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Analysis of interaction of murine 66cl4 and 67NR breast carcinomas with tumor-associated macrophages

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ABSTRACT

Breast cancer is the most common cancer among women in the world, and death is usually caused by metastasis. A tumor is a heterogeneous mass of different cells, and the tumor microenvironment is complex with extensive communication between the different cell types. Tumor cells are able to polarize cells of the microenvironment, like macrophages, by secreting different compounds including members of the TGF-β superfamily. Macrophages can be polarized towards classically activated M1 macrophages or alternatively activated M2 macrophages. Tumor-associated macrophages (TAMs) are mainly M2 macrophages and support tumor growth by promoting tumor cell survival and proliferation, matrix remodeling, angiogenesis and metastasis. The number of macrophages in a tumor is correlated with poor prognosis in breast cancer patients.

By utilizing the 4T1 breast cancer mouse model the communication between tumor cells and macrophages was studied. Transcriptome data of cell lines and primary tumors of the non-metastatic 67NR and the metastasizing 66cl4 showed a higher amount of M2 macrophage markers in 66cl4 primary tumors. 66cl4 cells also produce and secrete the TGF-β superfamily member BMP4, as well as its antagonist GREM1. GREM1 was produced even more in 168FARN cell lines, as well as found to be cell surface-associated. High amount of GREM1 is correlated to poor prognosis in breast cancer patients. By adding conditioned medium from the tumor cells to RAW 264.7 macrophages, it was seen that conditioned medium from 168FARN, 66cl4 and 4T1 potently inhibited both basal and rmBMP4-stimulated SMAD signaling. Conditioned medium from 66cl4 also upregulated the inflammatory signaling in RAW 264.7 macrophages by activating STAT1. In bone marrow-derived macrophages (BMDMs) it was seen that both conditioned medium from 67NR, 168FARN and 66cl4, as well as the presence of them in a transwell changed the morphology of the BMDMs. The presence of 67NR, 168FARN and 66cl4 cells also activated the SMAD pathway of the BMDMs.

Further research is however needed to see if the regulation of the SMAD pathway in macrophages is related to the different functions of macrophages in tumors.

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Camilla Izabel Wolowczyk

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ABBREVIATIONS

ACTR2	Activin receptor 2
ADCC	Antibody-dependent cellular toxicity
ALK 1/2	Activin receptor-like kinase 1/2
BAMBI	Bmp and activin membrane bound inhibitor
BMDM	Bone marrow-derived macrophages
BMP	Bone Morphogenetic Protein
BMPR1A/B	Bone morphogenetic protein receptor 1 A/B
CAFs	Cancer-associated fibroblasts
CSC	Cancer stem cell
CSF-1	Colony Stimulating Factor-1
DNA	Deoxyribonucleic acid
ECM	Extracellular matric
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
FGF	Fibroblast growth factor
GDF	Growth differentiation factor
GF	Growth factors
GREM1	GREMLIN 1
HER-2	Human epidermal growth factor-2 receptor
HIF	Hypoxia-inducible factor
IFN	Interferon
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IRF3	Interferon regulatory factor 3
JAKs	Janus kinases
LPS	Lipopolysaccharide
M-CSF1	Macrophage-colony stimulation factor 1
MET	Mesenchymal epithelial transition
MHC	Major histocompability complex
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cells
NO	Nitric oxide
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor

PR	Progesterone receptor
PRR	Pattern recognition receptor
PTI	Protein transport inhibitor
RNA	Ribonucleic acid
SMURF1/2	Smad ubiquitin regulatory factor 1/2
STAT1	Signal transducers and activators of transcription 1
TAMs	Tumor-associated macrophages
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

The nomenclature of genes and proteins mentioned in this thesis follow the rules and guidelines of the *HUGO Gene Nomenclature Committee* (HGNC) for human genes and proteins, and the *International Committee on Standardized Genetic Nomenclature for Mice* for mice genes and proteins.

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1 INTRODUCTION

1.1 CANCER

1.1.1 BREAST CANCER

Breast cancer is the form of cancer that is most frequent among women in the world, with an estimated number of 1.67 million new cases in 2012 [1]. Breast cancer incidence varies in different geographic regions and is more frequent in more developed areas like Northern America, Western Europe and Australia, however the mortality is much higher in less developed countries [1].

In 2014, breast cancer stood for 22% of all cancer incidences among women in Norway [2]. Breast cancer incidence has doubled since the beginning of observation (1955-1959). One of the reason for the higher incidence rate is the implementation of the Norwegian Breast Cancer Screening Programme in 1996, which invites women at the age of 50-69 for a screening every second year. This program along with other novel methods of diagnosis has made it easier to detect breast cancer at a much earlier stage. Because of better diagnostics and novel cancer treatment, breast cancer survival has increased tremendously and it is now right below 90%. Despite this, 663 women died of breast cancer in Norway in 2014 [2]. In the last decades it has become clearer that lifestyle also contributes to breast cancer incidence. Factors that might influence breast cancer incidence negatively are little or no physical activity, overweight, alcohol and diet. Factors that might decrease the incidence are having children early and breastfeeding.

