (PTHRP), enhancing osteoclast activity[15], or endothelin-1, inducing osteoblast proliferation[16]. This leads to enhanced bone turnover and therefore an enhanced release of potent growth factors from the bone matrix. These can then feed back to the tumor to increase cancer cell proliferation. This phenomenon has been termed the 'vicious cycle of bone metastasis'[10].

Bone tissue together with immobilized growth factors are an extremely fertile soil for tumor cells once they have gained a foothold in the bone microenvironment, rendering this stage of cancer incurable. The focus must therefore lie on understanding how initial metastases are formed in order to prevent them in a clinical setting. The problem is that although the processes occurring in bone metastasis growth are understood to a certain degree, the question of how tumor cells gain an initial foothold in the bone remains unresolved. One idea which is currently being discussed is the concept of the pre-metastatic niche[17]. It describes a phenomenon in which the primary tumor is able to prepare the site of metastasis for subsequent colonization. This could be potentially mediated by endocrine factors secreted by primary tumor cells rendering the bone marrow more susceptible for circulating tumor cells. PTRHP, for example, is produced by a number of primary tumors and at the distant site of the bone it enhances bone resorption and the release of the chemokine CCL2 by osteoblasts and endothelial cells[18]. CCL2, in turn, is known for its proliferative[19] and chemotactic[20] effect on prostate cancer cells. Another potential mechanism through which cancer cells could initiate colonization of the bone is by mimicking the homing process of hematopoietic stem cells (HSC) [21,22]. This process, in which HSC present in the blood circulation are guided into their stem cell niche in the bone, is regulated by chemotactic gradients secreted by MSC also residing in the HSC stem cell niche[21,22]. Although these mechanisms appear to play a role, the process of initial bone metastasis formation remains elusive and in need of further investigation.

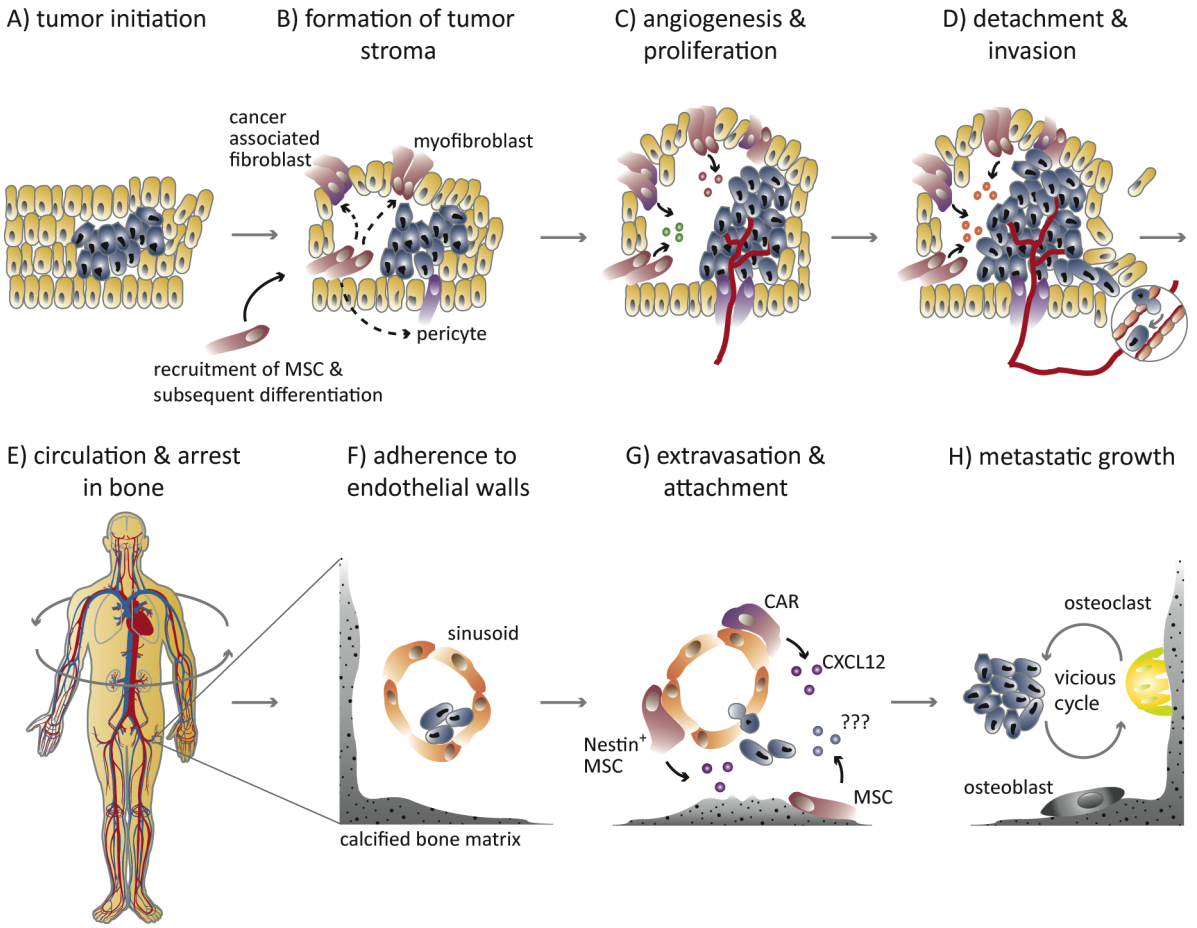


Figure 1: Steps in cancer progression and bone metastasis formation.
A: DNA damage induces cellular transformation of normal cells to cancerous cells leading to unbalanced cancer cell growth. **B:** During tumor growth, MSC are recruited to the primary tumor, participate in the formation of the tumor stroma and differentiate into numerous stromal cells. **C:** These stromal cells enable enhanced growth and vascularization of the tumor through secretion of growth and angiogenic factors. **D:** Invasion of tumor cells into the surrounding tissue is a three-step process requiring attachment of the cancer cells to, proteolytic digestion of and locomotion through the extracellular matrix of the tumor stroma and the adjacent tissue. This is enhanced by MSC-derived chemokines. Tumor cells can then enter the circulation through thin-walled venules of the blood or lymphatic system. **E:** Transport of tumor cells throughout the body. **F:** Tumor cells become entrapped in capillaries and sinusoids of the bone marrow and adhere to endothelial cells. **G:** Adhered cancer cells extravasate past the endothelial cells into the bone marrow space along gradients of chemotactic factors secreted by cells of the bone marrow niche. This extravasation might be very similar to the homing process of hematopoietic stem cells into the bone marrow niche. In this process (described in more detail in Figure 2) the chemotactic gradient attracting the hematopoietic stem cells is formed by the chemokine CXCL12. CXCL12 is secreted by various MSC including CXCL12 abundant reticular cells (CAR) and Nestin^{+} MSC. It is still a matter of debate which further factors, secreted by MSC or other cells, are also involved in cancer cell extravasation into the bone marrow niche. **H:** Once the metastasizing cancer cells have gained a foothold in the bone, metastatic tumor growth induces enhanced remodeling of bone tissue by bone degrading osteoclasts and bone forming osteoblasts. This causes the release of growth factors previously entrapped in the calcified bone matrix further driving tumor growth. This phenomenon is called the 'vicious cycle of bone metastasis'.

1.3. Mesenchymal stem cells in cancer

1.3.1. Mesenchymal stem cells

Mesenchymal stem cells (MSC), also called mesenchymal stromal cells, were initially isolated from human bone marrow[23] and later on from multiple tissues including synovia, muscle, fat, dermis and amniotic tissue. MSC are best known for their ability to differentiate into many tissues of mesodermal origin, such as bone, cartilage or connective tissue[24], as well as for their immunomodulatory characteristics[25]. Due to their diverse differentiation potential, the relative ease of their isolation from multiple tissues and the fact that they can be expanded *in vitro*, MSC are regarded as a promising tool for clinical applications. This is why MSC are currently subject of over 300 clinical trials (www.clinicaltrials.gov) although their true identity is still a matter of debate.

MSC cultivated *in vitro* are currently defined by their plastic adherent growth, their differentiation potential under specific conditions *in vitro*, and a panel of surface markers which should either be expressed (CD73, CD90, CD105) or not expressed (CD34, CD45, CD14 or CD11b, CD79a or CD19, HLA-DR), as proposed by the International Society of Cell Therapy[26]. Nonetheless, these criteria are not sufficient and fail to clearly define MSC and distinguish them from other stromal cells such as fibroblasts. Furthermore, the *in vivo* identity of MSC remains unknown[27-29].

Not only are MSC elusive regarding their true identity, but they also show a high degree of complexity regarding their functional properties, of which their differentiation ability is only part of their capabilities.

First and foremost, MSC have the ability to exit their niche and migrate to sites of inflammation or tissue damage allowing them to participate in tissue regeneration[29]. Once at the site of tissue damage, MSC can differentiate into a number of potentially required cell types. More importantly, MSC act as trophic mediators via the secretion of bioactive factors; initiating, aiding and enhancing the regeneration process. This is achieved by the inhibition of apoptosis and fibrosis, immune modulation, chemoattraction of immune cells to the site of damage, enhancing angiogenesis and finally stimulation of mitosis as well as differentiation of tissue specific progenitor cells[30]. Anti-apoptotic effects of MSC were described in mouse models of ischemia or acute kidney injury and the authors of these studies could show that these effects were based on MSC-derived factors including VEGF, HGF, bFGF or TGF-β amongst others[31,32]. HGF and bFGF not only have an anti-apoptotic effect but also are important in the inhibition of fibrosis[33,34]. The immune modulatory properties of MSC were initially shown in studies describing the inhibition of T-cell proliferation[35]. Since then, MSC were shown to modulate B-cell proliferation[36,37], inhibit NK cell activation[38] and alter the cytokine secretion profile of macrophages[39] and dendritic cells[40]. All of these studies

showed an important role of prostaglandin 2 as well as TGF-β and HGF in regard to T- cells and TGF-β in regard to NK cells. It is known as well that MSC can secrete a vast array of chemoattractive factors which allow MSC to further aid in tissue regeneration or remodeling by attracting other cells such as monocytes or hematopoietic and endothelial progenitor cells to the site of injury[29,41]. In addition to attracting endothelial precursor cells, MSC were also shown to secrete a number of angiogenic factors including bFGF, VEGF, MCP-1, IL-6[42]. Finally, studies have described that MSC stabilize vasculature formation *in vitro*[43] and *in vivo*[44]. Based on their abilities and complex secretome, MSC can therefore be seen as powerful mediators in processes related to tissue inflammation and damage.

1.3.2. Mesenchymal stem cells in tumor stroma formation and cancer progression

Studies have shown that bone marrow MSC migrate towards different types of primary tumors and integrate into the tumor microenvironment[45-47]. These tumors have been described as sites of chronic inflammation, constant tissue remodeling and wound healing. Indeed, many of those inflammatory factors found at sites of tissue damage can also be localized to tumors. Hence it seems plausible that the same tropism that guides MSC to the sites of tissue damage and inflammation is also believed to initiate the observed migration of MSC towards tumors[48,49].

There has been an ongoing discussion what effects the MSC exert once they reach the site of the tumor. Although there are studies showing anti-tumor effects of MSC[50,51], a growing body of evidence suggests that MSC promote cancer progression in many cases. They potentially do so by immune response suppression[52], stimulation of angiogenesis[46] and proliferation[53], inhibition of apoptosis[54] as well as enabling enhanced extravasation, migration and metastasis[4]. Based on their multi-lineage differentiation potential, they can furthermore differentiate into tumor stroma associated cells including pericytes, cancer associated fibroblasts and myofibroblasts which themselves can effect cancer progression[55-57]. Often, these described interactions between the tumor and its stroma are reciprocal, with cancer cells and MSC regulating each other and by this further driving the progression of cancer[4,58]

1.3.3. Mesenchymal stem cells and their bone marrow niche

Although the *in vivo* identity of MSC is still a matter of debate[29], it is believed that MSC found in the bone marrow reside in a niche termed the bone marrow stem cell niche or hematopoietic stem cell niche. What this niche actually looks like and how it functions is still not fully understood. Regardless of the unanswered questions, this commonly accepted concept of a bone stem cell niche describes an area in the bone marrow in which mesenchymal and hematopoietic stem cells localize and interact[59]. HSC are multi-potent progenitor cells that give rise to all mature blood and various immune cells[60]. Tracing experiments with HSC revealed that they localize to the bone endosteum lined by osteoblasts and additionally localize close to sinusoidal endothelium[61,62]. These findings initiated the idea of multiple niches in the bone, including a so-called endosteal and a perivascular niche. Nonetheless, a clear distinction of separate niches seems problematic as multiple cellular components of either niche are found in the other[63]. It also needs to be noted that HSC are believed to change their localization in the niche in response to stress, making the concept of the HSC niche even more complex[64]. For the sake of simplicity, here the bone marrow niche will be considered as one entity (Figure 2).

The bone marrow stem cell niche contains a number of cell types with functional importance. Among these are osteoblasts which line the bone endosteum and can be found in close proximity to HSC under physiological conditions[65] and after bone marrow transplantation[66]. Osteoblasts secrete several factors including the extracellular matrix (ECM) protein osteopontin, binding to HSC via β_1 integrin resulting in enhanced HSC quiescence[67]. Furthermore, they secret CXCL12 and N-cadherin involved in HSC maintenance and retention as well as angiopoetin 1, membrane bound stem cell factor and thrombopoietin which aid in regulation of HSC quiescence[68,69].

The vasculature of the bone marrow is comprised of sinusoids, which are lined by a special type of endothelial cells termed bone marrow sinusoidal endothelial cells (BMSEC). These are fenestrated and have a marginal basement membrane with minimal pericyte coverage. Sinusoids are in close proximity to mesenchymal reticular cells and are embedded in extracellular matrix proteins secreted by this subpopulation of MSC. In addition to acting as a barrier between niche and vasculature, it has been suggested that BMSEC might influence HSC, based on co-localization of these cells[70]. Nonetheless, it is still not clear whether BMSEC have a direct influence on HSC, which is distinct from the reticular cells and the general surrounding[71].

MSC are the major player in the bone marrow stem cell niche. For one, MSC in the endosteum can act as progenitor cells of the functionally important osteoblasts. This could be shown for a subclass of MSC termed Nestin$^+$ MSC. Furthermore, these cells not only have osteogenic differentiation potential but also express high levels of HSC maintenance factor transcripts including CXCL12, SCF,

angiopoetin 1, IL-7, vascular cell adhesion molecule 1 (VCAM1) and osteopontin[63]. A further subclass of MSC found residing close to sinusoids and expressing high amounts CXCL12 are termed CXCL12 abundant reticular (CAR) cells. CAR cells actively regulate HSC mobilization into the bloodstream and homing back into the bone niche by secretion of CXCL12 and SCF, with elevated concentrations in the niche supporting HSC retention and homing or reduced concentrations allowing the exit of HSC from the niche[71,72].

Interestingly, MSC do not necessarily execute their functions in an isolated fashion but interact with and are regulated by their environment. Nestin$^+$ MSC undergo crosstalk with adrenergic nerve fibers of the sympathetic nervous system which has been shown to play a role in mobilization and release of HSC by secreting noradrenaline, targeting the β_3-adrenergic receptor of MSC and altering the secretion of CXCL12 by MSC[72]. Macrophages, also present in the bone marrow stem cell niche, were found to be key regulators of HSC mobilization from the niche[73,74]. Previous studies suggested that this macrophage-triggered HSC mobilization is also mediated via altering the secretion of CXCL12 by MSC[75].

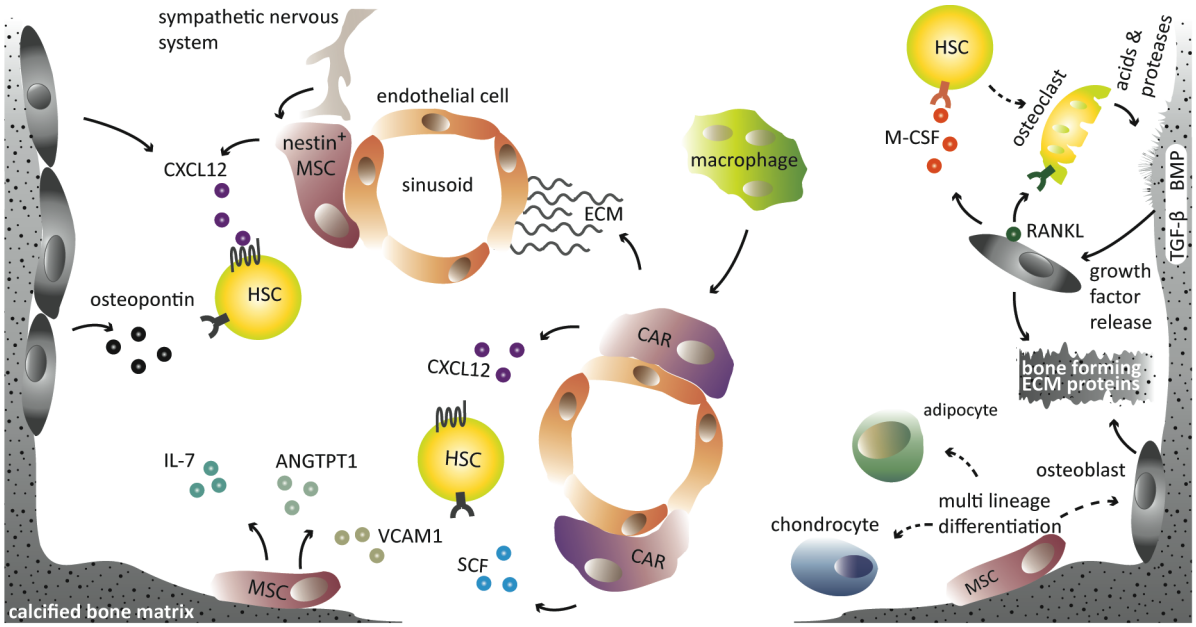


Figure 2: Cellular components of the bone marrow niche.
The bone marrow stem cell niche is home to the hematopoietic stem cells (HSC), a place in which their proliferation and differentiation is tightly regulated. Main regulators of HSC function and cellular status are different subtypes of mesenchymal stem cells (MSC) including CXCL12 abundant reticular cells (CAR) and Nestin$^+$ MSC as well as MSC-derived osteoblasts secreting various growth factors. Furthermore, MSC secrete CXCL12, enabling retention of HSC in and homing of HSC into the niche.
The spatial composition and the physical integrity of the bone is enabled by a balanced activity of MSC-derived bone forming osteoblasts and HSC-derived bone degrading osteoclasts. ANGPT1, angiopoietin-1; BMP, bone morphogenic protein; Il-7, interleukin-7; M-CSF, macrophage stimulating factor; RANKL, RANK ligand; SCF, stem cell factor; TGF-β, transforming growth factor-β.

1.3.4. Extracellular matrix proteins in the bone marrow niche

Despite progress on understanding the cellular components of the bone marrow stem cell niche, the extracellular matrix of this niche, synthesized by non-hematopoietic stromal cells, remains poorly understood. This stands in contrast to the suspected relevance of ECM molecules in the bone marrow niche as hematopoietic progenitor cells express over 20 different adhesion receptors which enable the cells to interact with a multitude of ECM proteins[76].

Among these are a number of fibrous ECM proteins which are present in the niche and secreted by cells of mesenchymal origin. These fibrous ECM proteins include type I, III, IV and VI collagen as well as fibronectin[77]. Matrix proteins, acting as structural components, make up the three-dimensional framework of the niche and define physical properties such as topography, porosity, stiffness and rigidity. These physical properties of the environment can influence general biological processes such as cell division, polarity or migration[78] as well as stem cell specific processes such as the equilibrium between quiescence, self-renewal and differentiation[79,80]. In addition to the physical cues, ECM proteins can bind growth factors and act as a reservoir, thereby regulating their availability and even establishing gradients[81]. In an environment, such as the bone stem cell niche, in which the distribution of growth factors requires spatial and temporal regulation, it seems plausible that ECM proteins may play a functional role in this context.

Besides their passive role as structural components and growth factor reservoir, ECM proteins also actively participate in the organization of the bone marrow stem cell niche. Homing of HSC into the bone marrow niche is primarily guided by growth factors and chemokines such as CXCL12. None the less, studies have also shown that after transplantation, the ECM glycosaminoglycan hyaluronan is required in the niche as it cooperates with CXCL12 guiding the HSC into their niche via the hyaluronan receptor CD44[82].

A similar study using osteopontin knockout mice showed that osteopontin was required for successful engraftment and localization of HSC to the endosteum after transplantation, further emphasizing the functional importance of ECM proteins in HSC homing and engraftment into the bone marrow stem cell niche[67].

The glycoprotein tenascin-C is an ECM protein restricted to sites of tissue damage[83] or inflammation[84] in an adult. Experiments in tenascin-C knockout mice showed that tenascin-C was not required for steady state hematopoiesis but was up-regulated in the bone marrow stroma and endothelial cells during hematopoietic recovery after bone myeloablation. These mice showed increased lethality and reduced hematopoietic regeneration as well, emphasizing the role of tenascin-C in hematopoietic regeneration[85].

The heterotrimeric laminins, in particular laminin 411, 421, 511 and 521, were also identified in human bone marrow and were shown to exert adhesive interactions with $CD34^+$ cell lines[86].

All in all, ECM proteins are an essential component of the bone marrow stem cell niche providing a spatial scaffold for cells as well as influencing these by physical cues and regulation of growth factor availability.

1.3.5. Mesenchymal stem cells in bone metastasis

MSC are an essential cellular component of the HSC niche in the bone marrow making it a hospitable environment. This not only assures the maintenance of the HSC progenitor pool throughout life, but could also make the bone marrow niche a perfect 'soil' for circulating cancer cells leading to cancer cell attachment and subsequent metastatic growth in the bone marrow niche.

In line with this hypothesis are observations describing the co-localization of transplanted HSC and prostate cancer cells to the bone marrow after lethal irradiation of mice. These results suggest a competition between homing HSC and cancer cells metastasizing into the bone marrow niche[21]. Homing into the niche is orchestrated by different subtypes of MSC, secreting a number of factors including the chemokine CXCL12. Like HSC, breast cancer[87] and prostate cancer[87,88] cells are reported to respond to CXCL12 gradients from the bone marrow niche, as neutralizing antibodies against the CXCL12 receptor CXCR4 or CXCR7 show a significant reduction in engraftment and growth of tumor cells in bone. In addition to CXCL12, prostate cancer cells are also known to respond to CCL2 gradients formed by MSC-derived osteoblasts in the niche, enabling homing and stable integration into the niche[18,19]. The struggle between cancer cells and HSC for niche predominance is not limited to the homing process. Cancer cells gaining a foothold in the bone marrow niche have also been shown to push HSC out of their niche, affecting HSC homeostasis which results in peripheral blood changes including infection or anemia during late stage progression of prostate cancer[88].

MSC are also being discussed in the concept of a pre-metastatic niche. This describes a phenomenon in which the primary tumor prepares the site of metastasis for subsequent colonization[89]. In this context, bone marrow-derived hematopoietic cells have been reported to migrate to sites of future metastasis and initiate fibronectin production[90] and inflammatory chemokine secretion[91], facilitating successful homing of cancer cells to the primed metastatic site. In the bone, similar processes can be witnessed with endocrine factors such heparanase[92] or PTHRP[15] being secreted by the primary tumor and altering functional behavior of bone marrow cells in the bone marrow stem cell niche. With MSC exerting key regulatory functions in the bone

marrow niche, it seems plausible that they could also be involved in the priming of the bone marrow niche for metastasis growth.

In addition to initial cancer cell homing, the cellular compartment of the bone marrow stem cell niche enables tumor dormancy in the bone. Dormancy describes a cellular state of growth arrest which allows the tumor cells to remain in the bone marrow for years and, most importantly, survive chemotherapeutic treatment, as this targets only the fast cycling cancer cells[93]. Tumor dormancy is therefore a major cause for cancer relapse after treatment and initial remission and occurs in about 20 to 40% of breast and prostate patients in a matter of years or even decades[94,95]. The topic of tumor dormancy is still in need of a large investigative effort. Nonetheless, initial accomplishments in understanding this phenomenon have been achieved. Whether HSC in the bone marrow are found in a quiescent or proliferative state depends on signals from the niche microenvironment. This key function of the HSC niche ensures the supply of blood and immune cell progenitors throughout life[68]. Cancer cells metastasizing to bone, seem to respond to the niche microenvironment similarly to HSC. Annexin II, for example, supplied by the niche microenvironment has been shown to induce growth arrest in prostate cancer cells and is believed to be a facilitator of tumor dormancy in bone[96]. The role of MSC in this context remains unknown.

The fact that cancer cells metastasize to the bone marrow stem cell niche, has become common ground in the field of bone metastasis research. The progression of metastatic disease has also been studied for some time and considerable insight has been gained into how cancer cells interact with the bone marrow niche. Nonetheless, the complete picture of how cancer cells interact with the bone marrow niche and its stromal constituents remains unclear, especially when it comes to the processes occurring during initial colonization of the bone by cancer cells.

With bone metastasis formation being one of the most detrimental steps in cancer progression, a better understanding of how bone metastases are initially formed is key to successfully targeting bone metastasis and by that helping patients suffering from breast or prostate cancer.

1.4. Aim of this thesis and experimental approach

In order to better understand the role of MSC in metastasis formation the present work was aimed to model how MSC induce migration of cancer cells. A fundamental idea was to choose an unbiased approach and to take the complete secretome of MSC into consideration. This approach allowed omitting guesses on previously described potential chemotactic factors. To address this idea, I used primary human bone marrow MSC isolated from healthy donors together with established prostate and breast cancer cell lines able to metastasize to bone. The experimental approach was initiated with the production of MSC cell culture supernatant under protein-free and chemically defined conditions. Cell culture supernatant then underwent multistep-processing including filtration, concentration, size exclusion and ion exchange chromatography in subsequent manner. After each processing step, migration-inducing activity towards cancer cells was determined. The use of transwell migration chambers combined with impedance measurements allowed quantitative analysis of cancer cell migration in a time-resolved fashion and, most importantly, high-throughput format. Active chromatography fractions were analyzed for protein content by antibody array or mass spectrometry and transferred to further processing. Finally, candidate proteins potentially mediating the observed migration of cancer cells towards MSC were tested and validated using recombinant proteins and RNAi approaches (Figure 3).