Around 5-10% of breast cancers are hereditary, meaning that they result directly from inherited mutations. The most common and known cause of hereditary breast cancer is an inherited mutation in *BRCA1* or *BRCA2* genes. These genes are tumor suppressor genes that are involved in DNA repair of double-stranded breaks. By inheriting a mutation in either of these genes, the risk of developing breast cancer is much higher. Other less common gene mutations are also causes of hereditary breast cancer, but they do not increase the risk as much as mutations in *BRCA1*/*BRCA2*. These include mutations in *ATM*, *TP53*, *CHEK2* and *PTEN* genes [3].

1.1.2 TUMOR HETEROGENEITY

For the past decades, it has been increasingly recognized that tumors are heterogeneous masses of different cells. This heterogeneity is due to both genetic and functional differences between the tumors (Figure 1) [4, 5]. Intertumoral heterogeneity is when patients have histologically similar tumors, but that differ in molecular subtype (Figure 1A). This is due to genetic aberrations in the tumor cells caused by the genome itself or external factors. Somatic mutations activate oncogenes and inactivate tumor-suppressor genes that will lead to uncontrolled cell growth and dysregulation of apoptosis and other cellular processes required for proper cell function [6]. The different combinations of mutations lead to heterogeneity between patients.

The cancer cells in the tumor might also have different phenotypes due to their genetic aberrations, resulting in different cancer cell subtypes within the tumor (Figure 1B). These differences are some of the hallmarks suggested by Hanahan and Weinberg in 2000 [6]. Some of these acquired abilities of tumor cells are sustained proliferative signaling and disruption of mechanisms that negatively regulate cell proliferation. Normal tissue cautiously control production and release of growth-promoting signals that guide cells through the cell cycle. Tumor cells disrupt this regulation, and either generate their own growth signals or direct the surrounding stroma to do it [7]. The different phenotypes among the cells make some of the cells better at escaping the normal growth control of the cell cycle.

Other differences might give some cells the ability to self-renew and initiate a tumor, while other cells differentiate or progress to apoptosis. Apoptosis is a natural barrier to tumor growth, however tumor cells evolve different strategies to avoid apoptosis; the most common is the loss of tumor-suppressor gene functions. Successful growth of tumors also requires an increasing demand for nutrients and oxygen. Tumors go through an “angiogenic” switch by changing the balance of pro-angiogenic and anti-angiogenic factors. This triggers sprouting of new blood vessels in to the tumor. Additionally, tumors have different abilities to invade local tissue and metastasize in other organs. Metastatic ability is obtained by further genomic alterations in genes encoding cell-cell adhesion molecules, integrins and proteases, thus changing the cells attachment to the extracellular matrix (ECM) and neighboring cells. Studies have also shown that tumor cells might get signals from the surrounding stroma that stimulates invasive behavior [7]. Of the disseminated cells, only a few are able to settle down and form macroscopic metastases. Metastatic cells will grow independently of the primary tumor and develop new genetic mutations and functions, thus creating intratumoral heterogeneity between primary site and the metastasis (Figure 1C).

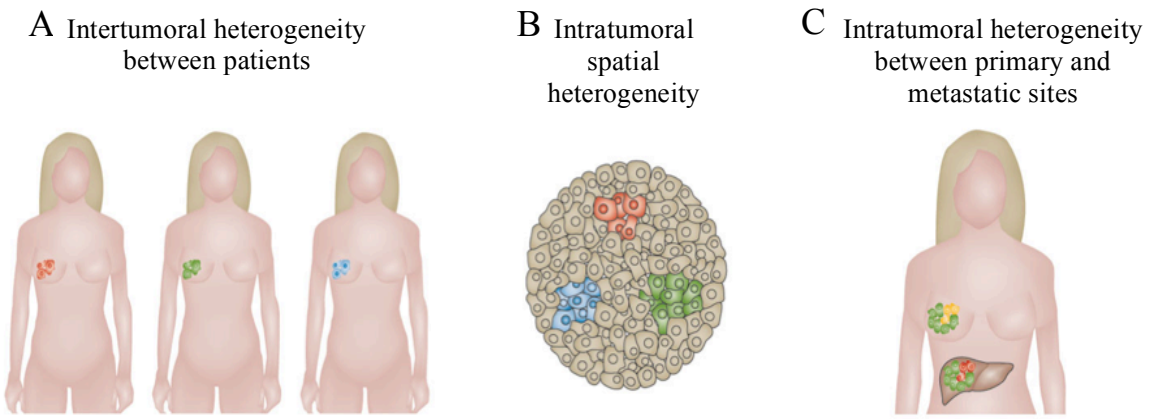


Figure 1 Tumor heterogeneity A. Intertumoral heterogeneity between patients that might have histologically similar tumors but different genetic mutations B. Intratumoral spatial heterogeneity due to different cell subpopulations in the tumor C. Intratumoral heterogeneity between primary and metastatic site due to different genetic mutation statuses. Modified from [4].