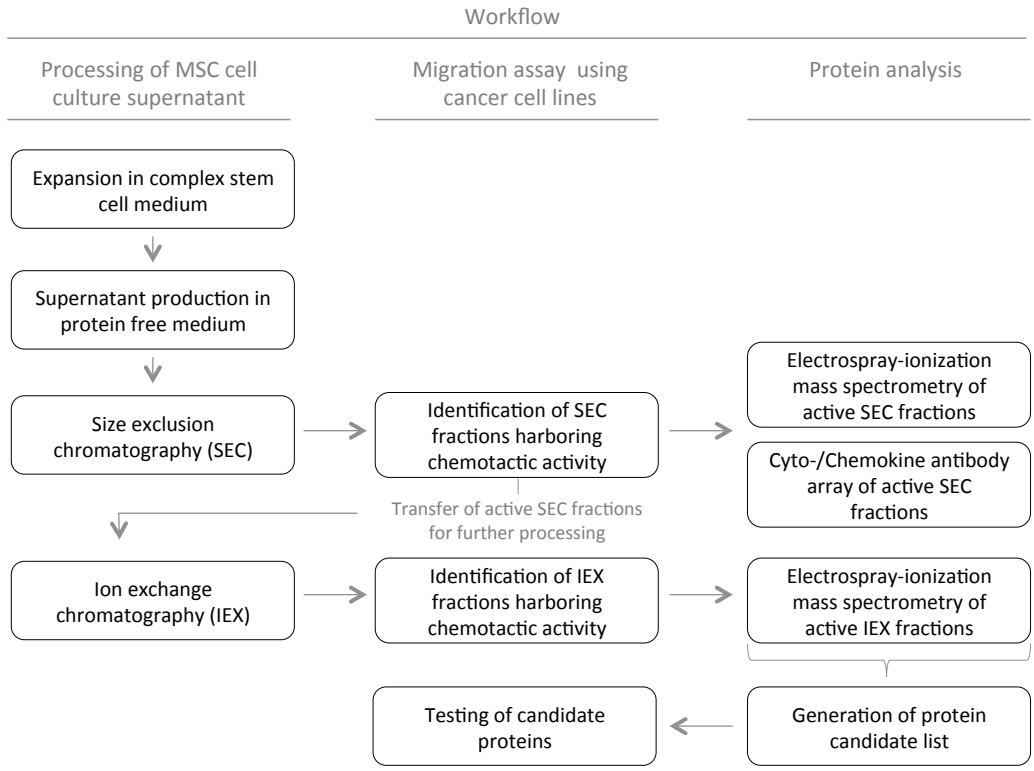


Figure 3: Workflow to identify secreted proteins inducing migration of cancer cells towards mesenchymal stem cells.

2. Results

2.1. MSC induce rapid migration of cancer cells

In order to investigate the interactions between MSC and cancer cells, initial experiments addressed the questions of whether MSC do induce cancer cell migration and which experimental set up is best suited to monitor this migratory interaction.

Trial experiments showed that the migration-inducing activity of MSC cell culture supernatant was best analyzed in transwell migration chambers of the Acea xCELLigence system. In this system, supporting up to 48 wells, cells migrating through the transwell membrane along a chemotactic gradient from one cell culture chamber to the other induced an electronic signal which accumulated over time. This device therefore allowed a non-invasive, high-throughput and real-time analysis of cancer cell lines migrating along a potential chemotactic gradient caused by the cell culture supernatant of MSC (Figure 15).

With this set-up it could be shown that MSC induced a rapid migration response of the prostate cancer cell line PC3 and to a somewhat lesser extent the breast cancer cell line MDA-MB-231 with a full migration response in as little as two hours (Figure 4 A). Both of these cell lines have in common that they have been reported as cancer cell lines to model bone metastasis formation *in vivo*[97,98].

In order to address whether this observed migratory ability of cancer cells was specific for cells able to form bone metastasis *in vivo*, additional prostate cancer cell lines were tested, known to have no or only a reduced potential to induce bone metastasis in animal models. In contrast to PC3 cells, the prostate cancer cell line LNCaP[99] and VCaP[100] did not show migration towards MSC supernatant (Figure 4 A).

The xCELLigence measurements are not only influenced by cell number, but also by cell adhesion, cell size and cell spreading[101-103]. These are characteristics differing between cell types. In order to reliably compare migration of the different cell lines investigated, migration dynamics were also analyzed as they are less influenced by cell adhesion and spreading. Analysis of dynamics in the first hour of migration confirmed the previous observations with PC3 and MDA-MB-231 cells showing high migration dynamics in contrast to LNCaP and VCaP cells (Figure 4 A').

As the xCELLigence system does not allow visual inspection of the migrated cells, results obtained with this system were confirmed with classic Boyden chamber transwell assays. A full migration response of PC3 cells after four hours was observed with cells showing full confluency on the membrane. MDA-MB-231 cells also showed a clear migration response after two hours compared to the negative control. Even after 48 hours, LNCaP cells failed to show directed migration towards the cell culture supernatant of MSC in comparison to a negative control (Figure 4 B).

As MSC are of heterogeneous nature[29,104] and the cells used in this study are primary cells, the observed potential of MSC to induce cancer cell migration required confirmation with several MSC donors. In comparison to donor 1, two further donors induced a migration response of approx. 70% and 85% for the prostate cancer cell line PC3 (Figure 4 C). These results could be confirmed when looking at the PC3 migration dynamics the respective cell culture supernatant samples induced. Donor 2 induced over 60% and donor 3 80% migration dynamics relative to donor 1 (Figure 4 C').

Stromal cells in general are discussed for their contribution to cancer progression and metastasis formation[3,105]. In order to understand whether the observed induction of migration was MSC specific or held true for other stromal cells as well, migration of PC3 cells towards two different fibroblast cell lines was investigated. Neither the cell line HS68 nor HFF1 was able to induce a migration response of PC3 cells comparable to MSC. An early migration response towards both cell lines was present but reached only 10% compared to MSC donor 1. These findings were confirmed when analyzing the dynamics of the early migration response towards the fibroblast cell lines which reached only 7% respectively compared to MSC donor 1. Interestingly, fibroblasts were able to induce a second phase of migration after 12 hours. Nonetheless, the two migration phases did not accumulate to more than 30% migration response compared to MSC donor 1 (Figure 4 C, C').

In conclusion, these initial experiments showed that cancer cell lines able to metastasize to bone migrate towards MSC in a rapid fashion. This migration response was induced by MSC from multiple donors, whereas fibroblasts were not able to induce comparable migration.

After establishing that the observed induction of cancer migration was reproducible and specific for MSC, the mediators required for this cancer cell – MSC interaction were isolated through a series of downstream processing steps of the MSC cell culture supernatant.

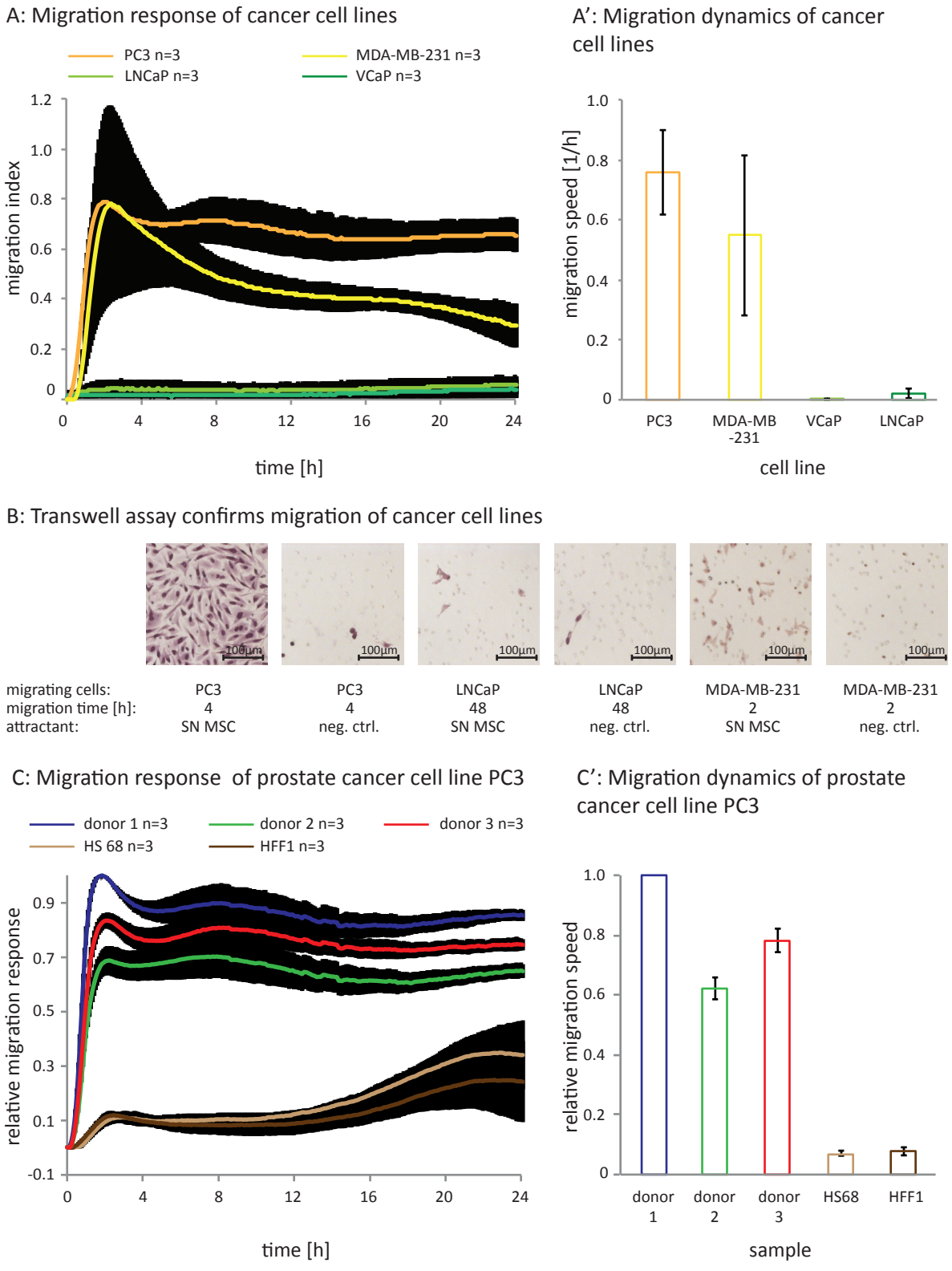


Figure 4: MSC induce rapid migration of cancer cells able to form bone metastasis.
A: Migration response of cancer cell lines towards MSC supernatant in xCELLigence transwell system. **A':** Average dynamics of early migration response displayed in (A) between 00:30 h and 01:15 h. **B:** Images of Boyden chamber transwell membranes with attached cells after migration towards MSC supernatant. Cell lines, duration of migration and attractant are indicated for the respective images. **C:** Migration response of PC3 cells towards fibroblast and MSC supernatant of multiple donors in xCELLigence transwell system. **C':** Average dynamics of early migration response displayed in (C) between 00:30 h and 01:15 h. Average values based on n=3 experiments, error bars indicate standard deviation (A – C').

2.2. Purification step 1: Size exclusion chromatography of MSC cell culture supernatant

In order to be able to isolate the migration-inducing factors of interest from the cell culture supernatant of MSC in a manageable time and work-load frame, reproducibility of the individual processing steps was crucial and the focus of process optimization. Reproducibility was also considered a key process parameter, as the MSC are primary cells isolated from individual donors. On account of this, variations in the secretome of the MSC populations were to be expected. To minimize variations in protein concentrations, cells were grown to full confluency at passage 5, cell culture supernatant was produced for 48 hours in protein-free as well as chemically defined medium and stored at -20°C. For downstream processing, supernatant was filtered and concentrated via ultrafiltration to allow size exclusion chromatography (SEC) as the initial downstream processing step.

2.2.1. Size exclusion chromatography yields highly reproducible fractionation despite MSC donor heterogeneity

Reproducible SEC fractionation was mandatory, as the protocol required pooling of several same molecular weight fractions of separate SEC runs for subsequent processing.

After testing various process parameters, the use of a Superdex 200 HR 10/30 column with 50 mM tris-buffer containing 150 mM NaCl as the fluid phase, combined with a loading of 500 µl sample volume and a flow rate of 250 ml/min allowed for SEC fractionation with highest reproducibility and reliability. Comparison of average FPLC chromatograms of three separate donors revealed variations in peak height but very low differences in the retention volumes at which the peaks eluted off the column (Figure 5 A). Low variations in retention volume were also observed when comparing retention volumes of separate FPLC runs of one MSC donor (technical replicates) and by comparing retention volumes of separate FPLC runs of two different MSC donors with each other (biological replicates) (Figure 5 B, C).

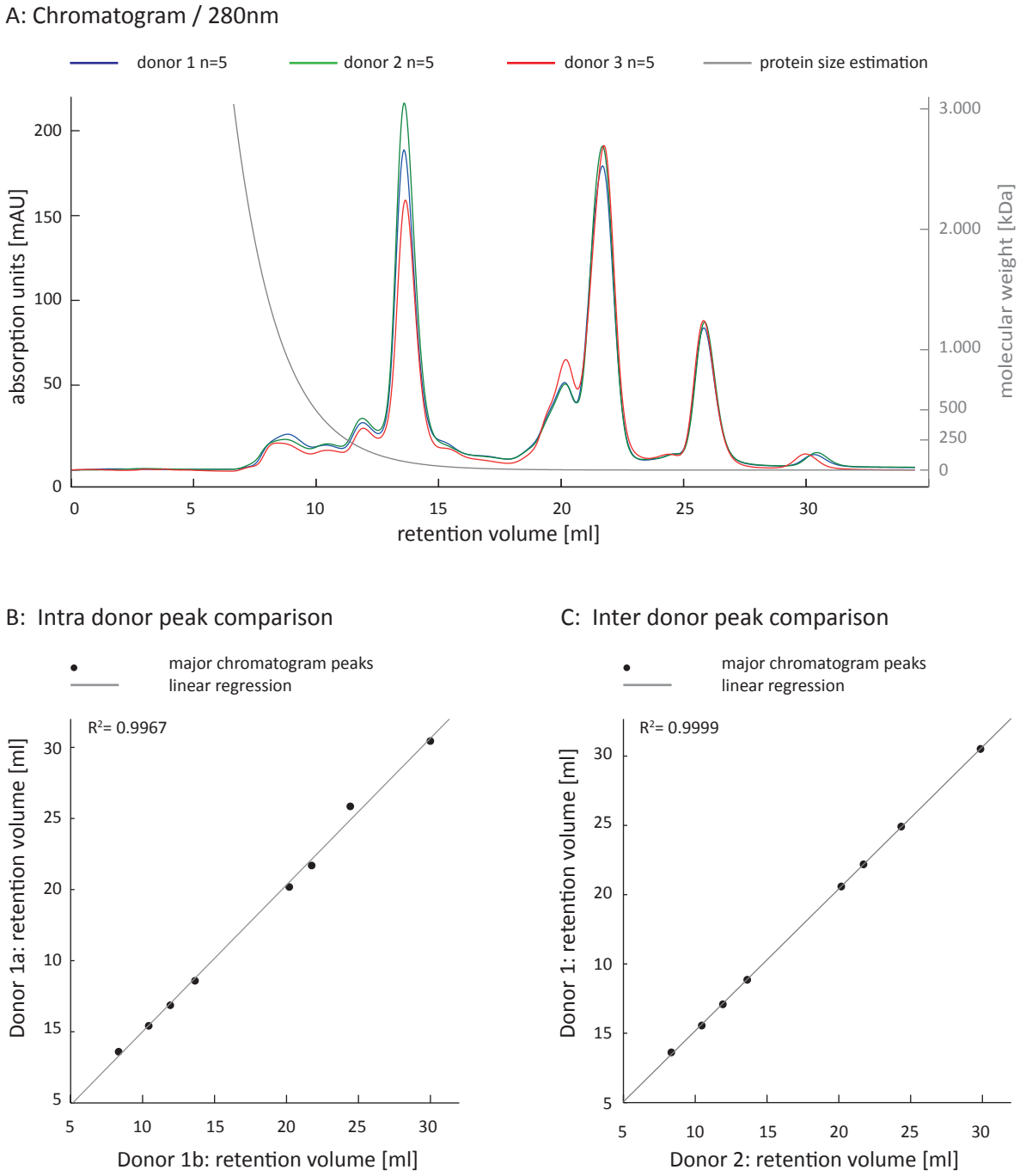


Figure 5: Size exclusion chromatography yields highly reproducible fractionation despite heterogeneity of primary MSC.
A: Size exclusion chromatograms of proteins detected at 280 nm during fractionation of cell culture supernatant of MSC. For each technical replicate 16 ml MSC cell culture supernatant were filtered, concentrated and fractionated by size exclusion chromatography. Results for each donor were confirmed by five technical replicates and are presented as average values. Protein size estimations are based on calibration experiments and extrapolation. **B,C:** Correlation plots of main peak retention volumes between technical replicates of the same MSC donor (B) and between biological replicates of two different MSC donors (C) (Pearson correlation is indicated).

2.2.2. High-molecular-weight proteins induce breast and prostate cancer cell migration

SEC fractions of three MSC donors were tested for their migration-inducing activity towards the prostate cancer cell line PC3. Fractions of up to 31 ml retention volume inducing a clear absorption signal in the chromatogram (Figure 5 A) were analyzed for their migration-inducing potential. These fractions harbored proteins, protein fragments and single amino acids in an estimated size of down to 100 Da determined by extrapolation calculations of column calibration experiments. Based on the column bed volume of 24 ml, the final fractions exceeding 24 ml retention volume were expected to be free of proteins or amino acids. The observed absorption signals must therefore be based on variations in salt ion compositions of the buffer. As calcium ions are known to play a role in homing of HSC to the bone[106] and can have a profound effect on cancer cell migration[107], proliferation[108] and metastasis to bone[109], all fractions up to 31 ml retention volume were investigated for their potential to induce cancer cell migration.

Migration response of PC3 cells towards the SEC fractions was analyzed in relation to the maximum response induced by the unprocessed cell culture supernatant of the respective donor. On average, a strong migration response of over 90% was seen towards SEC fractions harboring proteins or protein complexes with molecular weights ranging from 1500 to 800 kDa. This was followed by a steep decline in migration-inducing activity with a slight elevation in migration response in fractions of around 100 kDa reaching 35% migration response. Afterwards the migration response reached a plateau and fluctuated around 10 to 20% with a gap of no migration towards SEC fractions of around 1 to 2 kDa (Figure 6 A).

Single time point analysis of PC3 migration response after three hours towards the SEC fractions of three MSC donors individually confirmed strong migration-inducing activity towards high-molecular-weight fractions for all three donors (Figure 6 B). In the case of donor 2, the migration response even exceeded migration-inducing activity of the untreated cell culture supernatant. The slightly elevated migration-inducing activity of the 100 kDa fractions could only be confirmed for donor 1 and donor 2. Donor 3 failed to show activity in 100 kDa fractions but instead triggered a migration response at around 1 kDa, in contrast to donor 1 and donor 2. In conclusion, the results show that the migration-inducing activity found in cell culture supernatant of MSC towards PC3 prostate cancer cells was primarily based on high-molecular-weight proteins or protein complexes of around 1000 kDa in size.

In order to assess whether the migration of PC3 prostate cancer cells towards high-molecular-weight proteins was cell line or cancer specific, the breast cancer cell line MDA-MB-231 was also analyzed for its migratory behavior towards SEC fractions of MSC cell culture supernatant. MDA-

MB-231 cells, also known to form bone metastasis *in vivo*, showed overall a slower migration response towards SEC fractions in contrast to PC3 cells that required as little as two hours for a full migration response. In addition, the migration-inducing activity towards this cell line was more broadly distributed compared to PC3 cells that displayed migration towards a more defined set of SEC fractions. Nonetheless, MDA-MB-231 cells showed a similar migration pattern towards SEC fractions of MSC supernatant. For all three donors, high-molecular-weight fractions of 1500 – 800 kDa displayed the highest migration-inducing activity followed by SEC fractions of around 100 kDa (Figure 7). After identifying high-molecular-weight fractions as the main source of migration-inducing activity in MSC supernatant, respective SEC fractions were analyzed for protein content and applied to further downstream processing.

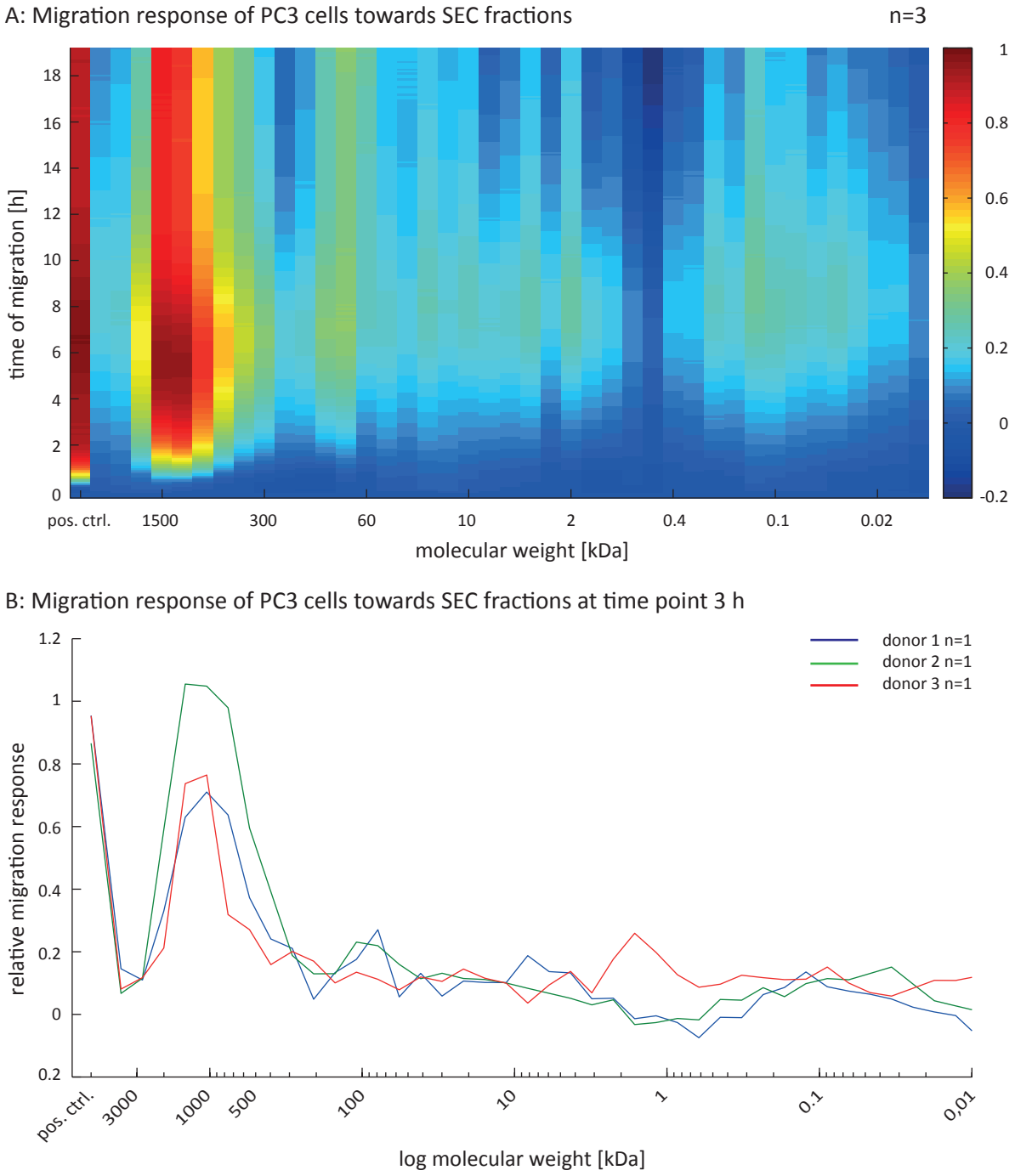


Figure 6: High-molecular-weight proteins induce prostate cancer cell migration.
A: Average migration response over time of prostate cancer cell line PC3 towards SEC fractions of cell culture supernatant of three individual MSC donors. **B:** Individual migration response of prostate cancer cell line PC3 towards SEC fractions of MSC culture supernatant after 3 h. Migration response, determined with the xCELLigence system, is displayed as relative to the maximum response observed towards the unprocessed cell culture supernatant of each donor (pos. ctrl.) respectively (A,B).