All these layers of heterogeneity present a clinical challenge when it comes to treating cancer. If tumors are viewed as a homogenous mass of cells that is treated with a standard treatment, many patients do not benefit from it while others would be over-treated. Tumors are classified based on how far the cancer has spread as well as the differences in hormone receptors on the cancer cell surface. Differences in protein and RNA expressions are also being used to characterize tumors [8]. These characteristics are the basis for personalized cancer therapy. The goal of personalized therapy is to give the right drug combination to the right person at the right time, ultimately improving survival and decreasing toxicity [4].

1.1.3 CLASSIFICATION OF BREAST CANCER

Classification and subtyping of breast cancer have been very important factors to assess prognosis and determine the right treatment for patients. Usually, several methods of classification are used in combination to better evaluate each individual patient. The American Joint Committee on Cancer (AJCC) TNM system is the most widespread staging system for breast cancer [3]. By utilizing both clinical and pathological staging techniques as biopsies, imaging and surgery results, it summarizes the information on how far the cancer has spread. TNM stands for tumor, nodes and metastasis, and ranks each stage after the size of the tumor, if the cancer has spread to the lymph nodes and whether metastases can be

found. After each category has been determined, they are combined in stage grouping. Tumors in the same group tend to have the same outcome and are treated similarly.

Tumor grading is a method that separates tumors into groups based on features of the cells [3]. A biopsy is taken from the tumor and the tumor is graded based on how much the cancer cells resembles normal cells, and on how many of the cells that are in mitosis. In Grade 1, the cancer cells in the tumor display a relatively normal phenotype and divide at a slow pace. Grade 2 is used for tumors harboring cancer cells that are less differentiated and that grow faster. Grade 3 means that the cancer cells look abnormal and grow very rapidly. Tumor grading directly correlates with prognosis, with Grade 3 giving poorest prognosis.

Breast cancer is also classified based on differences in hormone receptor expression, human epidermal growth factor 2-receptor (HER2) status and proliferation rate [3, 9, 10]. Most studies divide breast cancer into four subtypes, Luminal A, Luminal B, Her2 type and Basal. Luminal A is estrogen-receptor (ER) positive and/or progesterone receptor (PR) positive. It is HER2 negative and has a low proliferation rate. Luminal B is also ER and/or PR positive. It can be either HER2 positive with a low proliferation rate, or HER2 negative with a high proliferation rate. Luminal A and B account for approximately 70% of all invasive breast cancers. Luminal A tend to have a lower histological grading than Luminal B and the prognosis of it is often better. HER2 positive tumors can be both ER and PR negative and is usually of a higher histological grading and lymph node positive. HER2 type accounts for 15 % of invasive breast cancers, and generally has a poor prognosis. Basal subtype accounts for 15 % of invasive breast cancers and is also called triple negative because it does not express ER, PR or HER2. These patients are difficult to treat, and therefore have a poor prognosis. This classification is the basis of targeted treatment based on receptor inhibitors that are very effective for cells that are dependent on these receptors to grow. However, more research is needed to develop a similar treatment for the cells that do not express these receptors.

1.1.4 METASTASIS

Breast cancer death is usually caused by metastasis. Primary tumors are often treated with radiation or surgery, but if the tumor is detected at a late stage, tumor cells might have had time to spread to distant organs. Metastasis can be widely spread and thus difficult or impossible to locate and treat. Metastasis is a very ineffective process, where only a small percentage of the cells that are disseminated from the tumors are able to survive in a new environment and grow [11]. The initial step, intravasation, is dependent on a hallmark of

cancer, induction of angiogenesis. The newly developed blood vessels, which often are permeable and leaky, provide an escape route for tumor cells. However, tumor cells might also enter the circulation indirectly through the lymphatic system, and thus settle in the lymph nodes. If they survive in the circulation, they extravasate into surrounding tissue where they initiate and sustain growth to form macroscopic tumors. All the steps of the metastatic process are limiting. It was previously thought that surviving in the circulation was the major limiting step of the metastatic process because of the immune system and the hemodynamic forces [12, 13]. However, recent studies indicate that most tumor cells may survive in the circulation and the extravasation, suggesting that the growth after extravasation is the key regulator of metastasis [11, 14].

The tumor cells ability to metastasize is influenced by many factors. In 1889, Stephen Paget introduced the "seed and soil"-theory, proposing that the ability of cancer cells to metastasize to specific organs was dependent on the cancer cell (seed) and the secondary organ (soil) [15]. James Ewing challenged this theory in the 1920s, when he suggested that circulatory patterns were sufficient to explain organ-specific metastasis [16]. Since then there has been found evidence that support both theories. For instance, breast cancer has a tendency of metastasizing to bone, liver, brain and lungs [17]. The blood-flow pathways partially explain this since blood vessels from the tumor go through the heart to the lungs where cells get trapped in the thin capillaries. If some cells manage to pass, they can end up in other capillary beds in the body. Whether the cancer cells manage to settle and grow in these capillary beds is dependent on the interactions between the cells and the microenvironment in the new organ, as described in the "seed and soil"-theory.

1.1.5 TUMOR MICROENVIRONMENT

Tissues and organs consist of many different cell types that work together to maintain normal physiology. These microenvironments of tissues are tightly controlled by intercellular communication facilitated by the extracellular matrix (ECM). They are provided with nutrients and oxygen through the endothelial vasculature and protected by immune cells. Sometimes the normal context of tissue is temporary disrupted due to wounding. However, if inflammation is sustained for a longer period of time, a functional disorder occurs in the tissue due to higher number of immune cells, activated stroma, growth factors and enzymes, and DNA damage-promoting agents (Figure 2) [18]. In the 1860s, Rudolf Virchow proposed a link between inflammation and cancer when he hypothesized that tissue injury, and

inflammation due to the presence of some irritants, increased cell proliferation [19]. Since then, cancer has been described as “a wound that never heals” because it mobilize the same repair mechanisms and exploits it to support tumor cell growth.

Tumor cells in the body may be recognized as foreign and the immune system can therefore act on such to eradicate them. Resident immune cells at the site start to produce pro-inflammatory cytokines and chemokines to recruit more immune cells to the site of inflammation. Macrophages, dendritic cells and neutrophils are phagocytic cells that engulf and kill transformed cells. Granulocytes and natural killer cells lyse transformed cells by releasing toxic substances. These innate immune cells will also activate the adaptive arm of the immune system by presenting antigens to T-cells and B-cells. CD8+ T-lymphocytes are cytotoxic and lyse tumor cells by direct contact, while CD4+ T-lymphocytes are activated through contact with antigen-presenting cells. CD4+ T-lymphocytes produce a high amount of pro-inflammatory cytokines like interferon-γ (IFNγ) and interleukins (ILs) to recruit effector leukocytes to the tumor. Many have studied the complexity of the immune system in cancer and it seems that IFNγ is critical for the tumor-suppressing role of the immune system [20, 21]. The role of B-lymphocytes is however controversial. Some studies show that B-cells act tumor promoting by inhibiting the induction of T-lymphocytes, while others show that they are tumor suppressing by either working together with the T-lymphocytes or by sensitizing them for complement or by activating antibody-dependent cellular cytotoxicity (ADCC) [22-25].

However, if a tumor cell manages to evade the immune surveillance of the body, it starts to produce compounds that recruits and differentiates immune cells to support the tumor instead of fighting it [7]. Experimental and clinical data show that innate immune cells like macrophages, granulocytes, dendritic cells and natural killer cells act tumor promoting during cancer development [18]. High numbers of innate immune cells also correlate with poor clinical outcome or angiogenesis [26-28]. The increased population of innate immune cells produces a diversity of cytokines, chemokines and other cytotoxic factors like reactive oxygen species that create a microenvironment that facilitates cell proliferation, genomic instability and cancer development [29]. A family of cytokines that have proven to be important during tumorigenesis is the transforming growth factor-β (TGF-β) superfamily members. The TGF-β superfamily is a family of approximately 30 members that are divided into subfamilies, including BMP and activin/ inhibin subfamily. TGF-β is extensively studied and is a key regulator of many biological processes including development, cell proliferation, inflammation, wound healing and angiogenesis [30]. TGF-β has a dual role in tumorigenesis.

It is usually tumor-suppressive in the early stages of tumor development before it switches to a tumor-promoting role later during tumor progression [31]. TGF-β is expressed at high levels in breast cancers, and is correlated to the progression rate of the disease [32]. It also affects the microenvironment by both inhibiting the active immune response and increasing the immune cells repair mechanisms [31].