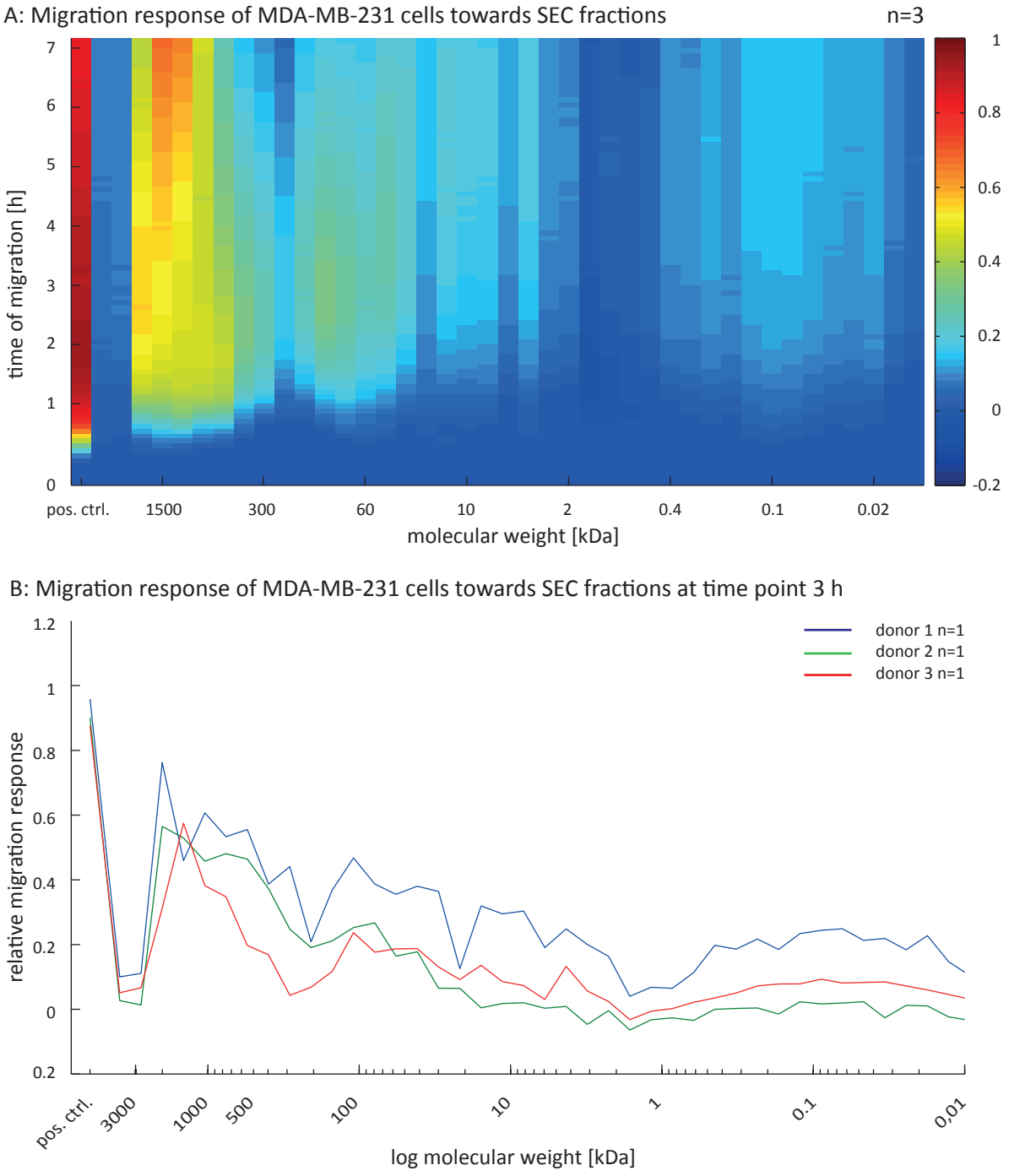


Figure 7: High-molecular-weight proteins induce breast cancer cell migration.
A: Average migration response over time of breast cancer cell line MDA-MB-231 towards SEC fractions of cell culture supernatant of three individual MSC donors. **B:** Individual migration response of breast cancer cell line MDA-MB-231 towards SEC fractions of MSC culture supernatant after 3 h. Migration response, determined with the xCELLigence system, is displayed as relative to the maximum response observed towards the unprocessed cell culture supernatant of each donor (pos. ctrl.) respectively (A,B).

2.2.3. Mass spectrometry identifies extracellular matrix proteins in high-molecular-weight SEC fractions

After establishing that MSC induce migration of prostate and breast cancer cells via high-molecular-weight proteins or protein complexes, the 1000 kDa SEC fractions of three MSC donors were analyzed by electrospray mass spectrometry[110]. These fractions mainly contained structural extracellular matrix proteins, matricellular proteins, glycoproteins, proteoglycans and other ECM associated proteins (Table 1).

Table 1: Proteins detected by mass spectrometry in high-molecular-weight SEC fractions of cell culture supernatant of MSC.
Proteins listed were identified in samples of two or all three (*) donors. Proteins of bovine origin such as remaining serum albumin or α-2 macroglobulin were discarded from the list as well as classic skin protein contaminations including proteins such as keratins and dermicidin.

Symbol	Name	Description
PGS1*	Biglycan	ECM Proteoglycan
CERU	Ceruloplasmin	Metalloprotein
COL1A1*	Collagen α-1(I) chain*	Fibrillar collagen
COL3A1*	Collagen α-1(III) chain*	Fibrillar collagen
COL6A1	Collagen α-1(VI) chain	Fibrillar collagen
COLCA1	Collagen α-1(XII) chain	Fibrillar collagen
COL1A2*	Collagen α-2(I) chain*	Fibrillar collagen
COL6A2*	Collagen α-2(VI) chain*	Fibrillar collagen
COL6A3*	Collagen α-3(VI) chain*	Fibrillar collagen
PGS2	Decorin	ECM proteoglycan
EMIL1	EMILIN-1	ECM glycoprotein
FBN1	Fibrillin-1	ECM glycoprotein
FINC*	Fibronectin*	ECM glycoprotein
LG3BP*	Galectin-3 binding protein*	ECM β-galactoside-binding protein
GPC1	Glypican-1	ECM proteoglycan
HEMO	Hemopexin	Acute phase plasma glycoprotein
LAMA4*	Laminin subunit α-4*	ECM glycoprotein of basement membrane
LAMB1*	Laminin subunit β-1*	ECM glycoprotein of basement membrane
LAMB2	Laminin subunit β-2	ECM glycoprotein of basement membrane
LAMC1	Laminin subunit γ-1	ECM glycoprotein of basement membrane
NID1	Nidogen-1	ECM matricellular protein
NID2	Nidogen-2	ECM matricellular protein
PTX3*	Pentraxin-related protein PTX3*	Immune response protein
PXDN	Peroxidasin homolog	Heme-containing peroxidase
HTRA1	Serine protease HTRA1	Serine protease
TSP1*	Thrombospondin-1*	ECM matricellular protein
TSP2	Thrombospondin-2	ECM matricellular protein
BGH3	Transforming growth factor-β-induced protein ig-h3	RGD containing ECM protein

2.2.4. Antibody array reveals cytokine-independent migration-inducing activity of high-molecular-weight SEC fractions

Chemokines, a family of cytokines of approx. 10 kDa in size, are extensively discussed in literature in the context of cancer cell migration and as mediators of metastasis[111-113]. Furthermore, it has been reported that chemokines can bind to bone and a number of extracellular matrix proteins, such as fibronectin, which were identified in the high-molecular-weight SEC fractions by mass spectrometry (Table 1)[114,115]. Additionally, it is known that MSC can secrete a number of different cytokines[29]. For these reasons, 1000 kDa and 100 kDa molecular weight SEC fractions of two donors, previously analyzed by mass spectrometry, were additionally investigated for the presence of chemo- and cytokines by antibody array. Of the 72 cytokines tested (Supplementary table 1) ten were identified in the unprocessed cell culture supernatant of both donors tested and an additional three in only one of the two donors. In contrast to this finding, no classic chemokines were found in the 1000 kDa or the 100 kD SEC fractions. Only a very faint presence of VEGFR3 could be shown in the 1000 kDa fraction of one of two donors. VEGFR3 as well as uPAR, TIMP1 and TIMP2 were detected in the 100 kDa SEC fraction of both donors (Table 2).

Table 2: Angiogenic and chemoattractive factors detected in untreated MSC cell culture supernatant and fractions of size exclusion chromatography.
Samples of two donors were analyzed for the presence of a total of 72 factors by antibody array. Factors marked by * were detected in one of two analyzed donors.

Cell culture supernatant (n=2)		
CCL2 (MCP1)	CXCL5 (ENA 78)	CXCL16*
TIMP1	CXCL1,2,3 (Gro)	CXCL10 (IP10)*
TIMP2	Angiopoetin 1	uPAR
IL6*	Angiopoetin 2	VEGFR3
CXCL8 (IL8)		

SEC fraction / 1000 kDa (n=2)	**SEC fraction / 100 kDa (n=1)**
VEGFR3*	VEGFR3
	uPAR
	TIMP1
	TIMP2

2.3. Purification step 2: Ion exchange chromatography of high-molecular-weight fractions of MSC cell culture supernatant

In order to reduce sample complexity and purify the migration-inducing factor(s) mediating cancer cell migration towards MSC, high-molecular-weight SEC fractions showing migration-inducing activity were pooled and further separated by ion exchange chromatography (IEX). A positively charged stationary phase bound negatively charged proteins to the column and allowed a direct elution of positively charged proteins. After loading the sample onto the column, the bound proteins were eluted off the column by gradually increasing the NaCl concentration of the fluid phase. Proteins eluted off the column depending on their ionic strength and on the amount of NaCl required to disrupt the interaction between the proteins and the stationary phase (anion exchange chromatography).

2.3.1. Ion exchange chromatography yields reproducible fractionation of high-molecular-weight proteins

The use of a MonoQ HR 5/5 column with 50 mM Tris-buffer containing 50 mM NaCl as the fluid phase, combined with 0.5 ml/min flow rate and an automated continuous NaCl gradient resulted in an anion exchange fractionation with high reproducibility and reliability. Prior to automated elution, the samples were manually loaded via a superloop and the initial flow-through was discarded. The initial flow-through did not require further analysis after establishing that it contained no observable migration-inducing activity towards PC3 cells.

Comparison of chromatograms of three separate donors revealed variations in peak height but very low differences in the retention volumes at which the main peaks eluted off the column (Figure 8 A). Low variations in retention volume were observed by comparing the retention volumes of chromatogram peaks of separate FPLC runs of two MSC donors (biological replicates) (Figure 8 B).

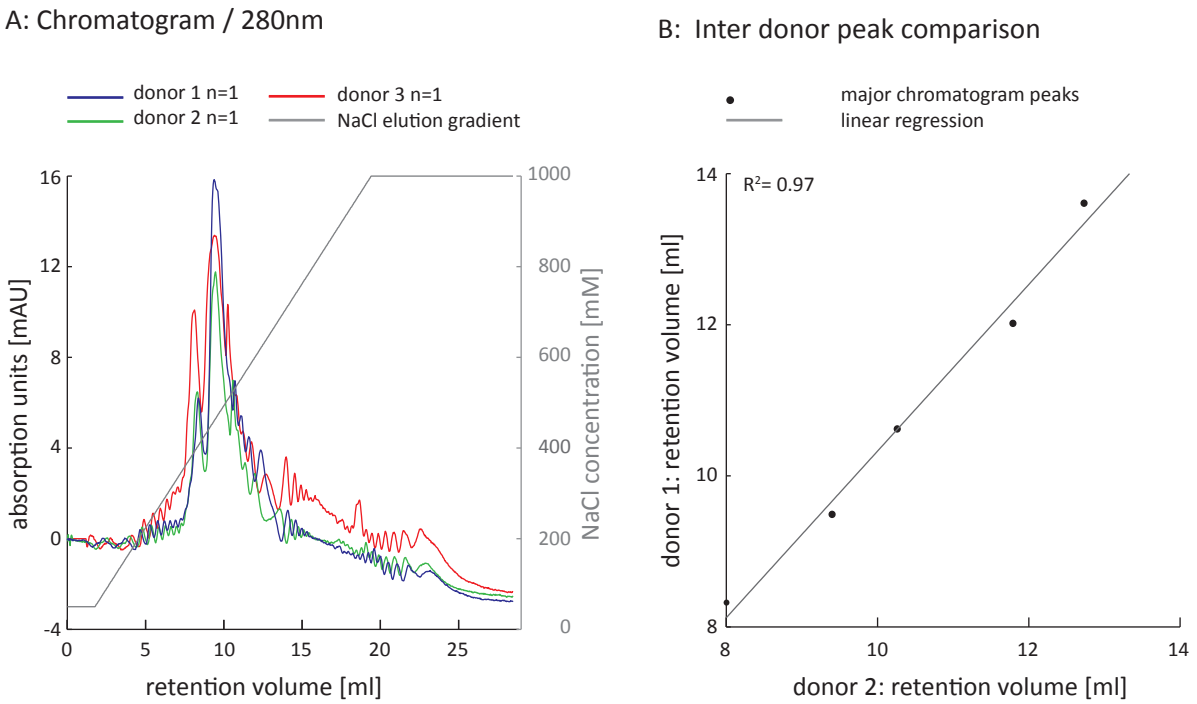


Figure 8: Ion exchange chromatography yields reproducible fractionation of high-molecular-weight proteins isolated from MSC cell culture supernatant.

A: Ion exchange chromatogram of proteins detected at 280 nm during elution. For each donor a total of 130 ml of MSC cell culture supernatant were filtered, concentrated and fractionated by multiple size exclusion chromatography runs. Resulting high-molecular-weight fractions, approx. 1500 – 800 kDa in size, with high migration-inducing activity were pooled, loaded onto an anion exchange column and eluted off the column by a NaCl gradient. **B:** Correlation plot of main peak retention volumes between samples of two different MSC donors (Pearson correlation is indicated).

2.3.2. High-molecular-weight proteins of distinct ionic strength show elevated migration-inducing activity towards prostate cancer cells

IEX fractions of three MSC donors eluted off the anion exchange column with NaCl concentrations ranging from 50 mM to 870 mM were tested for their migration-inducing activity towards the prostate cancer cell line PC3. Migration response of PC3 cells towards the IEX fractions was analyzed relative to the maximum response induced by the unprocessed cell culture supernatant of the respective donor. Fractions eluting off the column at around 250 mM showed migration-inducing activity of up to 35% compared to the unprocessed cell culture supernatant. On average though, the strongest migration response of up to 70% was seen for proteins eluted off the column at a NaCl concentration of approx. 340 mM (Figure 9 A). In contrast to SEC fractions (Figure 6 A), IEX fractions showed a more focused distribution of migration-inducing activity in fewer fractions.

Individual analysis of PC3 migration response after three hours towards the IEX fractions of three MSC donors confirmed strong migration-inducing activity towards 340 mM IEX fractions for all three donors ranging from 50% to 80% (peak 2). Donor 1 and donor 2 showed additional activity peaks (peak 3) in fractions eluted at approx. 380 mM NaCl with comparable migration-inducing activity. This peak was not observed for donor 3. Migration-inducing activity of fractions eluted at 250 mM NaCl (peak 1) could also only be confirmed for donor 1 and donor 2 of which donor 1 induced a migration response of approx. 60% whereas donor 2 only showed minor activity of approx. 20%. IEX fractions of Donor 3 showed an overall reduced migration-inducing activity towards the prostate cancer cell line PC3 (Figure 9 B).

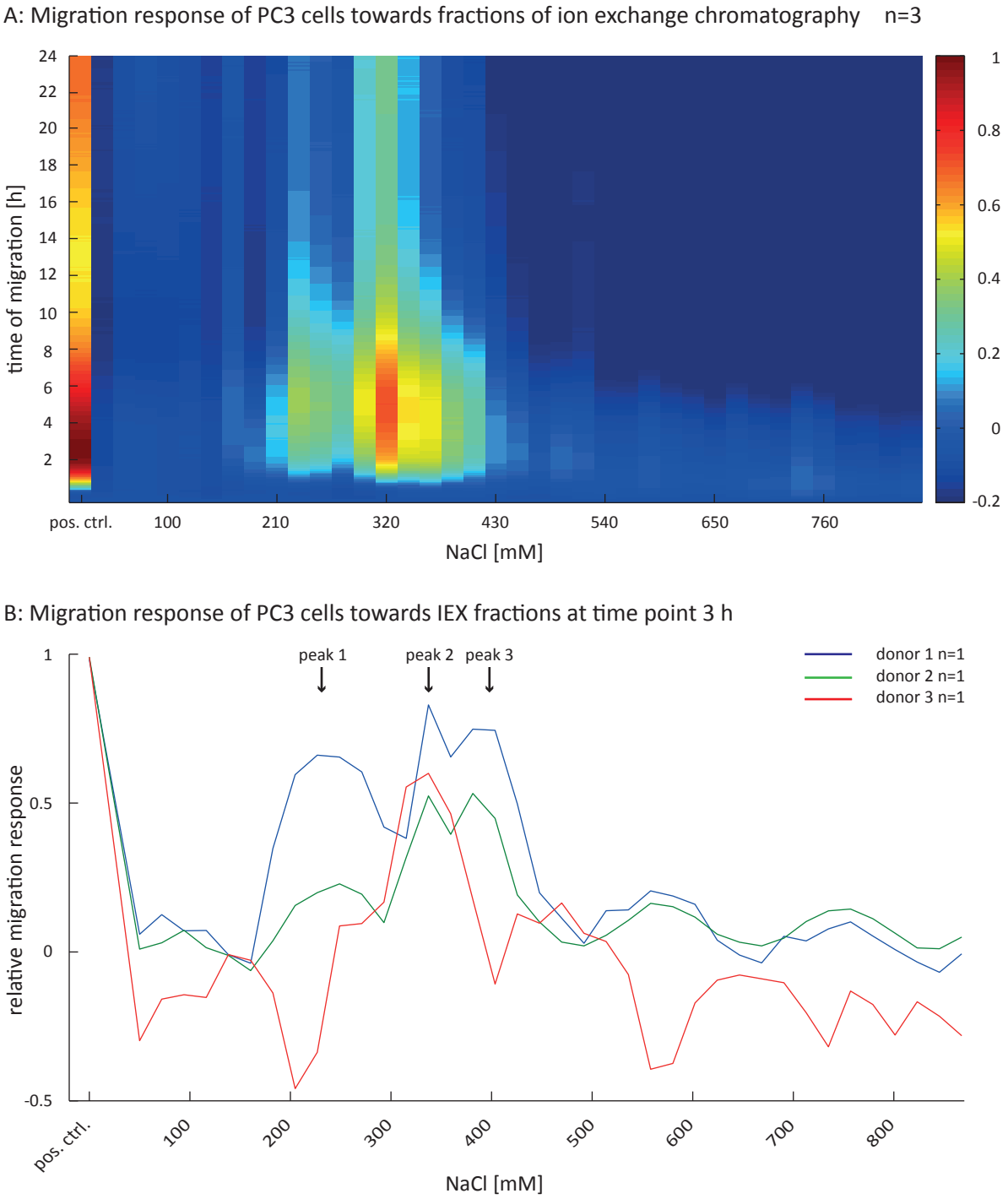


Figure 9: Proteins of distinct ionic strength show elevated migration-inducing activity towards prostate cancer cells.
A: Average migration response over time of prostate cancer cell line PC3 towards IEX fractions of cell culture supernatant of three individual MSC donors. **B:** Individual migration response of prostate cancer cell line PC3 towards IEX fractions of MSC culture supernatant after 3 h. Migration response, determined with the xCELLigence system, is displayed as relative to the maximum response observed towards the unprocessed cell culture supernatant of each donor (pos. ctrl.) respectively (A,B).

2.3.3. Mass spectrometry confirms the presence of extracellular matrix proteins in IEX fractions with elevated migration-inducing activity

Due to fluctuations in migration-inducing activity of IEX fractions of individual MSC donors (Figure 9 B), varying fractions were chosen for mass spectrometry analysis depending on the donor. All analyzed IEX fractions showed elevated migration-inducing activity and could be assigned to activity peak 1 (donor 1, donor 2), peak 2 (donor 1, donor 2, donor 3) as well as peak 3 (donor 1, donor 2) (Figure 9 B). A full list of proteins identified in IEX fractions with elevated migration-inducing activity towards the prostate cancer cell line PC3 can be found for peak 1 (Table 3), for peak 2 (Table 4) and for peak 3 (Table 5).

A number of proteins that were previously detected in migration-inducing SEC fractions (Table 1) were also identified in at least one of the IEX fractions showing peaks in migration-inducing activity (Table 3, Table 4, Table 5). These proteins included type I, III and VI collagen, fibrillin, fibronectin, galectin-3 binding protein, hemopexin, laminins with subunits β-2 and γ-1 such as laminin 421, serine protease and TGF-β induced protein.

Table 3: Proteins detected by mass spectrometry in IEX fractions of MSC cell culture supernatant; activity peak 1 found in MSC donors 1 and 2.
Migration-inducing proteins were eluted off the column at varying salt concentrations ranging from 200 mM to 250 mM NaCl depending on the MSC donor. Nonetheless, these fractions shared the first eluted migration-inducing activity towards PC3 cells. Proteins listed were identified in samples of both donor 1 and donor 2.

Symbol	Name	Description
CO1A1	Collagen α-1(I) chain OS	Fibrillar collagen
CO1A2	Collagen α-2(I) chain OS	Fibrillar collagen
DESP	Desmoplakin OS	Intra cellular protein of desmosome complex

Table 4: Proteins detected by mass spectrometry in IEX fractions of MSC cell culture supernatant; activity peak 2 found in MSC donors 1, 2 and 3.
Migration-inducing proteins were eluted off the column at salt concentrations of approx. 325 mM NaCl for all three MSC donors. Proteins listed were identified in samples of two or all three (*) donors.

Symbol	Name	Description
CD63*	CD63 antigen*	Transmembrane protein
COL1A1*	Collagen α-1(I) chain*	Fibrillar collagen
COL3A1*	Collagen α-1(III) chain*	Fibrillar collagen
COL6A1*	Collagen α-1(VI) chain*	Fibrillar collagen
COL1A2*	Collagen α-2(I) chain*	Fibrillar collagen
COL6A2*	Collagen α-2(VI) chain*	Fibrillar collagen
COL6A3*	Collagen α-3(VI) chain*	Fibrillar collagen
DSG1	Desmoglein-1	Cell adhesion protein of the desmosome complex
DESP*	Desmoplakin*	Intra cellular protein of desmosome complex
G3P	Glyceraldehyde-3-phosphate dehydrogenase	Energy metabolism enzyme
HEMO*	Hemopexin*	Acute phase plasma glycoprotein
PLAK	Junction plakoglobin	Cytoplasmic protein of desmosome complex
LAMC1	Laminin subunit γ-1	ECM glycoprotein of basement membrane
LAMP2	Lysosome-associated membrane glycoprotein 2	Cell membrane glycoprotein
PZP	Pregnancy zone protein	Proteinase binding macroglobulin
PIP	Prolactin-inducible protein	Secreted protein with aspartyl protease activity
RAB7A	Ras-related protein Rab-7a	Endosomal protein
TRFE*	Serotransferrin*	Iron binding protein
SDCB1	Syntenin-1	Intra cellular syndecan binding protein
BGH3*	Transforming growth factor-β-induced protein ig-h3*	RGD containing ECM protein
RS27A	Ubiquitin-40S ribosomal protein S27a	Ribosomal protein
ZA2G	Zinc-α-2-glycoprotein	Secreted stimulant of lipolysis

Table 5: Proteins detected by mass spectrometry in IEX fractions of MSC cell culture supernatant; activity peak 3 found in MSC donors 1 and 2.

Migration-inducing proteins were eluted off the column at salt concentrations off approx. 380 mM NaCl depending on the MSC donor. Proteins listed were identified in samples of both donor 1 and donor 2.

Symbol	Name	Description
CO1A1	Collagen α-1(I) chain	Fibrillar collagen
CO3A1	Collagen α-1(III) chain	Fibrillar collagen
CO6A1	Collagen α-1(VI) chain	Fibrillar collagen
CO1A2	Collagen α-2(I) chain	Fibrillar collagen
CO6A2	Collagen α-2(VI) chain	Fibrillar collagen
CO6A3	Collagen α-3(VI) chain	Fibrillar collagen
DESP	Desmoplakin	Intra cellular protein of desmosome complex
FBN1	Fibrillin-1	ECM glycoprotein
FINC	Fibronectin	ECM glycoprotein
LG3BP	Galectin-3-binding protein	ECM β-galactoside-binding protein
HEMO	Hemopexin	Acute phase plasma glycoprotein
LAMB2	Laminin subunit β-2	ECM glycoprotein of basement membrane
LAMC1	Laminin subunit γ-1	ECM glycoprotein of basement membrane
MYH9	Myosin-9	Intra cellular non-muscle myosin
LRP1	Prolow-density lipoprotein receptor-related protein 1	Plasma membrane receptor
TRFE	Serotransferrin	Iron binding protein
TARSH	Target of Nesh-SH3	SH3 domain-binding protein of NESH-SH3/Abi3
TENA	Tenascin	ECM glycoprotein
BGH3	Transforming growth factor-β-induced protein ig-h3	RGD containing ECM protein
RS27A	Ubiquitin-40S ribosomal protein S27a	Ribosomal protein

2.4. Functional analysis of extracellular matrix proteins secreted by MSC

Size exclusion chromatography of MSC cell culture supernatant revealed migration-inducing activity towards the prostate cancer cell line PC3 and the breast cancer cell line MDA-MB-231 to be mainly caused by high-molecular-weight proteins or protein complexes of approximately 1000 kDa in size. Mass spectrometry and antibody array analysis of these fractions revealed the presence of ECM proteins and the absence of classic chemokines. Ion exchange chromatography of SEC fractions with migration-inducing activity further enriched the migration-inducing factors and confirmed the presence of extracellular matrix proteins. In order to understand the effect of extracellular matrix proteins on the migration of cancer cells towards MSC, recombinant and isolated extracellular matrix proteins as well as chemokines were purchased and tested for their potential to trigger prostate cancer cell migration.

2.4.1. Prostate cancer cells do not migrate towards recombinant chemokines

Mass spectrometry and antibody array analysis failed to show the presence of chemokines in chromatographic fractions with migration-inducing activity. Nonetheless, three recombinant chemokines were tested for their effect on the migratory behavior of PC3 cells, since chemokines are intensely discussed in the process of metastasis formation[111,112]. Furthermore, CCL2 was detected in the unprocessed cell culture supernatant of MSC prior to chromatographic fractionation and CCL2[116], CCL5[117] as well as CXCL12[118] are reported to play a role in prostate cancer progression or cell migration. Therefore, these chemokines, in recombinant form, were analyzed for their ability to induce PC3 prostate cancer cell line migration in comparison to the cell culture supernatant of MSC.

CCL2, CCL5 and CXCL12 were tested in concentrations ranging from 10 ng/ml to 500 ng/ml and the most potent concentration was used to describe the chemotactic potential the respective protein had to induce prostate cancer cell migration. All three chemokines failed to induce a migration response comparable to the cell culture supernatant of MSC. After 24 hours CCL2 was able to induce a slight migration response of 10% but results varied strongly between experiments (Figure 10 A – C).

Some reports have suggested that bone-metastasizing cancers may mimic the process of homing of hematopoietic stem cells to their bone niche, in which MSC play a crucial role[21,22]. It has been demonstrated that CXCL12 secreted by MSC is a key driver of HSC migration and homing[119]. By comparing the migration response of freshly isolated primary HSC and PC3 cells towards a gradient of CXCL12 to their respective negative control, it was observed that CXCL12 induced a fourfold

average increase in HSC migration, whereas CXCL12 failed to induce an increase of PC3 cell migration (Figure 10 D).

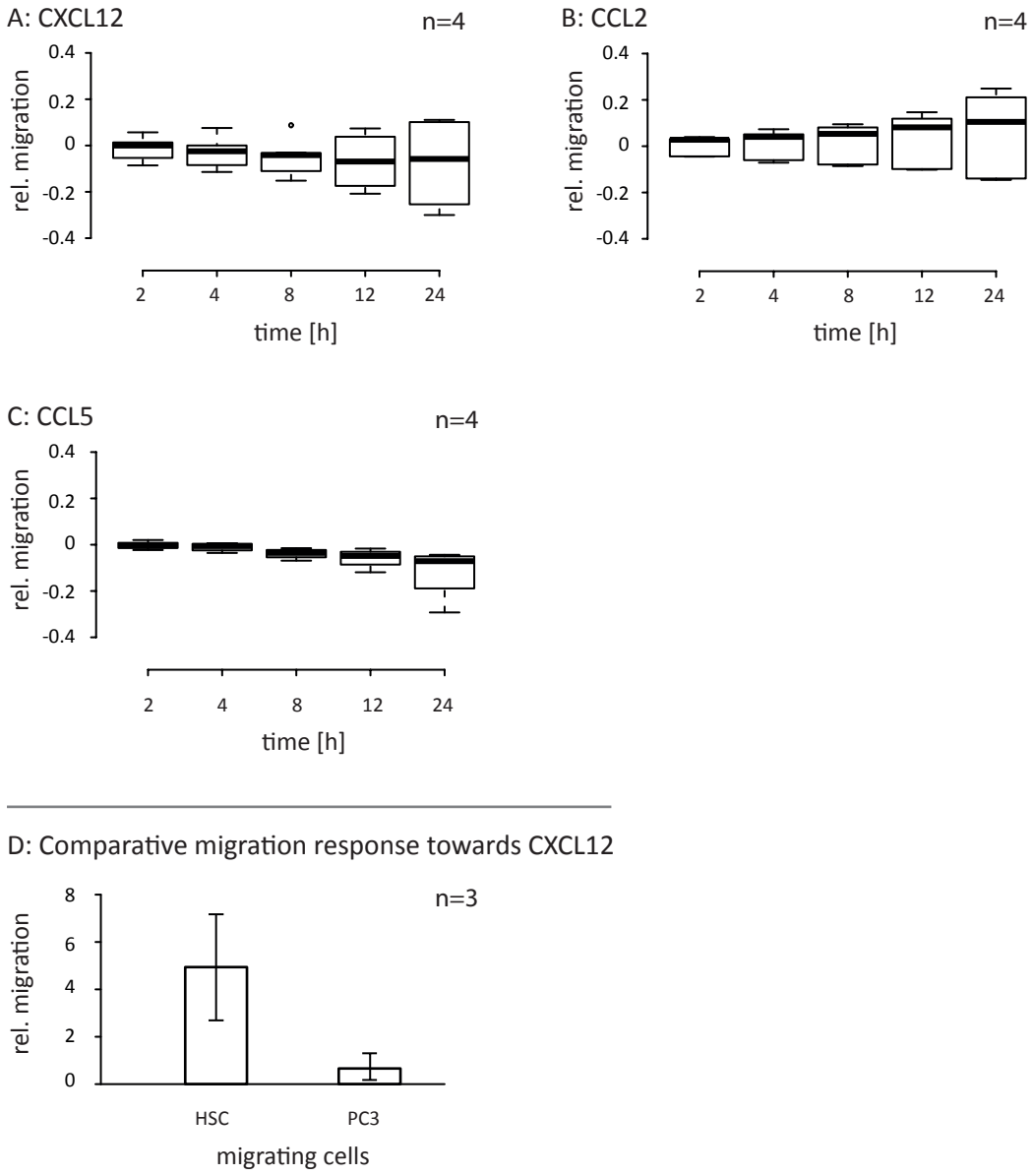


Figure 10: Prostate cancer cells do not migrate towards recombinant chemokines.
A – C: Migration response of PC3 prostate cancer cells towards chemokines is displayed relative to the migration towards MSC cell culture supernatant at the respective time points. CXCL12 (A), CCL2 (B) and CCL5 (C) were used at a concentration of 100 ng/ml. Error bars of boxplots are based on Tukey-whiskers which extend to data points that are less than 1.5 x inter quartile range away from 1st or 3rd quartile. **D:** Average migration response of primary hematopoietic stem cells (HSC) and PC3 cells towards CXCL12 (100 ng/ml) relative to the corresponding negative controls. Error bars indicate standard deviation. Migration response of PC3 cells was determined with the xCELLigence system, whereas non-adherent HSC migration was monitored in classic Boyden chamber transwells in combination with the Incucyte live-cell microscope.

2.4.2. Prostate cancer cells migrate towards recombinant and isolated proteins of the extracellular matrix

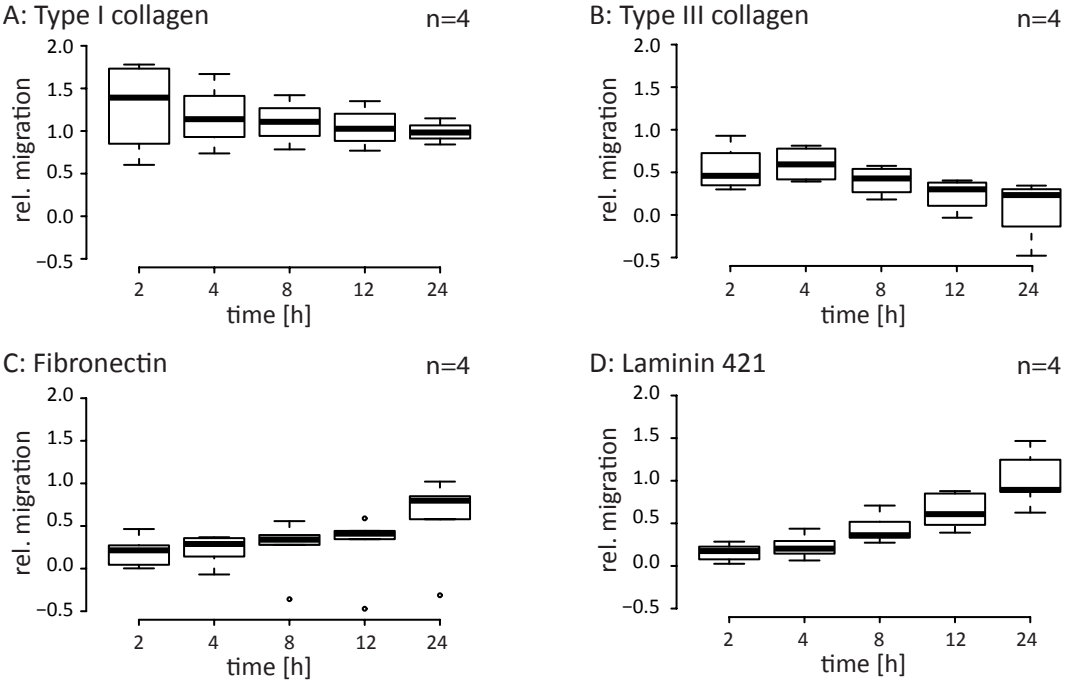
Extracellular matrix proteins identified by mass spectrometry in SEC or IEX fractions with migration-inducing activity, and which were previously discussed in the literature in the context of metastasis[120] or cancer progression[78], were investigated for their ability to trigger migration of the prostate cancer cell line PC3.

Commercially available recombinant and isolated ECM proteins were tested in concentrations ranging from 0.1 µg/ml to 10 µg/ml. The most potent concentration was used to analyze the migration-inducing potential the respective proteins had to induce prostate cancer cell migration (Figure 11). Fibrillar type I and III collagen induced a rapid PC3 migration response after only two hours. Type I collagen even exceeded the migration-inducing potential of the cell culture supernatant of MSC, triggering a 140% migration response after two hours (Figure 11 A). Type III collagen induced an early migration response of over 45% compared to MSC cell culture supernatant. Interestingly, type III collagen was not able to induce a sustainable migration response, with values declining down to 12% over time (Figure 11 B). Plasma fibronectin induced a linearly increasing migration response over time starting at 20% after two hours and reaching its maximum response after 24 hours of approx. 80% compared to the MSC cell culture supernatant (Figure 11 C). Laminin 421 showed similar dynamics in PC3 migration induction but reached up to 90% of migration response induced by the MSC cell culture supernatant after 24 hours (Figure 11 D). In contrast to laminin 421, Laminin 411, also found in mesenchymal tissues, did not induce a migration response with only a median response of 20% after 12 hours, which again diminished after 24 h (Figure 11 E). Matricellular proteins nidogen1 (Figure 11 F) as well as thrombospondin 1 & 2 (Figure 11 G, H) also failed to induce clear PC3 migration with only a slight migration response after 24h. Nonetheless, this late response towards thrombospondin 1 & 2 did not exceed 6% and 20% respectively and showed a high degree of variability between experiments. Both galectin-3 binding protein (Figure 11 I) and TGF-β induced protein (Figure 11 J) showed no induction of migration.

In summary, the tested proteins could be classified into three groups:

- Proteins inducing rapid migration, including type I and III collagen.
- Proteins inducing migration with slower or delayed dynamics, including fibronectin and laminin 421.
- Proteins, which do not induce migration, including laminin 411, thrombospondin 1 & 2, galectin-3 binding protein as well as TGF-β induced protein.

ECM proteins inducing migration



ECM proteins not inducing migration

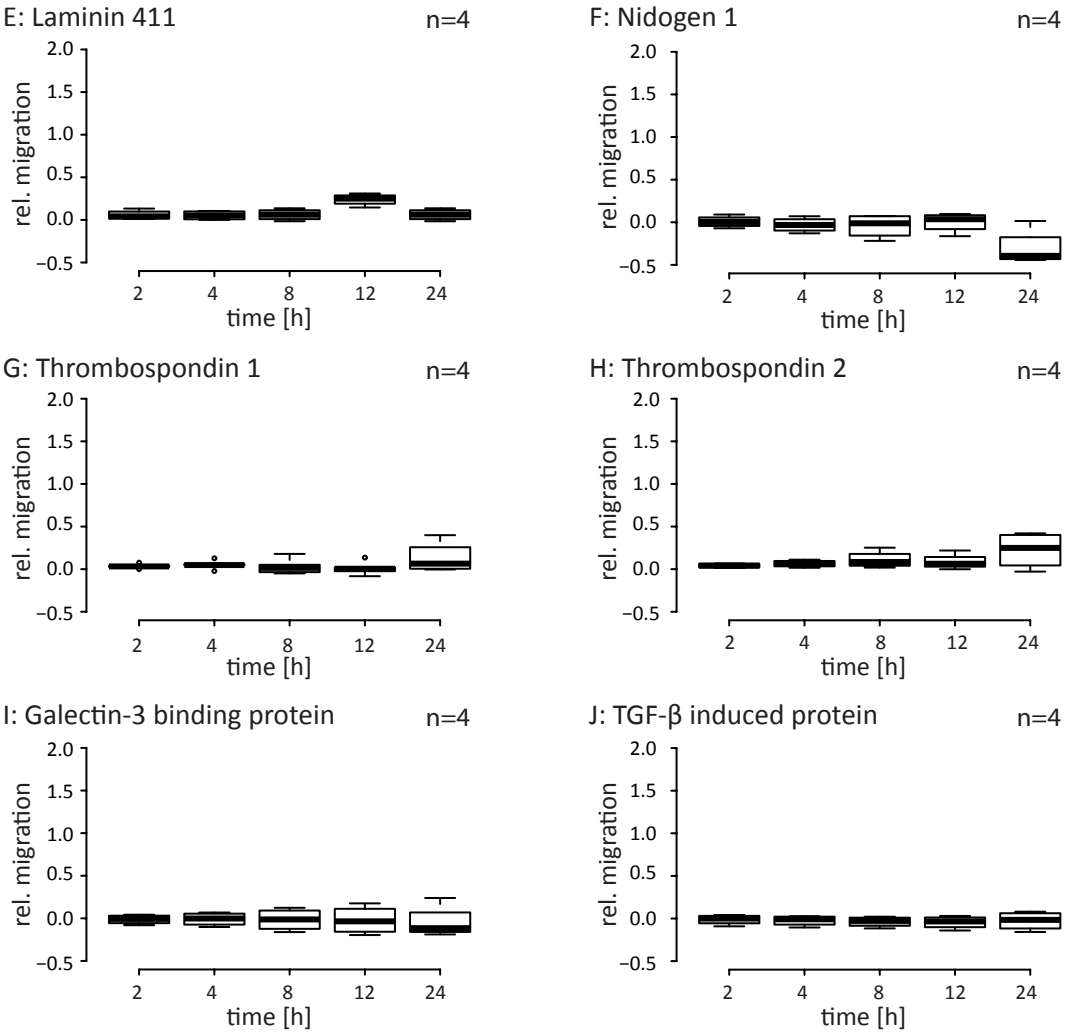


Figure 11: Prostate cancer cells migrate towards recombinant and isolated proteins of the extracellular matrix.
Migration response of PC3 prostate cancer cells towards ECM proteins is displayed relative to the migration towards MSC cell culture supernatant at the respective time points. All proteins were tested with concentrations ranging from 0.1 µg/ml to 10 µg/ml and the most potent concentration was chosen for analysis. **A:** Type I collagen isolated from human skin, 5 µg/ml. **B:** Type III collagen isolated from human placenta, 10 µg/ml. **C:** Fibronectin isolated from human plasma, 10 µg/ml. **D:** Recombinant human laminin 421, 10 µg/ml. **E:** Recombinant human laminin 411, 10 µg/ml. **F:** Recombinant human nidogen 1, 1 µg/ml. **G:** Recombinant human thrombospondin 1, 1 µg/ml. **H:** Recombinant human thrombospondin 2, 0.1 µg/ml. **I:** Recombinant human galectin-3 binding protein, 1 µg/ml. **J:** Recombinant human TGF-β induced protein, 1 µg/ml. Error bars of boxplots are based on Tukey-whiskers which extend to data points that are less than 1.5 x inter-quartile range away from 1st or 3rd quartile. Migration response of PC3 cells was determined with the xCELLigence system (A – J).

2.5. Knockdown of β_1 integrin in prostate cancer cells by RNAi impairs migration towards MSC

After establishing that ECM proteins triggered the observed prostate cancer cell migration towards MSC, integrin receptors potentially mediating the response of the migrating cancer cells were investigated. Integrins are essential cellular receptors of extracellular matrix proteins. They consist of an α- and β- subunit forming a functional dimer[121]. In order to better understand how prostate cancer cells migrate along a diffusible gradient of ECM proteins towards MSC, β-integrins (ITGB) expressed in PC3 cells were chosen for siRNA-mediated knockdown[122]. Integrins of interest were identified by in house RNAseq experiments on PC3 cells and current knowledge in the literature[123]. Here β_1, β_3, β_6, β_7 and β_8 integrins were chosen for knockdown in PC3 cells migrating towards the cell culture supernatant of MSC.

Targeting gene expression by siRNA mediated knockdown required a number of control experiments. As the general fitness of a cell has an impact on its motility and migration capacity, a control siRNA was included in the experiments to consider cell viability and fitness effects caused by the toxicity of the siRNA reagents and transfection procedure. As control siRNA siRLUC targeting *Renilla*-luciferase, which is not expressed in PC3 cells, was chosen. Furthermore, successful reduction of mRNA levels by siRNA knockdown of the respective genes was confirmed by qPCR for each experiment (Figure 12 F). As cellular migration depends on the cells ability to attach and spread, PC3 cells were also analyzed by phase contrast microscopy after siRNA treatment to assess the capability of the cells to attach and spread.

Among targeted β-integrins, only β_1 integrin knockdown showed a strong reduction in cancer cell migration. In comparison to siRLUC treated cells, siITGB1 treated cells showed a reduced migration response towards the cell culture supernatant of MSC over the complete time course of 24 hours. After two hours in which PC3 cells normally showed a full migration response, β_1 integrin knockdown fully inhibited PC3 migration. A gradual migration response of siITGB1 treated cells

could be observed in the following hours, reaching only 30% of siRLUC treated cells after 24 hours (Figure 12 A). Phase contrast imaging of PC3 cells after siITGB1 knockdown (Figure 13 B, Supplementary figure 4) showed a slight viability effect and cellular attachment deficits compared to siRLUC treated cells (Figure 12 A, Supplementary figure 3). Nonetheless, the strong reduction of cell migration after siITGB1 treatment was not accompanied by such strong morphological differences that could explain the decreased migration phenotype.

In contrast to siITGB1 treatment, siITGB3 treated PC3 cells showed the opposite effect. After only a slight reduction in migration of approx. 10% after two hours, a clear increase in migration response after four hours of over twofold was observed when comparing siITGB3 and siRLUC treated cells (Figure 12 B). Furthermore, a strong change in cellular morphology was observed with cells presenting reduced phase contrast, indicating increased cellular attachment and spreading (Figure 13 C, Supplementary figure 5).

A similar effect could be observed with PC3 cells undergoing siITGB8 treatment, albeit not quite as pronounced. Cells showed an increased migration response of up to twofold after eight hours (Figure 12 E). siITGB8 cells also showed morphological changes and reduced intensity in phase contrast microscopy compared to siRLUC treated cells. This difference was not as strong as in the case of siITGB3 treated cells, suggesting that the attachment and spreading did not increase as strongly as in the case of siITGB3 treated cells.

siITGB6 treated cells showed a slight delay in migration response with cells reaching full migration after four hours. These cells were therefore still able to migrate towards the cell culture supernatant of MSC, but were not able to show the high migration dynamics seen in siRLUC treated cells (Figure 12 C). No visible morphological changes could be observed after siITGB6 treatment (Figure 13 D, Supplementary figure 6).

siITGB7 treated cells showed a similar migration phenotype to siITGB6 treated cells with a stronger delay in early migration response reaching approx. 40% of siRLUC treated cells after two hours. Full migration response towards the cell culture supernatant of MSC was regained after eight hours (Figure 12 D). No morphological changes were observed compared to siRLUC treated cells (Figure 13 E, Supplementary figure 7).

In summary, the expression of β_1 integrin was essential for the observed migratory interaction of PC3 cells and MSC. PC3 cells lacking β-1 integrin not only failed to show the observed rapid migration response after two hours but also showed a strong decrease in migration after 24 hours. Cells lacking β_6 integrin, and even more, cells lacking β_7 integrin showed a reduction in early rapid migration response towards the cell culture supernatant of MSC. Nonetheless, a full migration response was observed after four and eight hours respectively. Loss of β_3 integrin and β_8 integrin did not alter the early migration response but led to an increase of migration response at later time

points. This observation was in line with morphological changes of cells undergoing siITGB3 and siITGB8 knockdown. In contrast to siITGB1, siITGB6 and siITGB7 treated cells, they showed morphological changes, including enhanced adhesion and cell spreading.

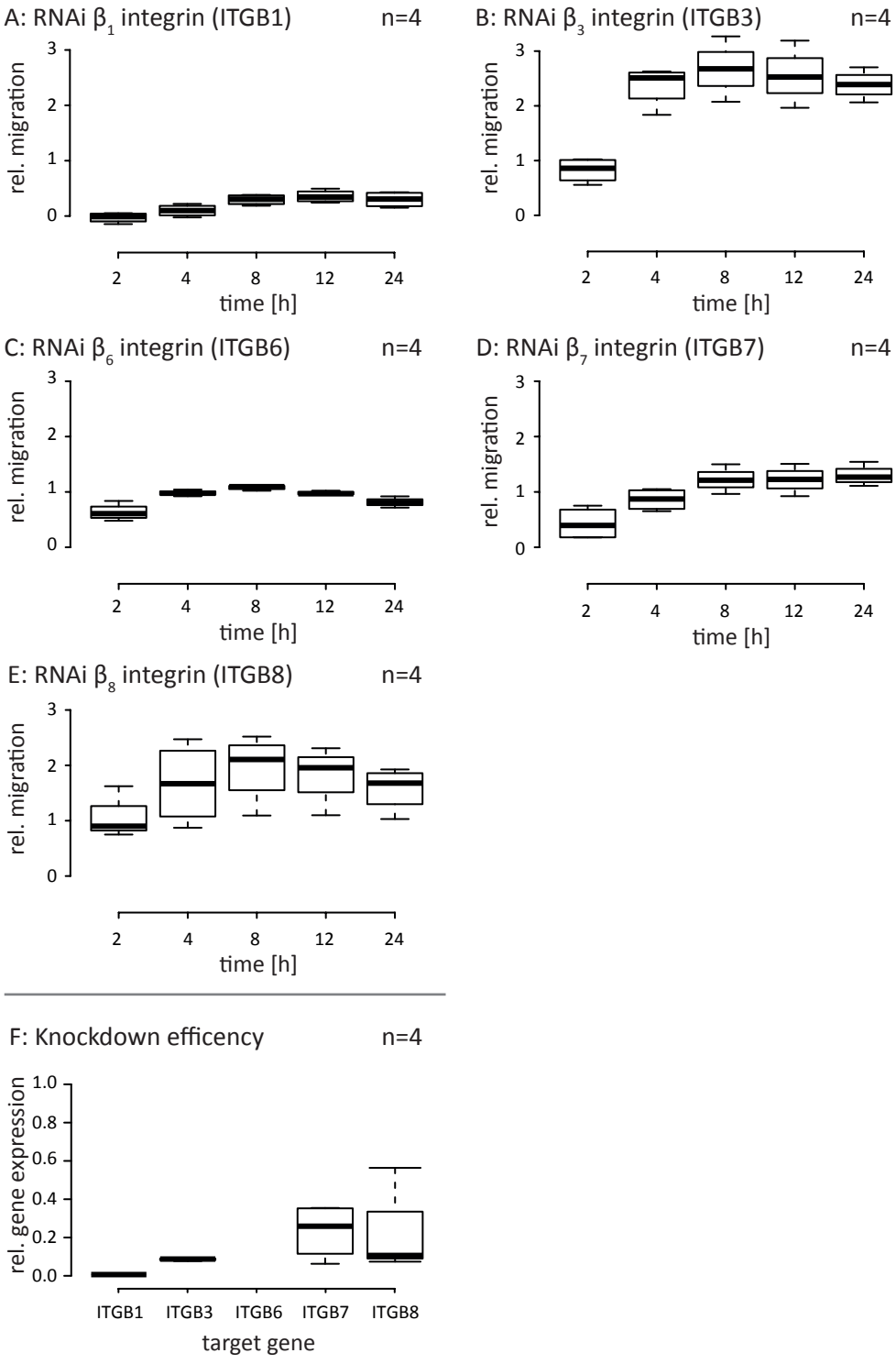


Figure 12: Migration of prostate cancer cell line PC3 towards cell culture supernatant of MSC after RNAi treatment.

PC3 prostate cancer cell migration response towards MSC cell culture supernatant after RNAi treatment is displayed relative to the migration response of PC3 cells undergoing treatment with a siRNA control (siRLUC) for each time point respectively. Migration experiments were conducted 72 h after reverse siRNA transfection. **A:** Migration of PC3 cells after β_1 integrin knockdown. **B:** Migration of PC3 cells after β_3 integrin knockdown. **C:** Migration of PC3 cells after β_6 integrin knockdown. **D:** Migration of PC3 cells after β_7 integrin knockdown. **E:** Migration of PC3 cells after β_8 integrin knockdown. **F:** Confirmation of successful knockdown of target genes. Target gene expression is normalized to the housekeeping gene *Gapdh* and relative to the

gene expression found in cells treated with siRLUC control. Missing values are based on siRNA mediated reduction of gene expression levels below the detection limit.
Error bars of boxplots are based on Tukey-whiskers which extend to data points that are less than 1.5 x inter quartile range away from 1st or 3rd quartile. Migration response of PC3 cells undergoing siRNA treatment was determined with the xCELLigence system (A – F).