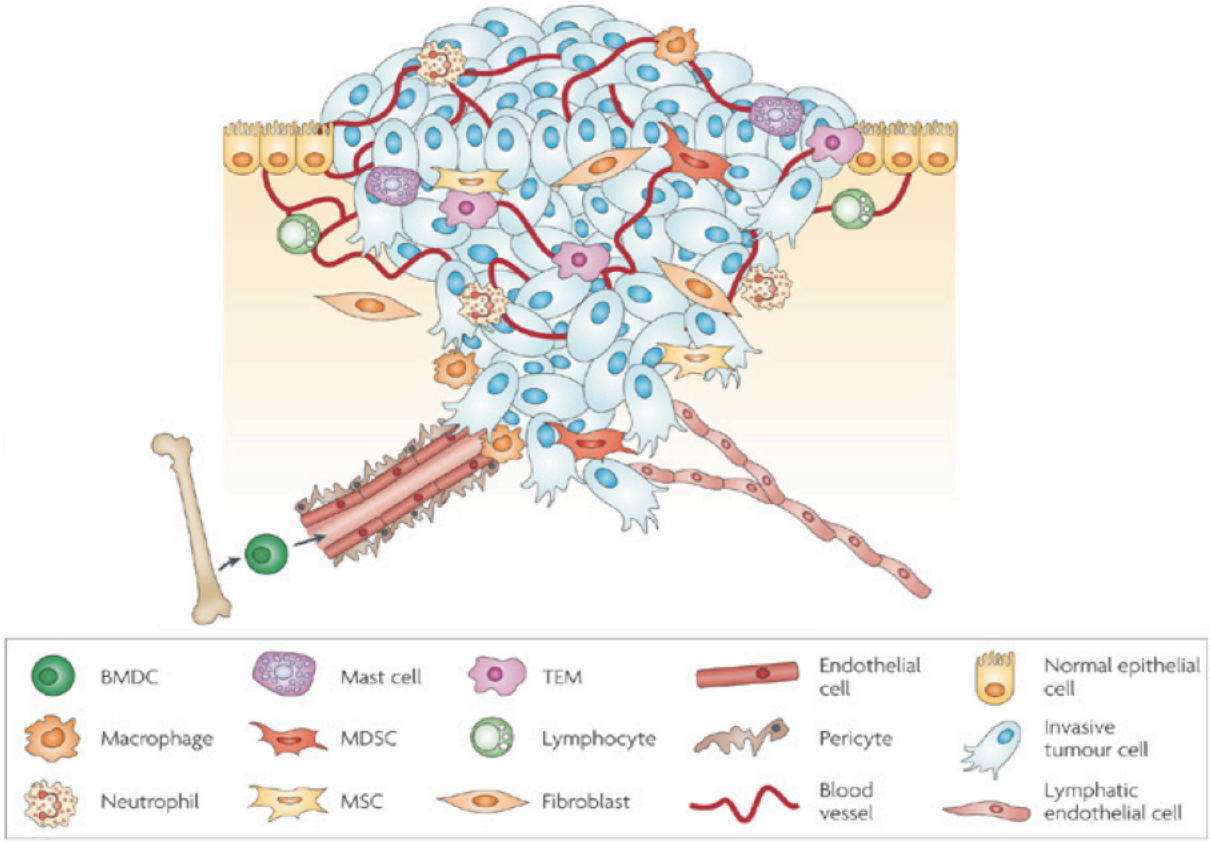


Figure 2 Tumor microenvironment Tumor cells in a tumor is surrounded by other cells like immune cells, bone marrow derived cells like macrophages and mesenchymal stem cells (MSCs), cells of the lymphatic and blood circulation and fibroblasts. Modified from [33].

When the tumor grows, there will be an extensive need for oxygen and nutrients. The hypoxic environment in the tumor, as well as activation of oncogenes and inactivation of tumor-suppressor genes, promotes angiogenesis through hypoxia-inducible transcription factors (HIFs), that upregulate the expression of many pro-angiogenic genes including the gene for VEGF [34]. VEGF, as well as platelet-derived growth factor (PDGF), stimulates growth and migration of endothelial cells and the supporting pericytes that forms new blood vessels. These create an escape route for tumor cells that can migrate to other tissues in the

body and form metastasis. Cells of the microenvironment also contribute by producing growth factors, cytokines and proteases that remodel the ECM [35-38].

Because of the importance of the microenvironment during the stages of tumorigenesis, different constituents of the microenvironment have been studied as targets for anticancer treatment. Targeting these components can be challenging since the components are still similar to normal cells, even though their functions are altered. The increased recruitment and number of innate immune cells like macrophages in the tumor is correlated to poor prognosis in patients, and more research is needed to study how this can be avoided or turned into the patients favor.

1.2 MACROPHAGES

For many years macrophages were recognized for their roles as immune effector cells, but research has made it clear that macrophages contribute to many other important functions in the body to sustain homeostasis. Macrophages are a part of the body's first line of defense, by quickly detecting endogenous danger signals and removing dead cell and debris as well as foreign particles and pathogens by phagocytosis. At the infected or injured site, macrophages colonize fast and start to produce a wide range of cytokines to attract other immune cells, and they also activate B- and T-lymphocytes by presenting them antigens.

Macrophages develop from myeloid progenitor cells found in the bone marrow. These cells mature into monocytes that are released into the peripheral blood where they continue to mature, before they enter different tissues to build up tissue macrophage populations. The monocytes in the blood stream are a heterogeneous population, but it is not clear whether they give rise to specific tissue macrophages [39]. The monocytes differ in size, granularity and nuclear morphology, and are recognized by markers as major histocompability complex (MHC) class II, CD14, CD64 and CC-chemokine receptors CCR2 and 5 [40].

The heterogeneity of macrophages is also seen in the specialized functions they adopt in different tissues. Due to their plasticity, signals from the environment can change their phenotype, giving specialized macrophages of the bone, lung, liver, brain, gut and eyes [40, 41]. Even though all macrophages depend on the same functions for phagocytosis and immunity, it seems like the local environments are crucial in the activation of the different functions necessary for that tissue.

1.2.1 POLARIZATION TOWARDS M1 AND M2 MACROPHAGES

Macrophages can change their physiology in response to the physiological or pathological situations they are recruited to. These signals can be cytokines from immune cells or other surrounding cells, or microbial substances. These changes lead to different subpopulations of polarized macrophages that differ in term of expression of receptors, cytokine production, effector function and in chemokine repertoire (Figure 3) [42]. The concept of classifying macrophages into M1 and M2 macrophages was based on T helper cell 1 and 2 nomenclature that separated T-cell populations based on the above-mentioned characteristics.

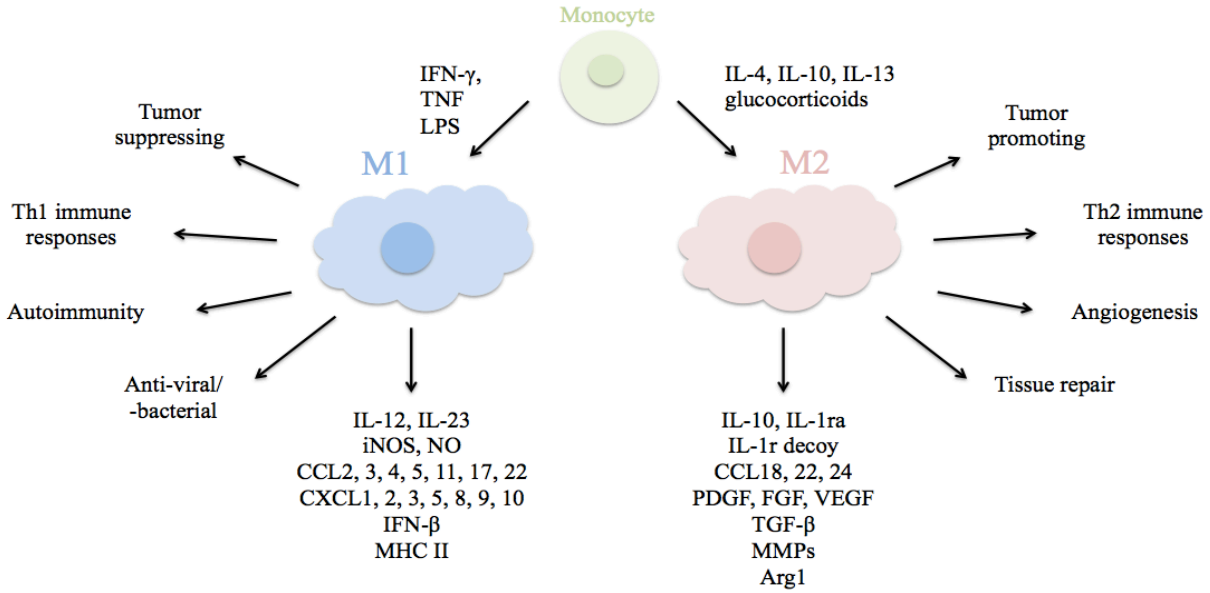


Figure 3 Properties of M1 and M2 macrophages. Monocytes will upon signals from the environment polarize into M1 or M2 macrophages. Stimulants like IFN-β, TNF and LPS lead to M1 macrophages that produce a large amount of pro-inflammatory cytokines and chemokines. They express MHC II that is required for antigen-presentation to immune cells, and produce reactive oxygen and nitrogen radicals that help in the killing of pathogens. Without tight regulation, the activity of M1 macrophages might lead to autoimmune diseases. Monocytes are polarized to M2 macrophages upon stimulation with IL-4, 10 and 13, and glucocorticoids. M2 macrophages produce a variety of factors that promote tissue repair and remodeling through Th2 immune responses, angiogenesis and act tumor promoting.

The term macrophage activation was introduced in the 1960s to describe the effector macrophages that are produced during immune responses towards bacteria and viruses [43]. This is today termed classical activation of macrophages, and the macrophages are called M1 macrophages. They are activated in response to IFN-γ, tumor necrosis factor (TNF) or microbial products like lipopolysaccharide (LPS), and are characterized by a high capability to present antigens to adaptive immune cells, large production of the pro-inflammatory cytokines interleukin-12 (IL-12) and interleukin-23 (IL-23), and an increased production of oxygen and nitrogen radicals [39]. When activated, they produce a wide range of chemokines

including CCL2, 3, 4, 5, 11, 17 and 22, and CXCL1, 2, 3, 5, 8, 9 and 10, as well as IFN-β due to activation of the transcription factor IFN regulatory factor-3 (IRF-3) [42]. However, M1 macrophages must be closely regulated since the large amount of cytokines and chemokines they produce can contribute to inflammatory immune pathologies found in autoimmune diseases like inflammatory bowel disease, cancer and rheumatoid arthritis [44, 45].