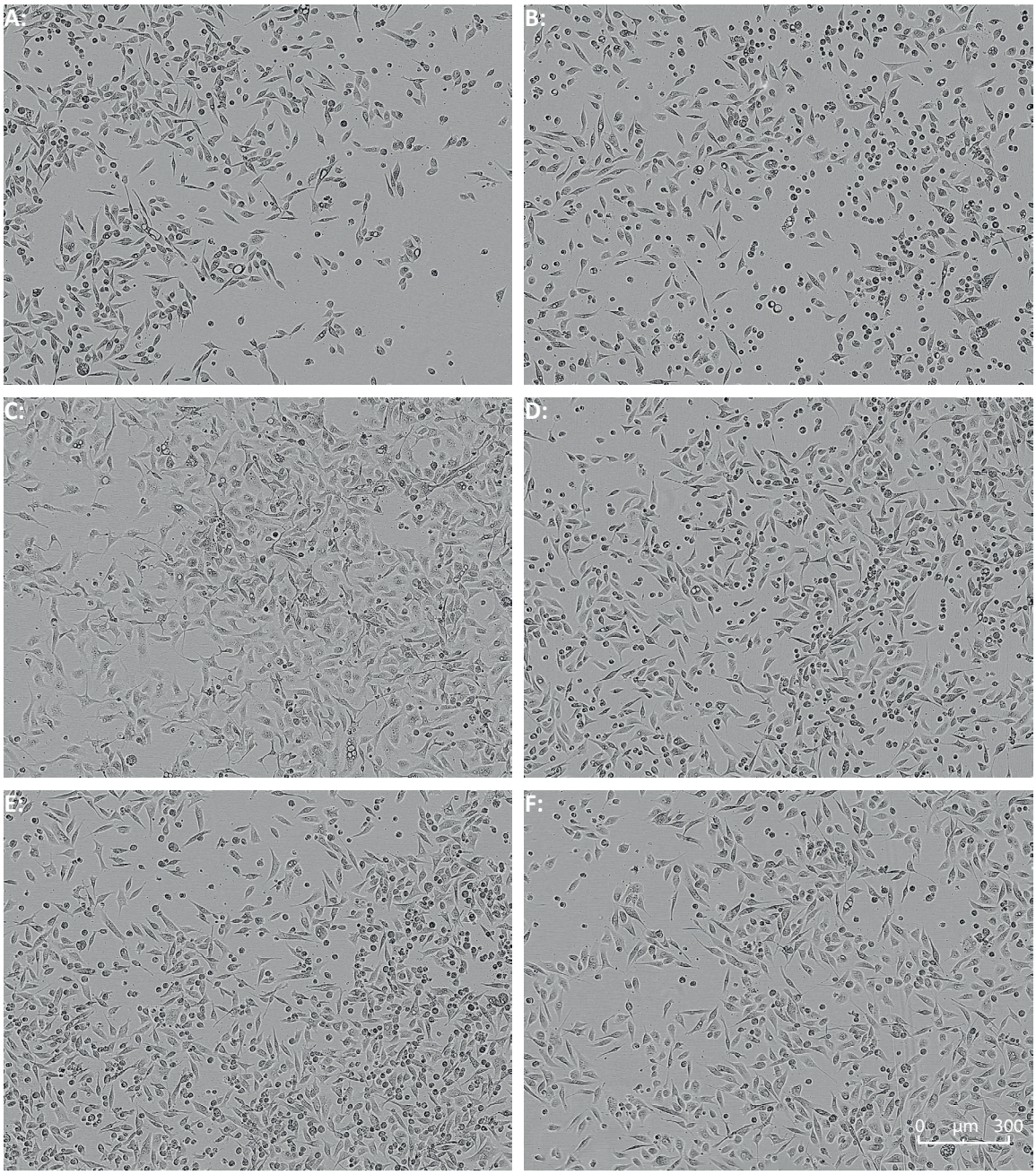


Figure 13: Imaging of RNAi treated PC3 cells.
PC3 prostate cancer cells were seeded 72 h post reverse siRNA transfection in 96-well tissue culture plates and imaged at 10 x magnification 12 h after seeding. **A:** siRLUC control. **B:** siITGB1. **C:** siITGB3 **D:** siITGB6. **E:** siITGB7. **F:** siTGB8.

3. Discussion

Previous work has linked mesenchymal stem cells and their progeny to the progression of cancer. As a cell type capable of migrating to sites of tissue damage and inflammation, MSC are shown to migrate towards the primary tumor and participate in tumor stroma formation, enabling a number of cancer hallmarks including tumor growth and angiogenesis[124,125]. The importance of the tumor microenvironment and its mesenchymal constituents has been appreciated, their role in cancer progression is being intensely studied and therapeutic strategies are being developed specifically designed to target the tumor stroma of the primary tumor[126].

When it comes to the role of MSC in bone metastasis, less is known. A limitation towards understanding the role of MSC in this step of tumor progression is that the *in vivo* identity and localization of bona fide MSC in the bone marrow niche remains a matter of speculation[27,29]. Nonetheless, three findings have suggested a potential role of MSC in bone metastasis formation and have sparked the interest in understanding how MSC – cancer crosstalk might mediate the formation of bone metastasis.

First, the chemokine CXCL12 is secreted by MSC and acts as a key regulator of HSC homing into the bone marrow niche[127-129]. It is also an important signaling molecule in cancer related processes[130,131] since it can induce cancer cell migration *in vitro*[132]. *In vivo* experiments confirmed the importance of this chemokine by showing that blocking the CXCL12 receptors CXCR4 and CXCR7 in cancer cells can reduce the incidences and severity of bone lesions[87,88]. Second, cancer cells and HSC have been shown to co-localize in the bone[21]. Lastly, the formation of bone lesions not only leads to skeletal complications regarding the structural integrity but also seems to affect the functional integrity of the bone marrow niche as HSC and cancer cells compete for this niche[133,134]. This can result in an increased amount of blood progenitor cells in the circulation, reduced immune cell count and even anemia in later stages of bone metastasis growth[135,136]. These findings have fueled a debate whether cancer cells might mimic the homing process of HSC into their bone marrow niche in which MSC play a critical role.

Since MSC are a known source of growth factors and cytokines, which are well described drivers of cancer progression[112,130,137], research has focused on how MSC influence cancer cells via these small molecular weight molecules[53,138]. However, systematic approaches taking all secreted proteins of MSC into consideration are lacking. I therefore decided to study the MSC – cancer cell cross talk by investigating the complete secretome of MSC in order to identify potential factors enabling cancer cells to metastasize to bone. This unbiased approach allowed me to identify extracellular matrix proteins as drivers of cancer cell migration and as acting independently of classic chemokines.

3.1. Strategy of experiments

To contribute to the understanding how MSC induce metastasis to bone, I investigated whether MSC mediate cancer cell migration in an *in vitro* setting. By combining high performance FPLC of MSC cell culture supernatant with the xCELLigence transwell migration system I was able to analyze and decipher the complete secretome of MSC for its ability to induce cancer cell migration.

Investigating biological processes in an *in vitro* setting allows for experiments to be conducted with a clear definition of experimental parameters, in a shorter time frame and with reduced complexity allowing for easier interpretation of the data. Furthermore, high-throughput experiments enable the analysis of large numbers of factors and parameters, which is not possible in an *in vivo* setting. On the other hand, one must consider that all cells are influenced and regulated by their surrounding (outside-in signaling) affecting a multitude of cellular processes. This is especially true for stem cells like MSC with physical and chemical cues affecting identity, stemness and differentiation[79,80,121]. One must therefore expect effects of the *in vitro* conditions on the cultured MSC and consider that they can only model the cells found *in vivo* to a certain extent[139,140]. For this reason, the interpretation of *in vitro* results requires caution and, ideally, subsequent confirmation in an *in vivo* setting, as this issue has potentially contributed to contradicting results and the uncertainty regarding the role MSC play in cancer progression[50]. To model the *in vivo* situation, I based the *in vitro* experiments on primary MSC freshly isolated from the bone marrow of healthy donors and characterized according to current standards of the International Society of Cellular Therapy by multi-lineage differentiation with the Stemflow hMSC Analysis Kit (BD)[26]. In addition, established cancer cell lines commonly used in bone metastasis mouse models were chosen to investigate the MSC – cancer cell cross talk. My results were in line with observations made in previous *in vivo* studies with prostate cancer cells lines. LNCaP cells, which do not form bone metastasis[141], and VCaP cells, which require direct injection into the bone marrow to successfully form an osseous tumor[142,143], failed to respond to signals from the MSC. In contrast, PC3 cells[97] and MDA-MB-231 breast cancer cells[98] readily migrated towards MSC (Figure 4 A, A').

3.1.1. Strategy of cell culture and migration assay

Cellular movement in a tissue always includes movement past other cells and through a framework of extracellular constituents. This can be partly mimicked *in vitro* with transwell migration chambers[144] equipped with a permeable membrane coated with cells such as endothelial cells[145], matrigel or specific ECM proteins [146-148]. During assay development I compared the migration through coated and uncoated membranes. I observed that coating the membranes of the transwell chambers with matrigel did not change the investigated migration response apart from the several

additional hours the cancer cells needed to surpass the added matrigel towards the MSC cell culture supernatant (data not shown). For this reason, I chose to conduct the high throughput migration assay without coating the transwell membranes to reduce experimental complexity and enhance reproducibility (Figure 15).

The presence of additives in the culture medium used can pose an experimental obstacle when analyzing the cell culture supernatant of cells. First, FCS or albumin, often used in high concentrations, can interfere with the purification process[149]. In addition, they can also mask the presence of proteins present in low concentrations or can lead to false positive results based on FCS-derived proteins in a mass spectrometry analysis. I therefore decided to conduct a whole secretome analysis on MSC that were cultured in a protein-free and chemically defined medium during the process of supernatant production. This allowed a reduced total protein load and ensured that all proteins present in the cell culture supernatant were of mesenchymal origin. Prior to supernatant production, MSC were expanded in low FCS medium according to Verfaillie[150] which aimed to reduce the potential impact that changes of FCS concentrations can have on the behavior of cultivated cells[151]. To ensure that the observed migration of cancer cells was not due to the culture conditions during supernatant production, MSC were cultured in multiple types of media, with and without additives (data not shown). In all cases, MSC induced a comparable rapid migration response of the prostate cancer cell line PC3 as seen for MSC cultured in protein free conditions (Figure 4 A). This confirmed that the observed cross talk between cancer cells and MSC was independent of *in vitro* culture conditions.

In addition to culture conditions, the general heterogeneity observed between primary cells of different donors did not pose an experimental obstacle with MSC of multiple donors inducing a strong migration response of PC3 cells. By contrast, fibroblast cell lines failed to induce a comparable migration of PC3 cells (Figure 4 C), further indicating that MSC, based on their secretion profile, are distinct of other stromal cells. Not only did all three MSC donors similarly induce cancer cell migration, but they also showed a highly comparable molecular weight distribution of secreted proteins (Figure 5 A), of which only the high-molecular-weight proteins induced a strong migration response (Figure 6, Figure 7).

3.1.2. Strategy of protein purification under native conditions

In order to identify the secreted proteins triggering the observed cancer cell migration towards MSC, they had to be purified from the complex MSC cell culture supernatant.

One important characteristic of the executed experimental approach to identify these mediators of cancer cell migration (Figure 3) was the fact that an unknown protein with a known functional property required purification. Therefore, the purification process needed functional testing of all

samples after each purification step to confirm the presence of the proteins of interest in a certain sample. This approach differs from classic downstream protein purification approaches in which the protein of interest is known and can be easily detected by classic methods such as antibody detection.

This situation resulted in two requirements which the experimental workflow needed to fulfill. For one, the functional migration assay had to be high-throughput compatible in order to allow analysis of a large number of samples in a reproducible fashion and in a manageable time frame. The xCELLigence transwell system enabled the analysis of 48 samples in parallel and due to the electrical impedance readout offered online and fast analysis of cancer cell migration.

The second requirement was to purify the protein of interest under native conditions, which caused a significant limitation in the purification techniques applicable. In initial experiments the cell culture supernatant was precipitated by salting out with ammonium sulfate. This approach separates proteins that easily aggregate from those that are more soluble. This technique can be a good initial purification step as it is cheap and does not require advanced technology. Nonetheless, this approach was not feasible as a clear separation of fractions with and without the ability to induce cancer cell migration was not possible. This was in part due to the fact that salting out is especially suitable for small soluble proteins and for bulk samples with very high protein concentration[152-154].

For increased reproducibility I based the final downstream processing design on semi-automated size exclusion and ion exchange FPLC using an Äkta Explorer system. In contrast to classic FPLC purification strategies[155,156], I chose size exclusion chromatography as the initial separation step. This was based on the fact that ion exchange chromatography failed to separate the proteins of interest from the crude cell culture supernatant, with a large number of resulting FPLC fractions harboring the potential to induce cancer cell migration after the initial purification step (data not shown). In contrast to ion exchange chromatography, size exclusion chromatography produced distinct FPLC fractions with the potential to induce cancer cell migration (Figure 6, Figure 7). Size exclusion chromatography, routinely applied as a final polishing step of a downstream processing strategy, requires samples of reduced volume and high protein load[157]. By initiating the purification process with a concentration step via ultrafiltration, the sample volume was reduced while the protein concentration increased, enabling the unconventional but successful establishment of size exclusion chromatography as the initial purification step. As the subsequent ion exchange chromatography allowed for the loading of large volumes onto the column, multiple size exclusion fractions harboring migration-inducing activity were pooled, addressing the issue that size exclusion chromatography causes dilution of the sample. Furthermore, by running both FPLC techniques with a tris buffer as the mobile phase, samples could be directly transferred from size exclusion

chromatography to the final downstream processing step of ion exchange chromatography after adjusting the salt concentration. Due to the fact that ion exchange chromatography leads to a concentration of the separated proteins[158], fractions could be diluted for the subsequent migration assay, circumventing the effect high salt concentrations can have on cells.

3.2. MSC induce cancer cell migration independent of cytokines

MSC are known to exert a number of physiological functions through their ability to secrete growth factors and cytokines. These include factors aiding hematopoiesis[59,63,159], modulating immune cell functionality[35-40] and facilitating tissue regeneration[30,160]. Furthermore, MSC are a known source of migration-inducing chemokines[161] enabling immune[162] and hematopoietic stem cell[163] migration. In regard to cancer, this characteristic of being a potent source of growth factors and cytokines makes MSC a prime suspect in the facilitation of cancer progression[3,49,105]. On that account I anticipated a role of these small signaling molecules in the observed ability of MSC to induce transwell migration of cancer cells (Figure 4).

Unexpectedly, size exclusion chromatography of MSC cell culture supernatant revealed that high-molecular-weight proteins exceeding 300 kDa in size were the main drivers of prostate (Figure 6) and breast cancer cell migration (Figure 7). In contrast, small molecular weight fractions potentially harboring chemokines (5 – 20 kDa) failed to induce a comparable migration response. Mass spectrometry analysis of the active size exclusion fractions revealed multiple ECM and ECM-associated proteins but no cytokines (Table 1). As cytokines and growth factors are known to bind to ECM proteins[114,115] I speculated that chemokines might be bound to ECM proteins causing their elution in fractions of high molecular weight. In addition, the failed detection by mass spectrometry might have been based on a masking effect of the abundant high-molecular-weight ECM proteins. To address these questions, the cell culture supernatant and the active size exclusion fractions were further analyzed by cytokine and chemokine antibody array. While the unprocessed cell culture supernatant of MSC contained several cytokines, the antibody array failed to detect any in the high-molecular-weight fractions, with the exception of uPAR (Table 2). This suggested that the small molecular weight proteins had been indeed removed by the chromatographic separation process and that the chemokines secreted by MSC were not responsible for the observed rapid migration of prostate and breast cancer cells towards MSC.

As failure of detection is only insufficient proof for absence, I decided to further investigate the potential role of the chemokines CCL2, CCL5 and CXCL12 on the migratory behavior of PC3 prostate cancer cells. These chemokines were chosen for further investigation, as CCL2 was detected in the unprocessed MSC supernatant and it could have been potentially missed in the high-molecular-weight fractions. In addition, CCL5 detection in the antibody array failed due to a strong

background signal (Supplementary figure 1). Lastly, CCL2[164-166], CCL5[117,167] and CXCL12[131] are all being studied as mediators of bone metastasis and have been reported to induce cancer cell migration.

For further analysis, the recombinant chemokines were investigated for their potential to induce PC3 prostate cancer cell migration. To assure functionality, only chemokines were chosen that had been successfully applied in published and peer reviewed studies[168,169]. To prove functionality of the protein, CXCL12, a known chemoattractant for HSC[127], was successfully tested to induce primary HSC migration (Figure 10 D) comparable to previous reports[170]. Although chemokines are classic mediators of cell migration, none of the investigated chemokines were able to induce a migration response comparable to the cell culture supernatant of MSC (Figure 10). However, due to the limitation of this *in vitro* study the isolation and culture conditions of MSC could have had an effect on the secretion of specific chemokines[171,172]. The portfolio of secreted chemokines will therefore differ from the secretome of MSC found *in vivo*. Nonetheless, my results emphasize that MSC can induce a very strong and rapid migration responses of prostate and breast cancer cells independent of chemokines. This contradicts the notion that chemokines are essential mediators of cancer metastasis causing them to be the focus of a number of clinical trials[173]. Moreover, my findings are supported by studies showing that blocking chemokine function reduces but not necessarily fully inhibits metastasis formation[174]. This suggests that other factors have the potential to mediate the formation of new lesions at the metastatic site synergistically with or independent of classic chemokines.

3.3. MSC induce cancer cell migration by extracellular matrix proteins

The ECM is a non-cellular constituent found in all tissues and organs. It comprises roughly 300 different ECM and ECM-associated proteins, the majority being fibrous proteins and proteoglycans[175]. These proteins are secreted by cells into the space surrounding them. Assembly and further modifications result in the formation of a mechanical framework providing a structural entity to which cells can adhere[176]. In addition to providing anchorage, ECM proteins affect cellular function and differentiation[177,178]. What is more, ECM proteins act as signaling molecules. In this context they can act alone by binding to integrin receptors, inducing subsequent signaling cascades via integrin linked kinases[179] or Src kinases[180]. A further mode of signaling is through synergistic crosstalk between integrin activation and growth factor signaling including examples such as PDGF, c-Met and EGF signaling[181]. Lastly, ECM molecules can act as signaling factor precursors which are activated upon enzymatic degradation of the ECM molecule. Examples include the plasma membrane protein laminin 322 and tenascin containing an EGF-like domain which upon enzymatic degradation is released and able to activate the EGF receptor[182,183].

As an interaction partner with cells, ECM proteins influence virtually all cellular processes including cell adhesion, shape, movement, proliferation and viability[176]. Due to their ubiquitous role, the homeostasis of ECM proteins is tightly regulated under physiological conditions and often unbalanced in pathological situations. This is also the case for cancer in which increased ECM synthesis or excessive enzymatic breakdown can be observed in multiple steps of cancer progression[184,185].

For example, correct cellular polarity and architecture in epithelial tissues is vital to organ formation as well as function and depends on ECM proteins. Correct polarity is lost upon ECM deregulation which can lead to epithelial-mesenchymal transition of cells and loss of basement membrane barrier function enabling tumor invasion into the surrounding tissue[186-188]. Upon tumor growth, the demand for oxygen and nutrients cannot be met by diffusion through the tissue alone, but instead requires angiogenesis. This can be facilitated by ECM proteins and protein fragments acting as pro-angiogenic factors[189], enabling endothelial cell proliferation or by establishing the biomechanical structures required for vessel formation[190]. In the end, ECM proteins also play functional roles at the sites of cancer metastasis. In the final steps of cancer progression, enhanced fibronectin deposition and increased activity of ECM modifying matrix metalloproteases have been reported to aid in the formation of a pre-metastatic niche[90].

All in all, my investigation regarding the role of MSC in cancer cell migration supports the notion that ECM proteins can be powerful drivers of cancer progression. My results show that MSC from

the bone marrow can mediate a strong and rapid migration response of cancer cells by diffusible gradients of ECM proteins.

During the multi-step purification of MSC cell culture supernatant, proteins inducing migration were eluted off the size exclusion column at high-molecular-weight ranges exceeding 300 kDa (Figure 6, Figure 7). Subsequent fractionation of these active fractions by ion exchange chromatography showed the elution of migration inducing proteins at 250 mM and 340 mM NaCl (Figure 9). Mass spectrometry analysis identified a number of ECM and ECM associated proteins that were present in FPLC fractions harboring the activity to induce PC3 prostate cancer cell migration (Table 1, Table 3, Table 4, Table 5). ECM proteins that were present in these fractions and that have been previously discussed in the context of cancer progression were tested for their ability to induce prostate cancer cell migration. These included structural proteins type I and III collagen as well as fibronectin, mesenchymal α-4 laminins laminin 421 and 411[191], matricellular proteins nidogen, thrombospondin 1 and 2[192,193], TGF-β induced protein[194] as well as galectin-3 binding protein[195,196]. Of these, type I and III collagen, fibronectin and laminin 421 induced strong migration of prostate cancer cells.

3.3.1. Type I & III collagen

In the transwell migration experiments, isolated fibrillar type I and III collagen induced a rapid PC3 prostate cancer cell migration response in as little as two hours. Type I collagen even exceeded the migration-inducing potential of the cell culture supernatant of MSC, triggering a 140% migration response after two hours compared to the MSC cell culture supernatant (Figure 11 A,). Type III collagen induced an early migration response of over 45% compared to MSC cell culture supernatant. In contrast to type I collagen, type III collagen was not able to induce a sustainable migration response with values declining down to 12% over time (Figure 11 B).

Although collagens have been traditionally seen as physical barriers against cancer growth at the primary tumor requiring their enzymatic degradation for cancer cell invasion and migration[197,198], it has become clear that fibrillar collagens can play an important tumorigenic role in driving cancer progression[199].

Fibrillar collagens are an important part in the tumor microenvironment in general, showing increased deposition during tumor growth[200-202]. In breast cancer, collagen mediated tissue stiffness is an indicator of cancer risk[203] as collagen can enable invasion into the surrounding epithelium[204] and cancer cell migration along collagen bundles[205]. As collagen fragments are potent chemoattractants for stromal cells[206-208], monocytes[209-211] and neutrophils[212] fibrillar collagens may not only exert tumorigenic functions through physical cues but may also act as diffusible chemoattractants upon enzymatic degradation[213]. These fragments may then mediate the

infiltration of stroma and immune cells into the tumor microenvironment, thus helping facilitate the hallmark of tumor inflammation.

As a major constituent of the bone, type I collagen has been repeatedly discussed in context of bone metastasis. During bone metastasis formation, remodeling of the bone tissue by osteoclasts and osteoblasts is a key hallmark in which the calcified bone matrix is degraded leading to a release of collagen fragments into the bloodstream. As collagen fragment concentration in the blood correlates with osseous spread, these fragments are used as a biomarker with prognostic value for patients suffering from breast and prostate cancer[214,215].

Although type I collagen fragments have been reported to induce migration of several cell types including breast[216] and prostate[210] cancer cells, fibrillar collagen fragments are predominantly seen as a bio marker and not as drivers of bone metastasis formation. Concerning the mechanisms which drive bone metastasis, attention is strongly focused on chemokines, cytokines and growth factors as the key mediators of cancer metastasis[111,132,217,218].

Although chemokines are clearly important mediators of cancer cell metastasis to the bone, my results show that breast and prostate cancer cells migrate rapidly towards type I and type III collagen, suggesting that these collagens which are released from the bone can aid in attracting metastasizing cancer cells to the bone independent of chemokines.

Our observations are further supported by studies suggesting that the ability to bind type I collagen may be a prerequisite for prostate cancer cells metastasizing to bone. This ability was shown to be limited to prostate cancer cell lines harboring the potential to metastasize to bone with PC3 cells readily adhering to type I collagen whereas LNCaP cells, derived from lymph node metastasis[99,219], failed to do so[220-222]. This is in agreement with my observations in which PC3 cells migrated strongly towards MSC whereas LNCaP cells failed to show a migration response (Figure 4 A).

Research on fibrillar collagens is mainly focused on the adhesive and proliferative effects collagens can exert on cancer cells forming a suitable 'soil' for cancer cell growth[223]. My results show that fibrillar collagens can act as strikingly potent attractants of cancer cells suggesting that further research efforts in this direction are required for understanding the process of bone metastasis formation.

3.3.2. Fibronectin

In comparison to type I and III collagen, fibronectin did not induce such a rapid cell migration but induced more of a gradual response. Over a period of 24 hours the migration response linearly increased up to 80% compared to the unprocessed cell culture supernatant of MSC (Figure 11 C).

Fibronectin is a glycoprotein expressed as soluble plasma fibronectin or as less soluble cellular fibronectin acting as a structural protein[224]. As an ECM protein important in wound healing[225], fibronectin was reported to be up regulated in the stroma of breast cancer tissue and to aid in multiple steps of tumor progression[226]. Additionally, studies suggest that fibronectin might cause resistance to chemo- and radiation- as well as ionization therapy in lung and breast cancer, indicating that expression of fibronectin is a bad prognosis marker[227-230].

Fibronectin is involved in ovarian cancer metastasis[231,232]. A potential role of fibronectin in bone metastasis is supported by the fact that its receptor $\alpha_4\beta_1$ integrin is involved in homing of blood progenitor cells to the bone marrow[233-235], a process potentially mimicked by metastasizing cancer cells[236]. Taken together, including its potential to induce migration of several cell types such as stromal cells[237] and monocytes[238] as well as enhanced attachment of prostate cancer cell lines[239], my results suggest that prostate cancer cells metastasizing to bone might interact with fibronectin to establish a foothold in the bone marrow. A role of fibronectin in cancer metastasis is further supported by the fact that fibronectin expression is increased in pre-metastatic niches observed in the lungs and other organs[17,90].

3.3.3. Laminin 421

In contrast to MSC supernatant, recombinant laminin 421 did not induce a rapid migration response of PC3 prostate cancer cells requiring only several hours but induced a gradual and linear migration response. Nonetheless, the response exceeded the migration induced by fibronectin and reached a similar response compared to the MSC cell culture supernatant after 24 hours (Figure 11 D).