More recently, it was shown that IL-4, IL-10, IL-13 and glucocorticoid hormones, which are all anti-inflammatory compounds, induce another activation of macrophages (Figure 3). This is termed alternative macrophage activation, and the macrophages are called M2 macrophages. These macrophages produce chemokines like CCL18, 22 and 24, as well as high levels of IL-10 and IL-1 receptor antagonist (IL-1ra) and type II IL-1 decoy receptor [42]. They regulate adaptive T helper cell 2-type (Th2) response, and promote angiogenesis, tissue remodeling and repair [46]. During wound healing, M2 macrophages produce PDGF and TGF-β that stimulates fibroblasts and epithelial cells to proliferate and differentiate, as well as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) that regulate ECM turnover [41]. As for M1 macrophages, it is important that M2 macrophage responses are tightly controlled. An inappropriate regulation of their function might lead to fibrosis, allergy and asthma, and increased susceptibility towards some intracellular infections [39].

It is however important to remember that M1 and M2 macrophages are at the very ends of a large spectrum of activated macrophages. All macrophages share functions like phagocytosis, cytokine secretion, and microbial killing, thus implying that the contribution of M1 and M2 macrophages responses are modulating effects. These effects are also seen in a tumor, modulated by tumor-associated macrophages. However, these functions are complex and often conflicting, and more research is needed to fully understand their function.

1.2.2 TUMOR-ASSOCIATED MACROPHAGES

Many studies have focused on the stability of polarized macrophages, and in vivo studies have suggested that macrophages can change their phenotype and function in tissues over time [39]. An example of a case where it seems to be a switch in macrophage phenotype is cancer. As with other pathologies, tumors are also able to recruit macrophages. The role of tumor-associated macrophages (TAMs) are various and conflicting. It was initially thought that TAMs were present to kill and clear tumor cells and debris, but now it is well established that macrophages act both tumor suppressing and tumor promoting.

Macrophages take part in the immune surveillance of tissues and clear out transformed cells, but cancer cells able to evade this can eventually form a tumor. Tumor cells release chemokines like CCL2, 3, 4, 5 and 8, as well as cytokines like colony stimulating factor-1 (CSF-1) and the angiogenic factor VEGF, which all recruit macrophages to the tumor [47]. M1 macrophages are recruited to site of infection and in a growing tumor where there is a high amount of pro-inflammatory molecules. The macrophages will in response to this increase their production of cytokines and chemokines to attract other cells of the immune system to form an immune response [47]. The increased production of nitrogen and oxygen radicals will not only help in the killing of tumor cells, but might also contribute to the early stages of tumorigenesis by causing DNA damage and mutations.

The plasticity of macrophages makes them capable of adapting to the changing environment in the tumor, and as the tumor grows there is a shift in macrophage phenotype towards M2 macrophages. This might be due to the tumor cells ability to polarize other cells, but it is not completely known how this equilibrium changes [48]. These TAMs can either directly stimulate tumor growth by the production of growth factors, or indirectly by stimulating endothelial cells and promoting angiogenesis (Figure 4) [49].

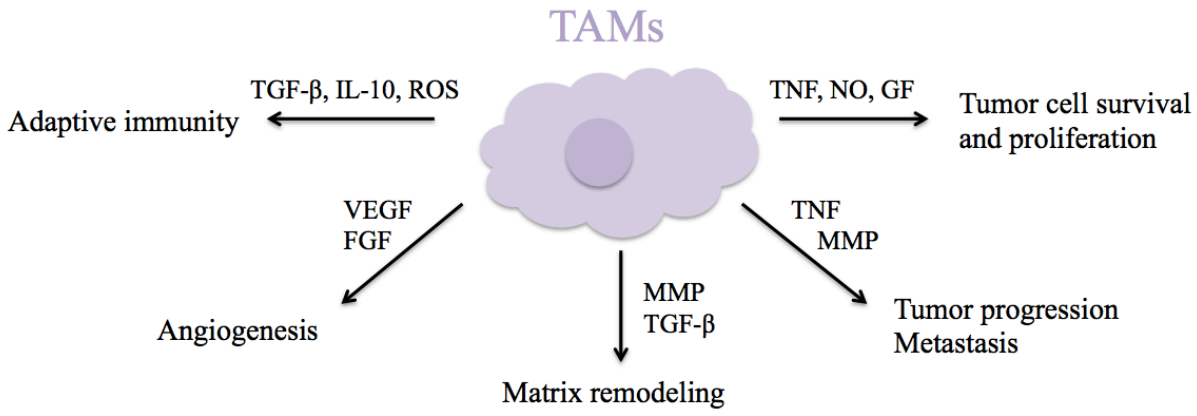


Figure 4 Functions of tumor-associated macrophages (TAMs) TAMs produce a wide range of chemokines and other factors that stimulate the growth of tumors. TNF, growth factors (GF) and nitric oxide (NO) stimulate tumor cell proliferation. MMPs and VEGF induce matrix remodeling, angiogenesis and metastasis. TGF-β also contributes to remodeling of the matrix as well as the initiation of an adaptive immune response

Tumors are tissues with an uneven distribution of vasculature and thus hypoxia. TAMs tend to accumulate in these hypoxic areas due to suppression of migration by tumor necrosis factor-α (TNF-α) [50]. In these hypoxic areas, TAMs work together with the tumor cells to produce pro-angiogenic factors including VEGF, PDGF, hypoxia inducible factor-1 (HIF-1)

and fibroblast growth factor (FGF) [49]. Expression of proteases like MMPs is increased, as well as the production of TGF- β, thus upregulating the remodeling of ECM, making it easier for epithelial cells to proliferate and form new vessels and tumor cells to migrate. TGF- β also induces adaptive immune system, thus favoring an anti-inflammatory response.