Laminin 421 (α4-β2-γ1) belongs to the protein family of laminins comprising 16 different glycoproteins each composed of an α, β and γ chain. Laminins are involved in the assembly and the structural integrity of basement membranes interacting with type IV collagen, nidogen, fibulin and other laminins. Failure of correct assembly may result in a number of diseases ranging from skin blistering diseases[240] to muscular dystrophy[241]. In addition to its important structural function, laminins interact with cells via integrins[242] and a number of different non-integrin receptors[243]. In the context of cancer, a number of laminins are known to potentially contribute to several cancer

hallmarks including proliferation[244] and angiogenesis[243]. These include the well studied examples laminin 111, laminin332[245,246] and laminin 511[247].

However, less is known about laminin 421, a laminin protein belonging to the α4 laminins including laminin 411 and 421, which are both found in the bone marrow[86]. At the primary tumor α4 laminin chains were overexpressed in the stroma of squamous cell carcinoma[248] and were upregulated in renal carcinomas[249]. In glioma, the switch from laminin 421 expression to 411 is reported to drive the progression of the disease and is predictive for patient survival[250]. A similar switch can be seen in breast cancer with a suspected role in tumor angiogenesis[251,252]. This ratio of pro-tumorigenic potential is not mirrored in my experiments which describe laminin 421 to be the more powerful inducer of cancer cell migration compared to laminin 411 (Figure 11 D, E). In contrast, previous reports describe the ability of laminin 411 to facilitate chemotaxis of endothelial cells[253], blood cells[254] and inflammatory cells[255] as well as hapotaxis of several cancer cell lines[191].

In contrast to laminin 332[256] and 511[247], laminin 421 has not been linked to cancer metastasis. Moreover, little is known about the potential of laminin 421 to induce migration with only few studies describing induction of melanoma cell migration[257] and hapotaxis of a number of cancer cell lines[191]. My *in vitro* experiments confirm these results showing that laminin 421 induced a strong migration response of prostate cancer cells. Furthermore, laminins in general are reported to play a role in the bone marrow niche including adhesive[258], mitogenic[86] and mobilizing[259] function for blood progenitor cells. I therefore propose that laminin 421 might act as an important driver of prostate or breast cancer metastasis to bone and is in need of further investigation.

3.4. Cancer cell migration towards MSC requires β_1 integrin receptor

After establishing that the observed cancer cell migration was primarily mediated by ECM proteins, I investigated the role of ECM-binding integrin receptors in the observed crosstalk between MSC and PC3 prostate cancer cells.

The binding of ECM proteins through transmembrane glycoprotein integrin receptors is enabled by integrins forming heterodimers consisting of one of 18 α and one of eight β subunits. Ligand affinity is determined by up to 25 combinations of the α and β subunits. Therefore, the expression of integrins determines how well a cell can adhere, migrate or invade a certain surface. The cellular response of a cell to an ECM ligand is initated by the receptors undergoing conformational changes and inducing cellular signaling pathways (outside-in signaling). This signaling also affects proliferation, survival, and cellular architecture[260-262]. Further complexity arises through the fact that integrins can interact with chemokine receptors, immunoglobulin membrane receptors such as CD47, growth factor receptors and tetraspanins[263-265]. This enables integrins to initiate a multitude of signaling pathways and cellular responses[121]

Our investigations showed that, in contrast to β_3, β_6, β_7 and β_8 integrin, only the knockdown of β_1 integrin strongly reduced the responsiveness of PC3 prostate cancer cells to migrate towards MSC. This correlates with the fact that β_1 integrin can be involved in the binding of all four ECM proteins identified as potential mediators of PC3 prostate cancer migration towards MSC. β_1 integrin can form heterodimers with a multitude of α subunits mediating binding of type I collagen through $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrin, binding of laminins through $\alpha_1\beta_1$, $\alpha2\beta_1$ and $\alpha_6\beta_1$ integrin as well as binding of fibronectin through $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_9\beta_1$ and $\alpha_v\beta_1$ integrin[266,267].

The fact that β_1 integrin knockdown abolished migration towards MSC is most probably based on two facts. First, PC3 cells were hindered to sense the migration-inducing stimulus due to the missing receptor. Second, PC3 cells required β_1 integrin mediated signaling to initiate a migratory response towards the stimulus secreted by the MSC. I can exclude an unspecific side effect of impaired cell adhesion of PC3 prostate cancer cells since these cells readily adhered to a cell culture dish after β_1 integrin knockdown (Figure 13 B). In agreement with my results, a different study showed that PC3 cells have higher β_1 integrin activity than LNCaP cells, potentially contributing to the differences these two cell lines displayed in my experiments and in their metastatic potential in general[268].

The importance of the observed β_1 integrin dependent cancer cell migration is reflected by various studies describing a role of β_1 integrin in cancer. In these studies $\alpha_2\beta_1$ integrin was reported to promote bone metastasis[269]. This is potentially mediated by talin 1 which in turn enables essential β_1 integrin activity to form bone metastases in prostate cancer[270]. These studies suggest that β_1 integrin is required to mediate attachment to collagen in the bone. Accordingly, my studies suggest that β_1 integrin can also mediate the interaction with bone-derived MSC. Furthermore, induction of $\alpha_4\beta_1$ integrin in Chinese hamster ovary cells facilitated bone metastases of cells otherwise unable to form bone lesions[271]. Moreover, fibronectin binding $\alpha_5\beta_1$ integrin plays a role in invasion and migration of ovarian cancer cells[231,272] and melanoma cells[273] as well as metastasis enabling adhesion to the bone marrow stroma in general[274].

In contrast to these reports, β_1 integrins including $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin have also been suggested to act as tumor suppressors and are down regulated in some primary tumors. This tumor suppressing activity is derived from the fact that these integrins can facilitate adhesion to the basement membrane, quiescence, growth inhibition and differentiation of cells[229,275-278]. These contradictions might be explained by the fact that the role of integrins is probably tissue and context dependent, allowing for both an anti-tumorigenic role in the primary tumor and a pro-metastatic role in metastasis formation. All in all, these contradicting results emphasize the importance of better understanding the role of integrins in cancer progression. In regard to MSC – cancer cell crosstalk, RNAi mediated knockdown of α integrins could help identify which α-β integrin

heterodimers are required for the PC3 prostate cancer cells to migrate towards MSC. Validation of these results by antibody inhibition would further substantiate these results.

In line with contradicting reports regarding the role of β_1 integrin in cancer, my observations indicate that β_3 integrin is not required to migrate towards MSC, although $\alpha_V\beta_3$ integrin has been reported to play a role in bone metastasis formation[279,280]. The discrepancy between these reports and my results requires further investigation and might be based on limitations my *in vitro* approach causes. Nonetheless, it is important to note that hampering β_3 integrin receptors may cause unexpected effects such as increased cell adhesion (Figure 13 C, Supplementary figure 5) and potentially stronger interaction with stromal cells (Figure 12 B) upon β_3 integrin inhibition. This is possibly mediated by compensatory mechanisms upon integrin inhibition[281] and should be considered as $\alpha_V\beta_3$ integrin inhibitors are already in clinical trial.

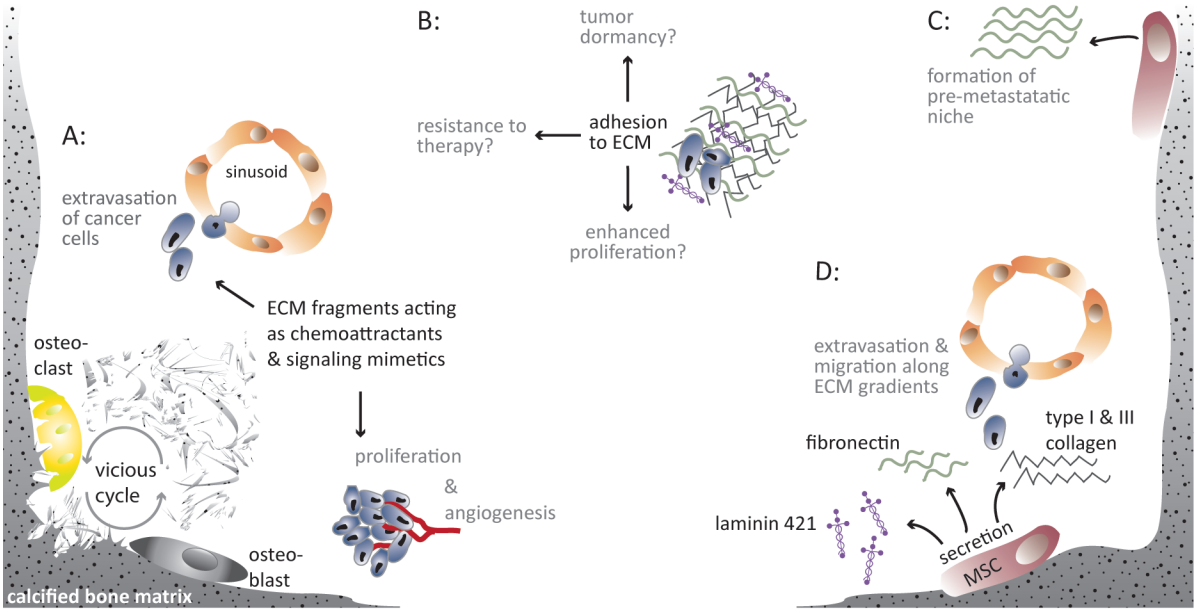


Figure 14: Possible roles of ECM proteins in bone metastasis formation.
Studies suggest numerous roles of ECM proteins in bone metastasis formation. **A:** Remodeling of the bone matrix by osteoblasts and osteoclasts causes the release of ECM fragments from the bone tissue. These ECM fragments can act as signaling mimetics inducing cancer growth and angiogenesis. They can also act as chemoatractants possibly enabling cancer cells to extravasate into the bone marrow. **B:** ECM proteins have been shown to induce either enhanced tumor cell proliferation or tumor cell dormancy and may cause resistance to cancer therapy. **C:** ECM proteins, such as fibronectin may be involved in the formation of a pre-metastatic niche. **D:** The analysis of the complete secretome of MSC confirmed the importance of ECM proteins in cancer progression as laminin 421, fibronectin and type I and type III collagen were identified to be powerful inducers of prostate cancer cell migration. These results suggest a possible role of these ECM proteins in cancer cell migration, extravasation and bone metastasis formation.

3.5. Conclusion

The English surgeon Lou Paget was one of the first to propose in 1895 that cancer metastasis is a non-random event[6]. Since then the understanding of what triggers and drives cancer metastasis has increased dramatically[2,7]. Nonetheless and despite all the achievements and efforts in cancer research, metastasis formation still causes severe morbidity and mortality of cancer patients. With worldwide 14 million new cancer cases, 8 million cancer deaths and 32 million people living with cancer (within 5 years of diagnosis) in 2012[9], further progress in understanding the principles and processes of metastasis formation is of great importance.

Chemokines are known mediators of cancer metastasis and the focus of attention in a number of clinical trials (www.clicialtrials.gov)[217]. Nonetheless, studies in breast cancer have shown that blocking chemokine function does reduce but not necessarily fully inhibits metastasis formation[174]. This suggests that other factors have the potential to mediate the formation of new lesions at the metastatic site synergistically with or independently of chemokines. In the context of MSC as mediators of cancer metastasis to the bone, my results showed that MSC were able trigger cancer cell migration via secreted ECM proteins independent of small molecular weight signaling molecules including cytokines and chemokines.

The aggressiveness which PC3 prostate cancer and MDA-MB-231 breast cancer cells display in animal models might therefore be based on their ability to respond to multiple ECM proteins. This could make them especially adaptive to different sites in the body allowing them to efficiently form metastasis. My results support the idea that the metastatic potential of a cancer cell is derived from its ability to interact with the cells and the matrix found at the site of metastasis. In the case of prostate cancer cells metastasizing to bone, I have shown that PC3 prostate cancer cells readily interact with bone marrow MSC and migrate to a variety of ECM proteins, found to be secreted by these MSC.

A further study using a high-throughput screening platform and hierarchical clustering analysis to measure the response of cancer cells to a multitude of ECM proteins showed that depending on their metastatic potential, lung adenocarcinoma cell lines clustered according to their tendency to interact with and adhere to certain ECM proteins and ECM protein combinations[282]. These results clearly show that the metastatic trait of a cancer cell is, at least in part, defined by its ability to interact with certain ECM molecules. Additionally, this study revealed that the combination of ECM proteins is relevant, with lung adenocarcinoma cells adhering best to fibronectin in combination with galectin-3 or galectin-8. This, in turn, could mean that MSC are such powerful inducers of breast and prostate cancer cell migration due to the composition of secreted ECM proteins. This could be the reason why PC3 and MDA-MB-231 cells shared the tendency to migrate towards the

same size exclusion chromatography fractions (Figure 6, Figure 7) and why, even at higher concentrations, single recombinant proteins were not able to completely mimic the migration inducing potential of the MSC supernatant. Only type I collagen isolated from human tissue induced a similar migration response compared to MSC, which might have been due to the presence of remaining ECM impurities or artificially high concentrations (Figure 11 A). Importantly, one has to consider that ECM proteins are modified enzymatically making a multitude of conformational and fragmentation states possible[175]. The ECM proteins, produced recombinantly or secreted by *in vitro* cultured MSC, will therefore not necessarily reflect the exact proteins found *in vivo* in regards to conformation and concentration. Despite these limitations, my results do show that ECM molecules, including type I and type III collagen, fibronectin and laminin 421, not only act as an important 'soil' once the breast or prostate cancer cells have metastasized to bone but also harbor the potential to act as diffusible chemoattractants in their own right. Moreover, my results suggest that MSC secrete migration-inducing ECM proteins that are soluble and not necessarily require enzymatic degradation to matrikines[213,283], since high-molecular-weight fractions after size exclusion chromatography were most potent to induce cancer cell migration (Figure 6, Figure 7).

In patients suffering from bone metastasis, targeting bone turnover is standard care in order to reduce the release of growth factors entrapped in the bone tissue. My results suggest that targeting bone turnover and ECM degradations could also prove to be important in order to prevent the release of ECM fragments that could have the potential to drive cancer cell migration and initiate metastasis formation in the first place. This hypothesis is supported by studies showing that prophylactic treatment with bisphosphonates, inhibitors of bone turn-over[284], were able to reduce the formation of bone metastases *in vivo*[285,286].

I therefore postulate that solely targeting chemokine and growth factor receptors will not suffice and future treatments will have to consider multiple drugable targets including the interactions of cancer cells with ECM proteins and MSC in the bone.

This approach has been proven valid in studies considering that cancer cells might mimic the homing process of HSC to the bone. The combination of AMD3100 or GCSF, classic HSC mobilization agents, and $\alpha_4\beta_1$ integrin inhibition increased HSC mobilization out of their niche into the circulation[287]. This study has shown that targeting the chemokine receptor CXCR4 in combination with integrin receptors strongly inhibits interactions between homing cells and the bone marrow stem cell niche. Consistent with these results, further studies have demonstrated efficacy of combined AMD3100 and $\alpha_4\beta_1$ integrin antibody treatment in acute myeloid leukemia[288-290].

In conclusion, my results have contributed to a better understanding of how cancer cells interact with mesenchymal stem cells and extracellular matrix proteins of the bone marrow niche. In

addition, my work highlights that a better understanding of this crosstalk will ultimately contribute to the development of more advanced strategies that aim to prevent and treat bone metastasis.

4. Materials

4.1. Reagents & buffers

FPLC buffer A 50 mM Tris buffer (Sigma)

FPLC buffer B 50 mM Tris buffer (Sigma), 1M NaCl (Sigma)

Adjust pH of FPLC buffers to pH 7.5 by adding 10M NaOH. Buffers were sterile filtered before storage and degased with helium prior to use.

Fixation solution 4% paraformaldehyde in PBS (Sigma)

Heat to 70°C until dissolved; adjust pH after cooling to pH 7.4.

Low-FCS stem cell medium[150]

60% DMEM low glucose (PAA)
38% MCSB 201 basemedium (Sigma)
2% FCS (HyClone)
100 M l-ascorbic acid-2-PO4 in PBS (Sigma)
1x insulin transferrin selenium (Invitrogen)
2 mM l-glutamine (Life Technologies)
1x linoleic acid (Sigma)
20 nM dexamethasone in PBS (Sigma)
10 ng/ml PDGF-BB (Peprotech)
10 ng/ml EGF (Peprotech)

4.2. Primers

Table 6: List of primers.
Primers were designed with the Roche ProbeFinder V2.45 and synthesized by Eurofins.

Gene	Forward Primer	Reverse Primer	Probe #
ITGB1	cagttactgaagaatttcagcctgt	gcagataatttcctactgctgac	17
ITGB3	tgcaggctacagtctgtgatg	tggcatcagtggtaaacacc	19
ITGB6	ctgcctgcttattggacctc	gccaactccagatggatgag	2
ITGB7	gcttcgatgccattctgc	gaatgtgtcgtctgaagtgaaca	19
ITGB8	gcattatgtcgaccaaacttca	gcaacccaatcaagaatgtaact	19
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc	60

4.3. siRNA reagents

Table 7: List of siRNAs.
siRNAs were purchased from Dharmacon/GE Healthcare.

Target gene symbol	siRNA ID	Sequence - Sense
siRLUC	P-002070-1-20	aaacaugcagaaaaugcug
ITGB1 #1	D-004506-01	gaacagaucugaugaauga
ITGB1 #2	D-004506-02	caagagagcugaagacuau
ITGB1 #3	D-004506-03	gaagggaguuugcuaaauu
ITGB1 #4	D-004506-04	ccacagacauuuacauuaa
ITGB3 #2	D-004124-02	gaaaguccauccuguaugu
ITGB3 #5	D-004124-05	gaaaauccguucuaaagua
ITGB3 #6	D-004124-06	uuacugccgugacgagauu
ITGB3 #7	D-004124-07	cgucuaccuucaccaauau
ITGB6 #1	D-008012-01	gcuaaaggaugucaauuaa
ITGB6 #2	D-008012-02	gaacggcucuuuccagugu
ITGB6 #3	D-008012-03	caucucagcuuaugaagaa
ITGB6 #5	D-008012-05	gccaacccuugcaguagua
ITGB7 #2	D-008013-02	gaccugagcuacuccauga
ITGB7 #3	D-008013-03	gaugauggcuggugcaaag
ITGB7 #4	D-008013-04	ggacaguaauccucucuac
ITGB7 #5	D-008013-05	acaguaauccucucuacaa
ITGB8 #2	D-008014-02	cugcaaaccucaauaauuu
ITGB8 #4	D-008014-04	gcagaaacgugacgagcaa
ITGB8 #5	D-008014-05	uggaaacgauuuaucuaga
ITGB8 #6	D-008014-06	gaucagacgucucaucucg

4.4. Patient material

Table 8: List of primary mesenchymal stem cell donors.
MSC were collected by the Heidelberg University Clinic after written consent according to the guidelines approved by the Ethics Committee of Heidelberg University (348/2004).

Donor ID	Gender	Source	age
275 / Donor 1	male	Bone marrow of the Iliac crest	50
304 / Donor 2	male	Bone marrow of the Iliac crest	26
345 / Donor 3	male	Bone marrow of the Iliac crest	25

Table 9: List of primary hematopoietic stem cell donors.
HSC were collected by the Heidelberg University Clinic after written consent according to the guidelines approved by the Ethics Committee of Heidelberg University (cord blood: 257/2002, peripheral blood: 348/2204).

Donor ID	description
CB4090-CB4092	Pooled HSC from cord blood of two donors
mPB170	Mobilized HSC from peripheral blood of one donor
CB4496-CB4502	Pooled HSC from cord blood of four donors

4.5. Proteins

Table 10: List of recombinant and isolated proteins.

Protein	Source	Type	Cat #	Manufacturer
CCL2	*E. Coli*	Recombinant, human	279-MC-010/CF	R&D Systems
CCL5	*E. Coli*	Recombinant, human	275-RN-010/CF	R&D Systems
CXCL12	*E. Coli*	Recombinant, human	350-NS-010	R&D Systems
Type I collagen	Placenta tissue	Isolated, human	C5483-1MG	Sigma
Type III collagen	Skin tissue	Isolated, human	C4407-1MG	Sigma
Fibronectin	Human plasma	Isolated, human	F0895-1MG	Sigma
Galectin-3 binding protein	Mouse NSO cell line	Recombinant, human	2226-GA-050	R&D Systems
Laminin 411	HEK293 human cell line	Recombinant, human	LN411-02	BioLamina
Laminin 421	HEK293 human cell line	Recombinant, human	LN421-02	BioLamina
Nidogen 1	Mouse NSO cell line	Recombinant, human	2570-ND-050	R&D Systems
TGF-β induced protein	Mouse NSO cell line	Recombinant, human	3409-BG-050	R&D Systems
Thrombospondin 1	Mouse NSO cell line	Recombinant, human	3074-TH-050	R&D Systems
Thrombospondin 2	Mouse NSO cell line	Recombinant, human	1635-T2-050	R&D Systems

5. Methods

5.1. Cell culture and cell-based assays

5.1.1. Isolation and characterization of MSC

Human bone marrow samples from the iliac crest were collected by bone marrow aspiration after written consent using the guidelines approved by the Ethics Committee of Heidelberg University (348/2004). The mononucleated cell fraction was isolated after density gradient centrifugation using lymphocyte separation medium LSM 1077 (PAA). After washing, mononucleated cells were re-suspended according to Verfaillie[150] in low FCS culture medium consisting of low glucose Dulbecco's modified Eagle's medium (DMEM) (PAA) supplemented with 40% (v/v) MCDB201 (Sigma), 2% (v/v) fetal calf serum (FCS) (HyClone), 2 mM L-glutamine (Sigma), 100 U/ml penicillin/streptomycin (Pen/Strep) (Lonza), 1% (v/v) insulin transferrin selenium (Sigma), 1% (v/v) linoleic acid-albumin from bovine serum albumin (Sigma), 10 nM dexamethasone (Sigma), 0.1 mM l-ascorbic acid 2-phosphate (Sigma), 10 ng/ml of each PDGF-BB and EGF (PreproTech) and seeded in T75 vented filter cap tissue culture flasks (Greiner bio-one) at a concentration of about $1x10^6$ cells/cm^2. Medium was changed after 2-3 days to remove non-adherent cells. Initial colonies were separated and further cultured. After reaching 80% confluence the cells were detached with 0.25% Trypsin EDTA (PAA), washed and seeded at a density of 10,000 cell/cm^2 for expansion. MSC were further characterized for their ability to differentiate towards osteogenic and adipogenic lineages and their immunophenotype with the Stemflow hMSC Analysis Kit (BD). After passage two, cell banks of each donor were generated and stored in liquid nitrogen.

5.1.2. Expansion of MSC and production of MSC cell culture supernatant

MSC were thawed and further expanded under previously described conditions in T75 and T175 vented filter cap tissue culture flasks (Greiner bio-one). After expansion up to passage six, cells were grown to full confluence and washed with PBS followed by 48 h of cultivation in DMEM F12 medium (Invitrogen) without any further additives. Cell culture supernatant of MSC was harvested under sterile conditions and stored at -20C°.

5.1.3. Isolation, characterization and culture of HSC

Human HSC were either collected from fresh umbilical cord blood, or from granulocyte colony-stimulating factor mobilized peripheral blood. All samples were taken after informed consent using guidelines approved by the Ethics Committee on the “Use of Human Subjects” at the University of

Heidelberg. Mononuclear cells were isolated after density gradient centrifugation on Ficoll-Hypaque (Biochrom KG, Berlin, Germany). CD34+ cells were enriched by labeling with a monoclonal anti-CD34 antibody conjugated with magnetic MicroBeads and passing them over an affinity column in an AutoMACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany).
After isolation, HSC were stored up to several hours at 4° in MACS buffer containing 0.1% FCS. Cells were then directly transferred to the respective experiments in DMEM F12 medium containing 10% FCS.

5.1.4. Culture of cancer cell lines

PC3, VCaP and LNCaP prostate cancer cell lines as well as the MDA-MB-231 breast cancer cell line were obtained from ATCC. All cancer cell lines were cultivated in DMEM high glucose supplemented with 10% (v/v) FCS and 100 U/ml penicillin/streptomycin under standard conditions. Media was changed every other day and cells were passaged at 80-90% confluence.

5.1.5. Mycoplasma testing of cultured cells

All cells and cell lines cultured und utilized in preparation of this work were checked for mycoplasma contaminations on a regular basis and were shown to be free of mycoplasma. Mycoplasma tests were conducted according to Uphoff and Drexler[291].

5.1.6. Cell line authentication

The identity of all cell lines cultured und utilized in preparation of this work was confirmed by the Genomics and Proteomics Core Facility of the German Cancer Research Center Heidelberg according to Castro et al.[292].

5.1.7. siRNA transfections

siRNAs against genes of interest (Table 7) were obtained from Dharmacon. Cells were transfected in 6 well plates with 20 nm siRNA using 0.15% Viromer Blue transfection reagent (Lypocalix) in a reverse transfection protocol. 1.5µl siRNA stock (5 µM) was prepared in 98.5 µl siRNA buffer (Lypocalix) and applied to single 6-wells. 2 µl Viromer Blue transfection reagent was diluted in 98 µl siRNA buffer, briefly mixed, incubated for 10 min at room temperature, finally added to single 6-wells containing siRNAs and incubated for 20-30 min. Cancer cells were added to the transfections mix in 1.3 ml DMEM high glucose supplemented with 10% (v/v) FCS for a total of 1.5 ml and cultured for 72 h. After 48 h, 1 ml of fresh medium was added. To identify adverse effects caused by the transfection protocol, siRLUC was used as control.

5.1.8. Quantitative real-time PCR

Total RNA was extracted according to the RNeasy Mini Kit protocol (Quiagen) and RNA concentration determined prior to cDNA synthesis. The cDNA was prepared from 1 µg of total RNA, using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fischer Scientific) and oligodT primer according to manufacturer protocol.

Subsequent quantitative real-time PCR (qPCR) was performed using the Lightcycler 480 system (Roche) in a 384-well format. 5 µl of cDNA (5 µg/µl) per well was transferred to 384-qPCR plates. 6ul of reaction mix was then added to each well. Reaction mix consisted of 5.5 ml master mix (Roche), 0.22 µl forward and reverse primer mix (20 µM), 0.11 µl UPL-hydrolysis probe (Roche) and 0.17 µl ddH_2O. The combinations of intron spanning primer and probe for each query gene were designed using the Roche ProbeFinder V2.45. All samples were amplified as triplicates and differential gene expression was calculated using the delta delta Ct method. *GAPDH* was used as reference gene for relative quantification.

5.1.9. Transwell migration (xCELLigence) of cancer cell lines

Cancer cell migration was analyzed using transwell migration CIM-plates with the xCELLigence RTCA DP system (Acea Bioscience) in a quantitative and time resolved fashion. CIM plates are based on classic transwell migration chambers. The upper chamber has 16 single wells that are sealed at the bottom with a microporous polyethylene terephthalate membrane containing micro-fabricated gold electrode arrays conducting an electrical current on the bottom side of the membrane. The median pore size of this membrane is 8 µm. Cells surpassing the membrane along a chemotactic gradient will attach to the gold electrodes causing electrical impedance. The xCELLigence readout is defined by the manufacturer as 'cell index (CI)'. The cell index is dependent on the background resistance measured at time point zero (Rt0), resistance measured at time point 'n' (Rtn) and the frequency dependent constant of the instrument (Figure 15).

Prior to migration, 175 µl of chemoattractant was placed in the lower and 50 µl of fresh DMEM F12 medium pipetted into the top chamber. After assembly, a background measurement was conducted to determine the resistance at time point zero. Cells were then detached with 0.25% trypsin, resuspended in fresh DMEM F12 medium without additives and 50,000 cells introduced into the upper chamber. The measurement interval was set to 5 min.

Cells migrating towards a known or suspected chemoattractant were also analyzed in parallel for their migration towards clean buffer or clean medium without chemoattractant. This biological background measurement allowed for the differentiation between baseline motility and directed migration of the cells towards the respective chemoattractants. By subtracting the biological

background for each time point, variability caused by passage number, cellular fitness or toxicity effects caused by treatments was reduced and allowed better comparison between independent experiments. Migration dynamics were determined with the RTCA xCELLigence software version 1.2.

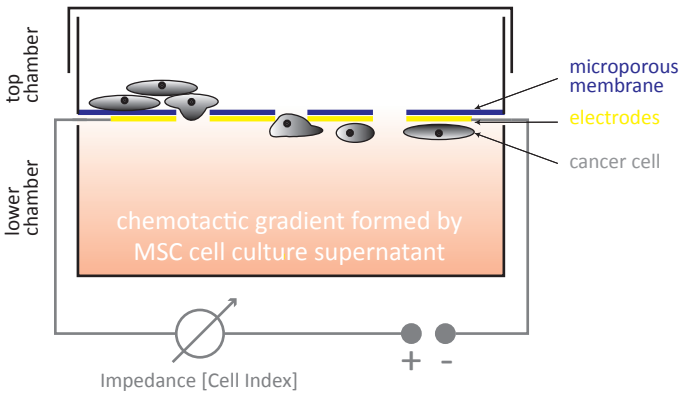


Figure 15: Graphical representation of single xCELLigence transwell.
Cancer cells seeded into the top chamber migrate along a chemotactic gradient towards the bottom chamber, surpassing the microporous membrane and attaching to the electrodes conducting an electrical current. This attachment influences the electrical current by causing impedance, which is directly dependent on the amount of migrated cells that have attached to the electrodes.

5.1.10. Transwell migration (classic Boyden chambers) of cancer cell lines

As the xCELLigence system does not allow visual inspection of the migrated cells, results obtained with the xCELLigence system were confirmed and complemented with classic Boyden chamber transwell assays (8 µm pore size; Corning Costar) using uncoated filters. Cells were detached with 0.25% trypsin, resuspended in fresh DMEM F12 medium without additives and 50,000 cells in 200 µl cell suspension were introduced into the upper chamber. 600 µl chemoattractant was placed in the lower chamber. Cells migrating towards a known or suspected chemoattractant were also analyzed in parallel for their migration towards clean buffer or clean medium without chemoattractant for comparison. After migration, transwells were washed in PBS, placed in 4% PFA fixation solution for 10 min, washed twice with PBS and placed in haematoxylin cell staining solution (Carl Roth GmbH) for 30 min. Transwells were then thoroughly rinsed in tap water and non-migrated cells remaining on the top side of the membrane were removed with a cotton swab. Afterwards the membranes were left to dry. Dry membranes were cut out and mounted on glass slides for microscopy analysis.

5.1.11. Transwell migration of HSC

In order to investigate the migration of HSC towards the recombinant chemokine CXCL12, cells were analyzed in classic 24-well Boyden chamber transwell assays (3 µm pore size; Corning Costar) using uncoated filters. Non-adherent HSC accumulated at the bottom of the well after migration through the filter inserts. This allowed them to be monitored using an IncuCyte Zoom live-cell imaging microscope (Essen Bioscience). Phase contrast and red fluorescence images were acquired every hour and the number of migrated cells per well were determined with the IncuCyte Zoom

software. Fluorescence imaging of the HSC required treatment with the life cell dye PKH26. Cells were stained with the PKH26 red fluorescent cell linker kit according to manufacturer protocol (Sigma). HSC were washed once in DMEM F12 medium without FCS, pelleted and resuspended in 500 µl diluent C. 500 µl staining solution (6 µM PKH26 in diluent C) was added and cells incubated for 2 to 5 min under constant rotation. The staining was stopped by the addition of 1 ml FCS. Cells were then washed three times with DMEM F12 medium containing 10% FCS resulting in a cell suspension of 10^7 cells/ml. Finally, the transwell migration experiments were set up by placing 600 µl DMEM F12 (10% FCS, 100 ng/ml CXCL12) and DMEM F12 (10% FCS) as a negative control into the bottom chambers. 200 µl HSC cell suspension were then introduced into the upper chamber and the 24-well plate set into the IncuCyte microscope for real time imaging. During the imaging process the IncuCyte system had problems choosing the correct focus plane resulting in occasional images of the filter insert instead of the cells on the well bottom. The addition of small amounts of inert agar beads into the bottom of the wells helped the imaging system to set the focus plane correctly onto the well bottom.

5.1.12. Processing of cell culture supernatant by FPLC

Complexity of SN MSC samples was reduced by fast protein liquid chromatography (FPLC) using an Äkta Explorer 100 system coupled with a Frac 950 fraction collector (GE Healthcare). System control and data analysis was conducted with Unicorn Software Version 4.0 (GE Healthcare). FPLC was run at RT with 50 mM Trizma® Base buffer (Sigma-Aldrich), pH 7.5 (Buffer A) and 50 mM Trizma® Base buffer, pH 7.5 containing 1M NaCL (Sigma-Aldrich) (Buffer B). Prior to use, buffers were filtered and degased.

5.1.13. Sample preparation

Prior to FPLC, remaining cells as well as cellular debris and precipitated protein was removed by passing the cell culture supernatant through a 0.22 µm sterile filter (Millipore). For each size exclusion chromatography run, 16 ml of filtered SN MSC was concentrated to approximately 700 µl by ultrafiltration using Amicon ultra-filter units with a molecular weight cut-off of 3 kDa (Millipore).

5.1.14. Size exclusion chromatography

700 µl of SN MSC concentrate was injected into a 500 µl sample loop ensuring an air bubble-free loading of the sample. After manually equilibrating the Superdex 200 HR 10/30 column (GE Healthcare), proteins present in SN MSC were separated based on their size and fractionated in an automated fashion (Table 11) into a low-protein-bind 96 well plate (Eppendorf).

In order to estimate the size of eluted proteins, the Superdex 200 column was calibrated with a size exclusion chromatography calibration kit containing a set of proteins of defined size (Bio-Rad). 500 µl of calibration standard was injected and fractionated under standard conditions (Table 11) and according to manufacturer instructions. Elution volume and protein size were then correlated by determining the void volume of the column V_0 and the retention volume V_R of each protein present in the protein standard solution. By plotting the known molecular size of each protein over its corresponding ratio of V_R/V_0, a logarithmic standard curve was determined.

Table 11: Äkta-Unicorn process parameters for automated SEC FPLC.

Process Block	Variable	Value
Main	Column	Superdex 200 10/30
Flow rate	Flow rate	0.25 ml/min
Column pressure limit	Column pressure limit	1.5 MPa
Start instructions	Wavelength 1	290 nm
	Wavelength 2 & 3	off
	Averaging time UV	2.56 s
Start with pump wash explorer	Wash inlet A1, A2, B1, B2	Off
Start conc. B	Start conc. B	15%
Column equilibration	Equilibrate with	0.2 cv
Sample injection	Empty loop with	1.5 ml
Isocratic elution before fractionation	Length before fractionation	0.2 cv
Volume fractionation	Eluate fraction size	0.6 ml
Isocratic elution with fractionation	Length with fractionation	1.2 cv

5.1.15. Ion exchange chromatography

After SEC, fractions of interest were pooled and further fractionated by ion exchange chromatography (IEX) with a Mono Q™ 5/50 GL ion exchange column (GE Healthcare). The column was manually equilibrated and the sample manually loaded with the help of a superloop (GE Healthcare). Flow-through while loading the column was discarded and bound protein was eluted off the column by a NaCl gradient in an automated fashion (Table 12) and fractionated into a low-protein-bind 96-well plate.

Table 12: Äkta-Unicorn process parameters for automated IEX FPLC.

Block	Variable	Value
Main	Column	Mono_Q_HR_5/5
Flow rate	Flow rate	0.5 ml/min
Column pressure limit	Column pressure limit	4 MPa
Start instructions	Wavelength 1	290 nm
	Wavelength 2 & 3	off
	Averaging time UV	2.56 s
Start conc. B	Start conc. B	5%
Column equilibration	Equilibrate with	5 cv
Sample injection	Empty loop with	24 cv
Wash out unbound sample	Wash column with	2 cv
Volume fractionation	Start fractionation at	5%
	End fractionation at	100%
	Eluate fractions size	0.5 ml
Linear gradient	Target concentration	100%
	Length of gradient	20 cv
Gradient delay	Gradient delay	2 cv
Clean after elution	Clean with	5 cv

5.2. Protein analysis

5.2.1. Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS)[110] experiments were conducted by the proteomics core facility of the German Cancer Research Center in collaboration with Dr. Martina Schnölzer and Ramona Mayer. Mass spectrometry analysis of FPLC fractions required the following preparation of the samples. In order to remove non-protein contaminants and prepare the sample for in-solution digestion a quantitative precipitation was performed. Protein precipitation was induced using a methanol-chloroform-water mixture according to Wessel and Flügge[293]. Protein precipitation was achieved by mixing 100 µl sample with 400 µl methanol followed by the addition of 100 µl chloroform and 300 µl ddH_2O. After each mixing step, the sample was vortexed thoroughly. Phase separation was then induced by centrifugation of the sample at 15,000 rpm for 2 min. The upper phase containing the chloroform was then removed without touching the interphase containing the precipitated protein. After the further addition of 300 µl methanol, vortexing and subsequent centrifugation at 15,000 rpm for 2 min the complete supernatant was removed and the precipitated protein pellet was dried for 10 min. After drying, the pellet was redissolved in 10 µl buffer containing 40 mM ammonium bicarbonate and 2 µl 10 mM dithiothreitol. Finally, proteins were alkylated by the addition of 1 µl 55 mM iodoacetamide and lastly the reaction quenched by the addition of further 2.5 µl dithiothreitol. Proteins were then enzymatically digested by the addition of 0.05 to 0.1 µg trypsin.

For ESI-MS, tryptic peptides were separated using a nanoAcquity UPLC system (Waters GmbH). Peptides were loaded on a C18 trap column (180 µm x 20 mm) with a particle size of 5 µm (Waters GmbH). Liquid chromatography separation was performed on a BEH130 C18 main-column (100 µm x 100 mm) with a particle size of 1.7 µm (Waters GmbH) at a flow rate of 0.4 µl/min. The chromatography was carried out using a 1 h gradient of solvent A (98.9% water, 1% acetonitrile, 0.1% formic acid) and solvent B (99.9% acetonitrile and 0.1% formic acid) in the following sequence: from 0 to 4% B in 1 min, from 4 to 40% B in 40 min, from 40 to 60% B in 5 min, from 60 to 85% B in 0.1 min, 6 min at 85% B, from 85 to 0% B in 0.1 min and 9 min at 0% B. The nanoUPLC system was coupled online to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Data was acquired by scan cycles of one FTMS scan with a resolution of 60,000 at m/z 400 and a range from 300 to 2000 m/z in parallel with six MS/MS scans in the ion trap of the most abundant precursor ions. Instrument control, data acquisition and peak integration were performed using Xcalibur software 2.1 (Thermo Scientific, Bremen, Germany).

Database searches were performed with the human SwissProt database using the MASCOT search engine (Matrix Science; version 2.2.2). Peptide mass tolerance for database searches was set to 5 ppm and fragment mass tolerance was set to 0.4 Da. Significance threshold was $p<0.01$. Carbamidomethylation of cysteine was set as fixed modification. Variable modifications included oxidation of methionine and deamination of asparagine and glutamine. One missed cleavage site in case of incomplete trypsin hydrolysis was allowed.

5.2.2. Cytokine antibody array

Unprocessed cell culture supernatant of MSC and SEC fractions of interest were analyzed for the presence of cytokines acting as potential chemoattractants. The membrane based c-series antibody arrays 'Human Angiogenesis Antibody Array C1000' and 'Human Chemokine Antibody Array C1' (RayBiotech) are based on a sandwich immunoassay principle and allowed for simultaneous detection of 70 cytokines with high sensitivity. Antibody arrays were used according to manufacturer protocol. In short, samples were incubated on the nitrocellulose membranes featuring a panel of capture antibodies printed on the solid support. This was followed by incubation with biotinylated antibodies and finally horseradish peroxidase-labeled Streptavidin. All incubation steps were conducted under gentle rocking motion and followed by washing steps. Finally, the chemiluminescence based read-out was visualized on x-ray film.

For each sample tested a corresponding negative control was also analyzed to avoid false positive results, due to unspecific binding. These included fresh cell culture medium for SN MSC as well as fresh cell culture medium undergoing FPLC for FPLC fractions of interest.

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7. Appendix

7.1. List of abbreviations

bFGF: Basic fibroblast growth factor

BMP: Bone morphogenetic protein

BMSEC: Bone marrow sinusoidal endothelial cells

ECM: Extracellular matrix

EGF: Epidermal growth factor

FCS: Fetal calf serum

FPLC: Fast performance liquid chromatography

HGF: Hepatocyte growth factor

HSC: Hematopoietic stem cell

ITGB: Integrin β-subunit

M-CSF: Macrophage colony-stimulating factor

MSC: Mesenchymal stem cell

PGF: Platelet-derived growth factor

qPCR: Quantitative real-time PCR

RANKL: Receptor activator of NF-κB ligand

TGF-β: Transforming growth factor β

SCF: Stem cell factor

SN: Supernatant

VEGF: Vascular endothelial growth factor

7.2. List of figures

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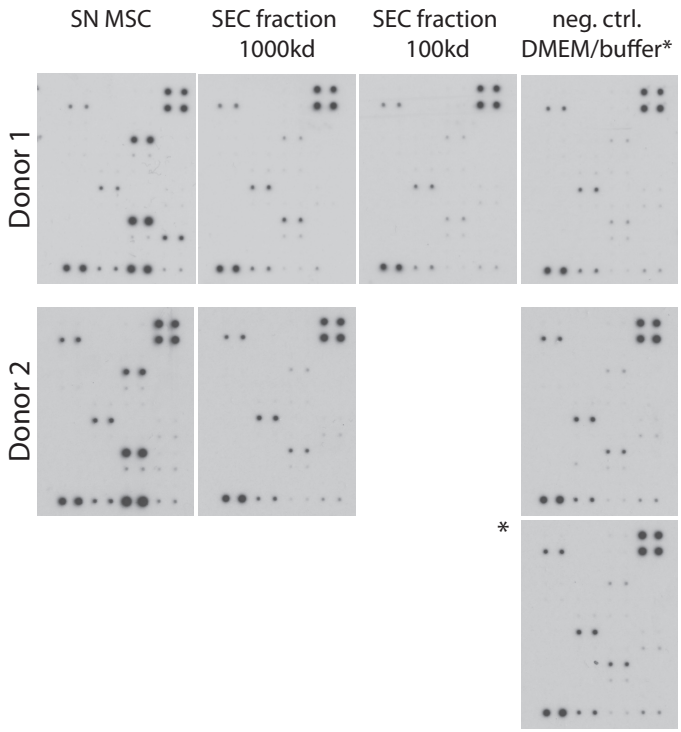
7.6. Cytokine arrays

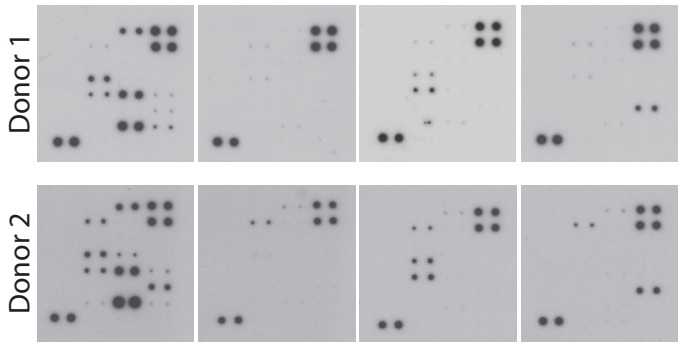
A: Chemokine array



array map

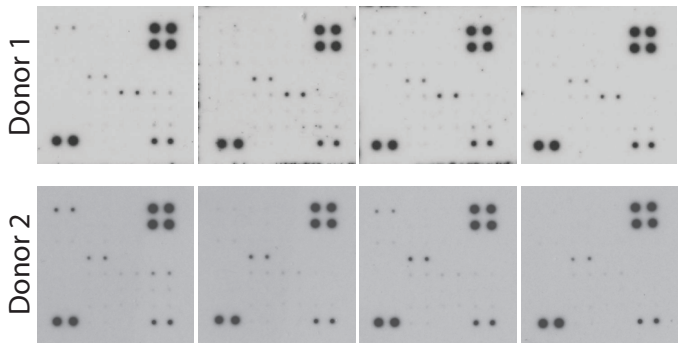
CCL18	CCL18	CCL8	CCL8	CCL26	CCL26	POS	POS
CCL5	CCL5	CCL2	CCL2	CX3CL1	CX3CL1	POS	POS
CXCL 12α	CXCL 12α	CCL13	CCL13	CXCL6	CXCL6	NEG	NEG
CXCL 12β	CXCL 12β	CCL22	CCL22	CXCL 1,2,3	CXCL 1,2,3	NEG	NEG
CCL17	CCL17	CXCL9	CXCL9	CXCL1	CXCL1	CXCL13	CXCL13
CCL25	CCL25	CCL3	CCL3	HCC4	HCC4	CCL28	CCL28
BLANK	BLANK	CCL4	CCL4	CCL1	CCL1	CCL23	CCL23
BLANK	BLANK	CCL15	CCL15	CXCL11	CXCL11	CCL27	CCL27
BLANK	BLANK	CCL20	CCL20	CXCL8	CXCL8	CXCL16	CXCL16
BLANK	BLANK	CCL19	CCL19	CXCL10	CXCL10	CXCL5	CXCL5
BLANK	BLANK	CCL23	CCL23	XCL1	XCL1	CCL11	CCL11
POS	POS	CXCL7	CXCL7	CCL2	CCL2	CCL24	CCL24

B: Angiogenic cytokine array 1



BLANK	BLANK	PLGF	PLGF	GRO	GRO	POS	POS
BLANK	BLANK	CCL5	CCL5	IFN-γ	IFN-γ	POS	POS
BLANK	BLANK	TGF-β1	TGF-β1	IGF-1	IGF-1	NEG	NEG
BLANK	BLANK	TIMP-1	TIMP-1	IL-6	IL-6	NEG	NEG
BLANK	BLANK	TIMP-2	TIMP-2	IL-8	IL8	ANG	ANG
BLANK	BLANK	THPO	THPO	LEPTIN	LEPTIN	EGF	EGF
NEG	NEG	VEGF	VEGF	CCL2	CCL2	CXCL5	CXCL5
POS	POS	VEGF-D	VEGF-D	PFGD-BB	PDGF-BB	bFGF	bFGF

C: Angiogenic cytokine array 2



uPAR	uPAR	CXCL11	CXCL11	G-CSF	G-CSF	POS	POS
VEGFR2	VEGFR2	CCL7	CCL7	GM-CSF	GM-CSF	POS	POS
VEGFR3	VEGFR3	CCL13	CCL13	CCL1	CCL1	NEG	NEG
BLANK	BLANK	MMP-1	MMP-1	IL-10	IL-10	NEG	NEG
BLANK	BLANK	MMP9	MMP9	IL-1α	IL-1α	ANGPT1	ANGPT1
BLANK	BLANK	PECAM1	PECAM1	IL-1β	IL-1β	ANGPT2	ANGPT2
BLANK	BLANK	TIE-2	TIE-2	IL-2	IL-2	PLG	PLG
POS	POS	TNFα	TNFα	IL-4	IL-4	ENDOST	ENDOST

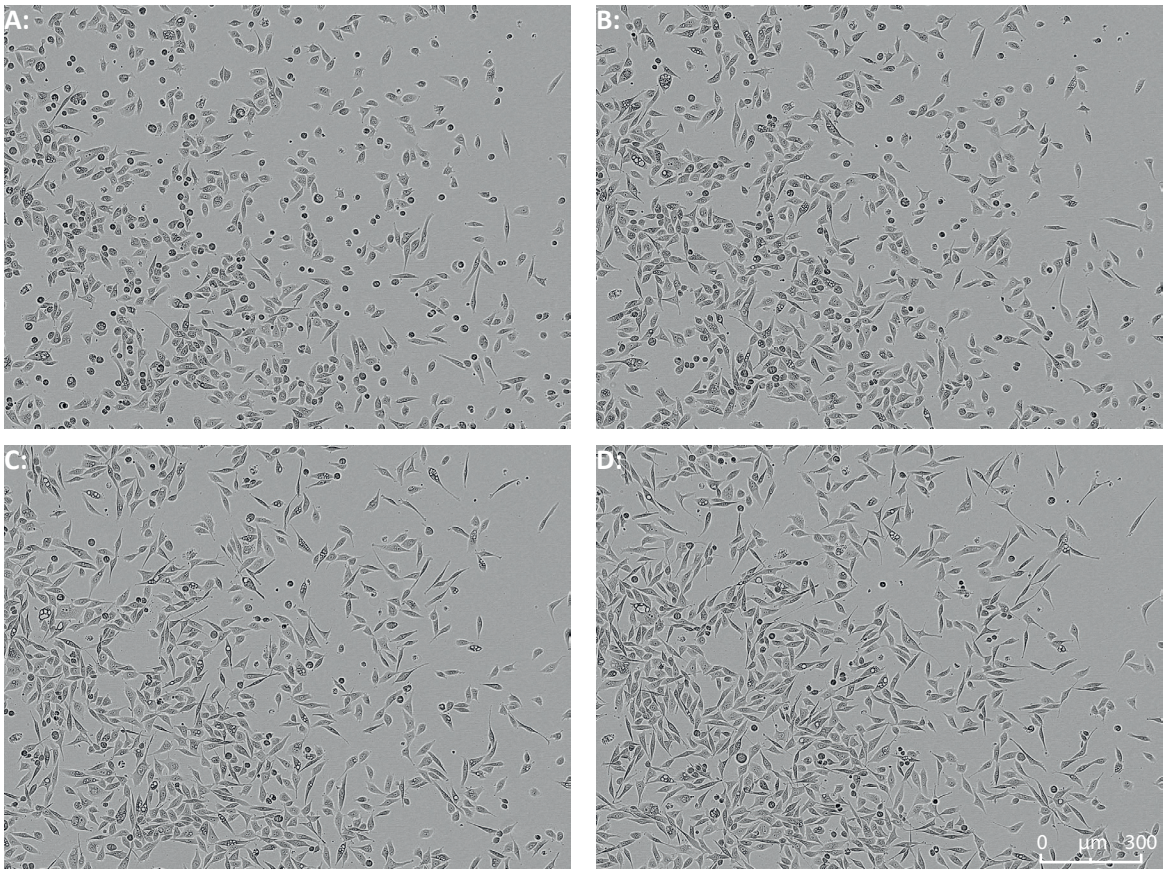
Supplementary figure 1: Antibody array exposures and positioning of spotted antibodies.
Detected chemokines **(A)** and angiogenic cytokines **(B,C)** in samples of the cell culture supernatant, 1000 kDa SEC fraction and 100 kDa SEC fraction of MSC donors 1 and 2. All three antibody arrays (A – C) were also tested with fresh DMEM F12 medium as negative control as well as tris-buffer (*) as an additional negative control in the case of the chemokine antibody array (A). Exposure time of all blots is 120s.

Supplementary table 1: List of all cytokines tested for their presence in cell culture supernatant and SEC fractions of cultured MSC by antibody array.
Detection of low (*) and medium (**) expressed and secreted cytokines potentially hampered by present background signal detected in neg. ctrl. samples.

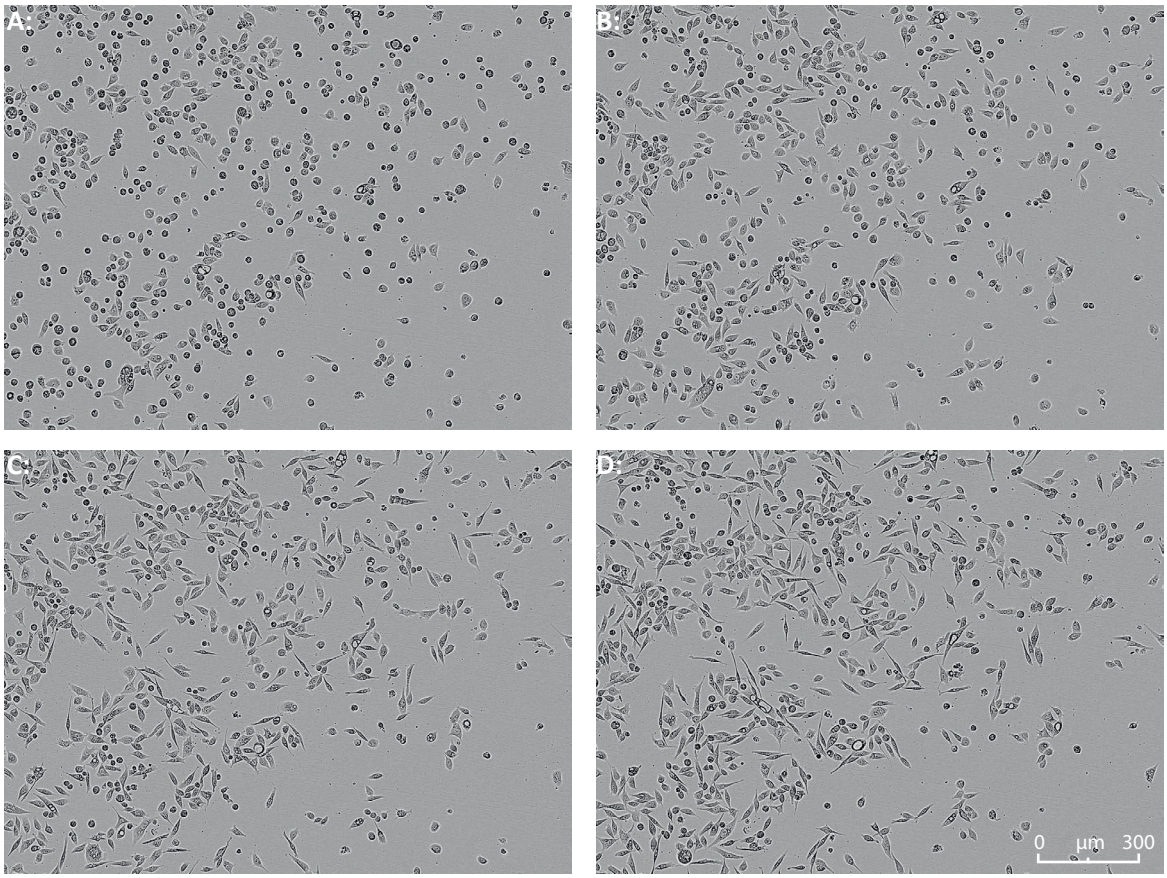
Chemokine array		Angiogenic cytokine arrays 1 & 2	
Chemokine symbol	**Name**	**Cytokine symbol**	**Name**
CCL1 (I-309)	T lymphocyte-secreted protein I-309	ANG	Angiogenin
CCL2 (MCP1)	Monocyte chemotactic protein 1	ANGPT-1	Angiopoietin-1
CCL3	Macrophage inflammatory protein-1α	ANGPT-2	Angiopoietin-2
CCL4 (MIP-1$^{\alpha}$) **	Macrophage inflammatory protein-1β	bFGF	Basic fibroblast growth factor
CCL5 **	RANTES	CCL1	T lymphocyte-secreted protein I-309
CCL7 (MCP3)	Monocyte chemotactic protein 2	CCL13	Monocyte chemotactic protein 3 (MCP-3)
CCL8 (MCP2)	Monocyte chemotactic protein 3	CCL2	Monocyte chemotactic protein 1 (MCP-1)
CCL11 (Eotaxin -1)	Eotaxin	CCL5**	Rantes
CCL13 (MCP4)	Monocyte chemotactic protein 4	CCL7	Monocyte chemotactic protein 2 (MCP-2)
CCL14	HCC-1	CD309 antigen	Vascular endothelial growth factor receptor 2
CCL15(MIP-1δ)	Macrophage inflammatory protein 5	COL18A1 **	Endostatin
CCL16 (HCC-4)	Hemofiltrate cc chemokine 4	CSF2	Granulocyte-macrophage colony stimulating factor
CCL17 (TARC)	Thymus and activation-regulated chemokine	CXCL1,2,3	Growth-regulated protein α,β,γ
CCL18 (PARC)	Macrophage inflammatory protein 4	CXCL11	Interferon-inducible T cell α chemoattractant
CCL19 (MIP-3β)	Macrophage inflammatory protein 3 β	CXCL5	Epithelial neutrophil-activating protein 78
CCL20 (MIP-3α)	Macrophage inflammatory protein 3	EGF	Epidermal growth factor
CCL22 (MDC)	Macrophage-derived chemokine	FIGF	Vascular endothelial growth factor D
CCL23 (MPIF-1) *	Myeloid progenitor inhibitory factor-1	GCSF	Granulocyte-colony stimulating factor
CCL24 (Eotaxin-2) *	Eotaxin-2	IFNG	Interferon γ
CCL25 (TECK)	Thymus-expressed chemokine	IGF1	Insulin-like growth factor-1
CCL26 (Eotaxin-3)	Eotaxin-3	IL10	Interleukin 10
CCL27 (CTACK) *	Cutaneous T-cell-attracting chemokine (CTACK)	IL1A*	Interleukin-1 α
CCL28	Mucosae-associated epithelial chemokine (MEC)	IL1B	Interleukin-1 β
CXCL1 (Gro α)	Growth-regulated	IL2	Interleukin 2

	α protein		
CXCL2 (Gro)	Growth-regulated protein β	IL4	Interleukin 4
CXCL3 (Gro)	Growth-regulated protein γ	IL6	Interleukin 6
CXCL5 (ENA 78)	Epithelial neutrophil-activating protein 78	IL8	Interleukin 8
CXCL6 (GCP-2)	Granulocyte chemotactic protein 2	LEP	Leptin
CXCL7 (NAP2) **	Neutrophil-activating peptide 2	MMP-9 *	Matrix metalloproteinase-9
CXCL8 (IL8)	Interleukin 8	MMP1 **	Interstitial collagenase
CXCL9 (MIG)	Monokine induced by γ interferon (MIG)	PDGFB	Platelet-derived growth factor subunit B
CXCL10 (IP-10)	Interferon-inducible protein-10	PECAM-1	Platelet endothelial cell adhesion molecule
CXCL11 (I-TAC)	Interferon-inducible T cell α chemoattractant	PGF	Placenta growth factor
CXCL12α (SDF-1^{α})	Stromal cell-derived factor 1α	PLAUR	Urokinase plasminogen activator surface receptor
CXCL12β (SDF-1 β)	Stromal cell-derived factor 1β	PLG	Angiostatin
CXCL13 (BLC)	B-lymphocyte chemoattractant	TEK	Angiopoietin-1 receptor
CXCL14	Chemokine BRAK	TGFB1	Transforming growth factor β-1
CXCL16	Transmembrane chemokine CXCL16	THPO	Thrombopoietin
CX3CL1 (Fractalin)	Neurotactin	TIMP-1	Metalloproteinase inhibitor 1
XCL1 (Lymphotactin)	Lymphotactin	TIMP-2	Metalloproteinase inhibitor 2
		TNF	Tumor necrosis factor
		VEGF R3	Vascular endothelial growth factor receptor 3
		VEGFA	Vascular endothelial growth factor A

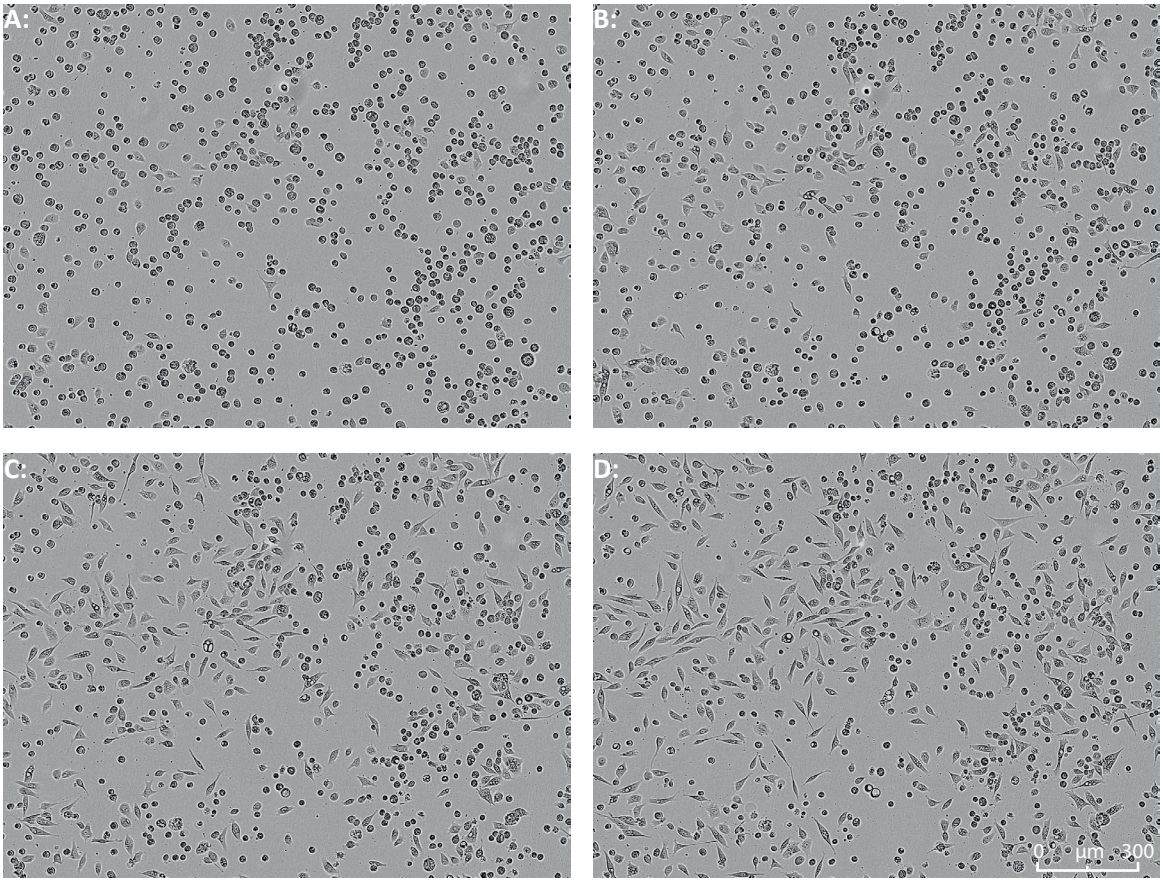
7.7. Images of prostate cancer cells undergoing RNAi treatment



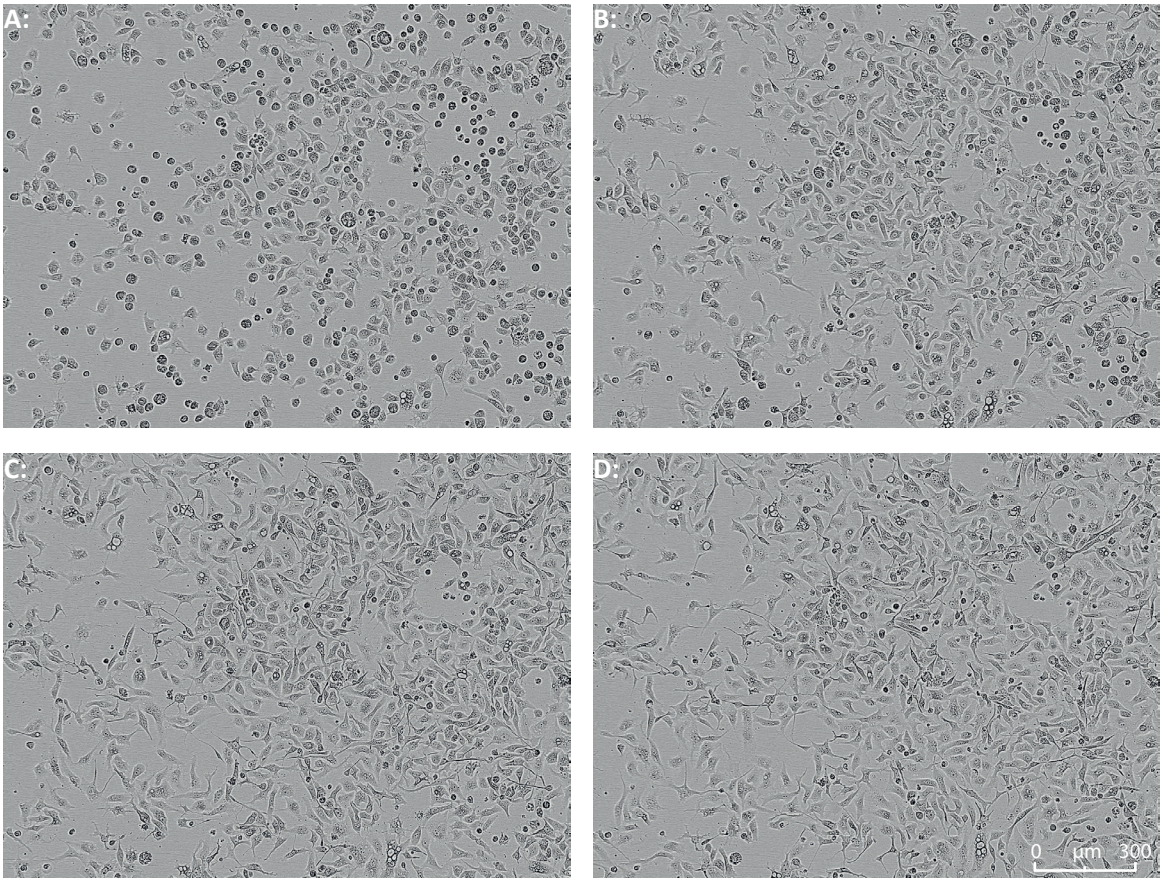
Supplementary figure 2: Phase contrast images of untreated PC3 cells.
Cells were seeded 72 h post reverse siRNA transfection in 96-well tissue culture plates and imaged at 10 x magnification 2 h (**A**), 4 h (**B**), 8 h (**C**) and 12 h (**D**) after seeding.



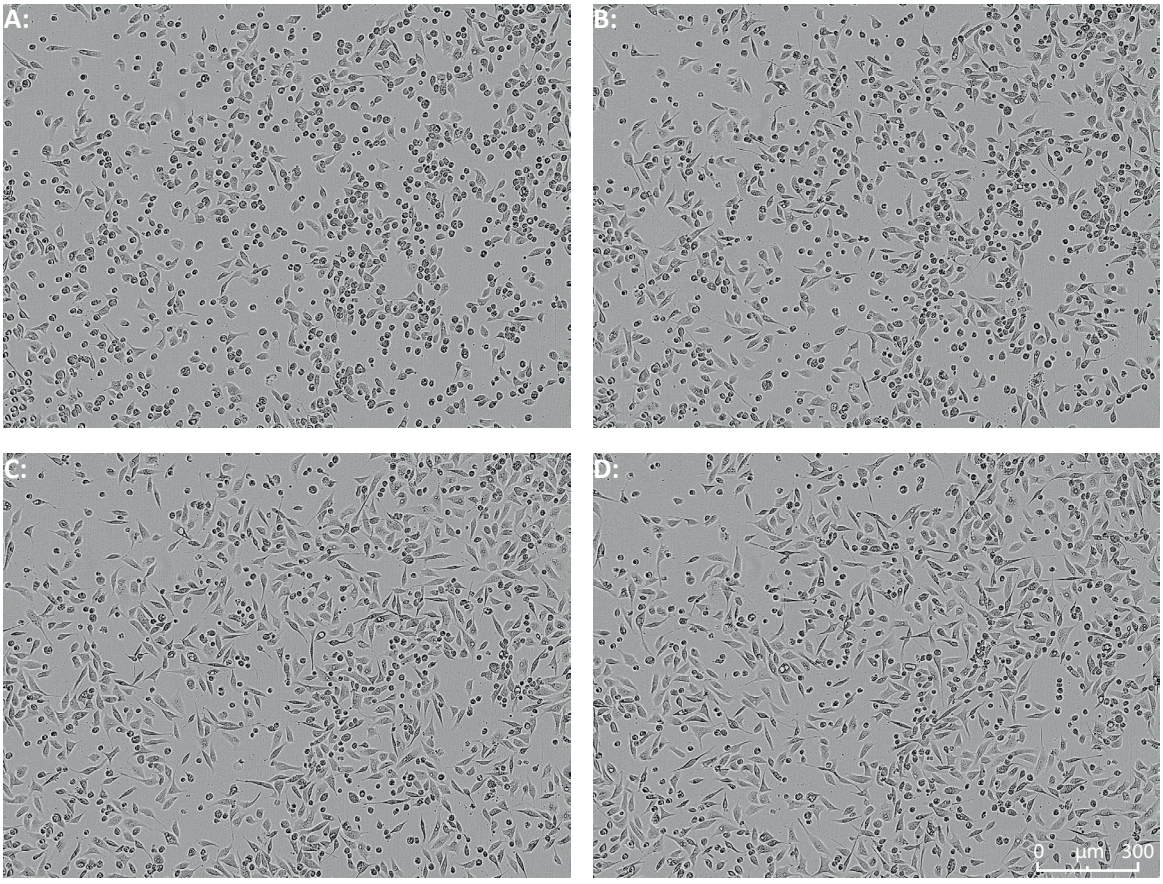
Supplementary figure 3: Phase contrast images of siRLUC treated PC3 cells.
Cells were seeded 72 h post reverse siRNA transfection in 96-well tissue culture plates and imaged at 10 x magnification 2 h (**A**), 4 h (**B**), 8 h (**C**) and 12 h (**D**) after seeding.



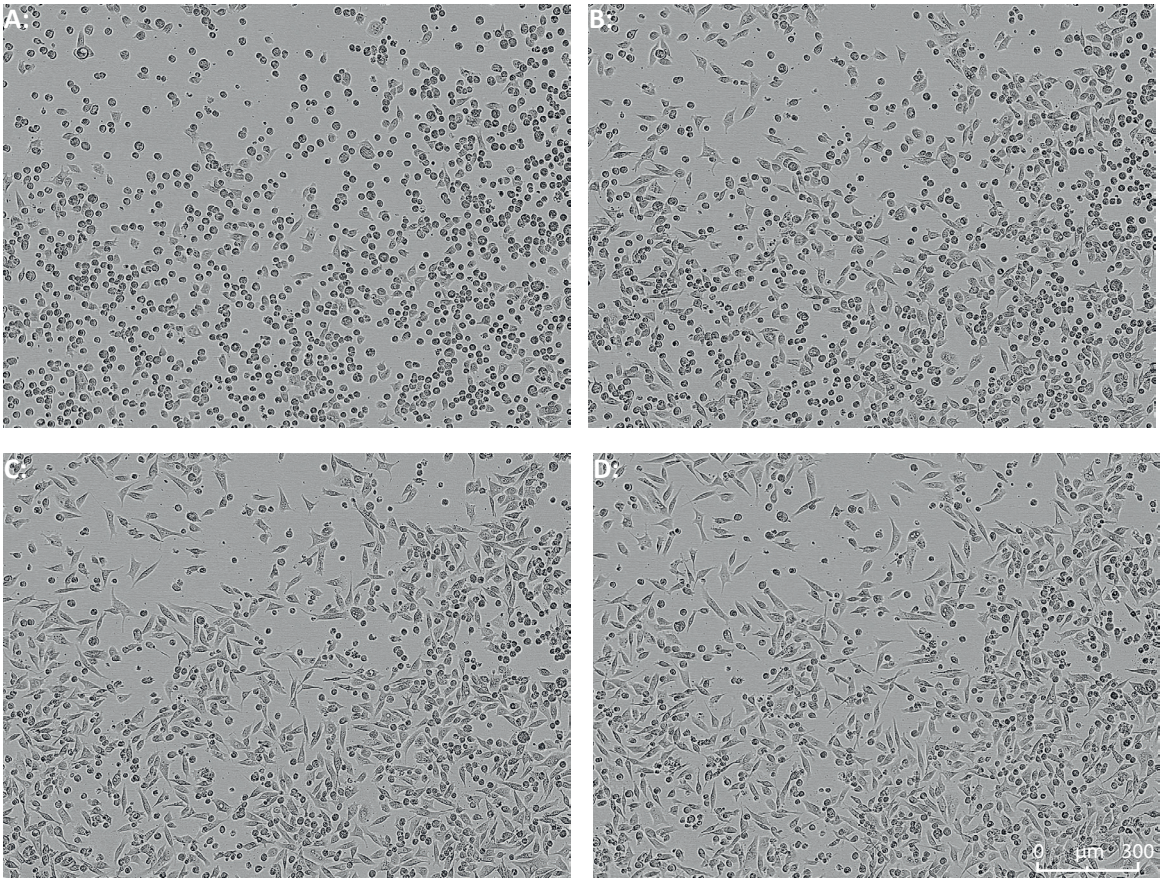
Supplementary figure 4: Phase contrast images of siITGB1 treated PC3 cells.
Cells were seeded 72 h post reverse siRNA transfection in 96-well tissue culture plates and imaged at 10 x magnification 2 h (**A**), 4 h (**B**), 8 h (**C**) and 12 h (**D**) after seeding.



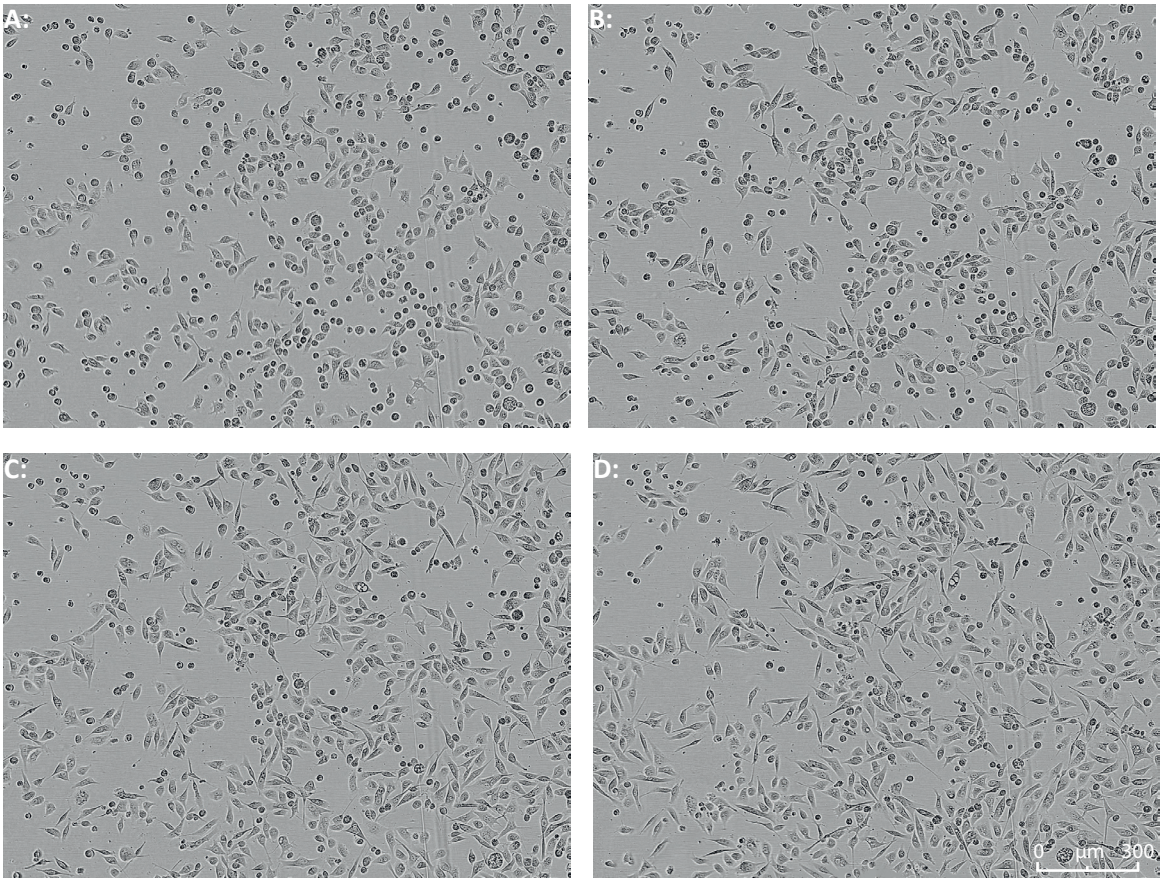
Supplementary figure 5: Phase contrast images of siITGB3 treated PC3 cells.
Cells were seeded 72 h post reverse siRNA transfection in 96-well tissue culture plates and imaged at 10 x magnification 2 h (**A**), 4 h (**B**), 8 h (**C**) and 12 h (**D**) after seeding.



Supplementary figure 6: Phase contrast images of siITGB6 treated PC3 cells.
Cells were seeded 72 h post reverse siRNA transfection in 96-well tissue culture plates and imaged at 10 x magnification 2 h (**A**), 4 h (**B**), 8 h (**C**) and 12 h (**D**) after seeding.



Supplementary figure 7: Phase contrast images of siITGB7 treated PC3 cells.
Cells were seeded 72 h post reverse siRNA transfection in 96-well tissue culture plates and imaged at 10 x magnification 2 h (**A**), 4 h (**B**), 8 h (**C**) and 12 h (**D**) after seeding.



Supplementary figure 8: Phase contrast images of siITGB8 treated PC3 cells.
Cells were seeded 72 h post reverse siRNA transfection in 96-well tissue culture plates and imaged at 10 x magnification 2 h (**A**), 4 h (**B**), 8 h (**C**) and 12 h (**D**) after seeding.