Even though the role of macrophages in tumors is conflicting and still not completely investigated, the presence of macrophages is important for tumor growth and invasiveness. Some researchers have studied this in macrophage-colony stimulating factor-1 (M-CSF-1) deficient mice, and found that this deficiency diminished macrophage recruitment, and reduced invasiveness and metastasis [51, 52]. Administration of recombinant M-CSF-1 restored the impairment. Other studies have shown that the number of macrophages in a tumor correlates with poor prognosis [53, 54]. There is a complex communication between TAMs and tumor cells, and more investigation is needed to further unravel the functions of macrophages in tumors. As high macrophage numbers correlate with poor prognosis in patients, there might be a possibility of targeting the macrophages by increasing the number and tumoricidal activity of M1 macrophages. It might also be possible to target cytokines and chemokines like TGF-β and others that promote polarization of M2 macrophages.

1.3 TGF-B/ SMAD SIGNALING PATHWAY

1.3.1 THE TRANSFORMING GROWTH FACTOR-B FAMILY

The transforming growth factor-β (TGF-β) superfamily is a family that consists of approximately 30 glycosylated cytokines that bind specific receptors on target cells. The superfamily is divided into several subfamilies including the bone morphogenetic (BMP) subfamily, activin/inhibin subfamily, the TGF-β subfamily and the growth differentiation factor (GDF) subfamily. The TGF-β superfamily members have a wide range of functions including the regulation of cell proliferation and differentiation, maintenance of stemness, as well as their involvement in wound healing and regulation of immune responses, and pathologies as fibrosis and cancer [55]. The subfamily of BMPs was initially discovered as regulators of bone formation, but has been found to be of importance in many other processes.

1.3.2 THE BONE MORPHOGENETIC PROTEIN FAMILY

In 1965, Marshall Urist discovered a protein that would become the first BMP, by its role in bone and cartilage formation [56, 57]. He showed that demineralized bone matrix could induce ectopic bone formation when implanted in the muscle of rats [58, 59]. The protein

responsible for this remained unknown until late 1980s, when it was purified, sequenced, and later cloned [60-62]. Later it was discovered that BMPs are involved in many other developmental and pathological processes, including muscle homeostasis, regulation of metabolism in the liver, kidney, adipose tissue and pancreas, and formation of new blood vessels [63-69]. BMPs also limit self-renewal, promote differentiation and specify cell fate in stem cells [70]. An aberrant BMP regulation is shown to lead to diseases including skeletal diseases, vascular diseases and cancer [71-73]. These findings have led to the introduction of the term Body Morphogenetic Protein as a more suitable name for the BMPs [74, 75].

BMPs are synthesized as large propeptides with a signal peptide at N-terminus that directs the protein to the secretory pathway, a mature polypeptide at C-terminus and a pro-domain in the middle that ensures proper folding [76]. The mature polypeptide gets cleaved and thereby activated. The active BMP contains seven cysteine residues that form intramolecular disulfide chains and an interchain bridge with another BMP monomer, establishing a biologically active dimer. The different BMP dimers are predominantly homodimers, but BMP2/7 and BMP4/7 heterodimers have shown to be potent for mesoderm induction [77]. BMPs act as ligands and transmit signals through two different types of serine/threonine kinase receptors, the type I receptors BMP receptor 1A and 1B (BMPR1A and BMPR1B) and activin receptor-like kinase 1 and 2 (ALK1 and ALK2), and the type II receptors BMP receptor 1 (BMPR1), activin receptor 2 (ACTR2) and activin receptor 2b (ACTR2B) [78]. BMPs bind the type I receptor that recruits the type II receptor. The type I receptor gets phosphorylated and thus activated, which lead to phosphorylation of intracellular proteins in the SMAD family. They oligomerize with SMAD4, and the complex translocates to the nucleus and acts as a transcription factor of BMP target genes (Figure 5).

The role of BMPs in cancer is complex. They act both suppressing and promoting towards tumor growth, and they act pro-metastatic. Elevated levels of BMPs have been correlated to tumor progression in prostate cancer, while low levels of BMPs are found in gastric cancer [73]. A survey of breast cancer cell lines and primary tumors showed elevated expression of many BMP ligands and receptors [79]. This was especially seen in the levels of BMP4 and BMP2. BMP2 and BMP4 have evolved from a common ancestor gene and share more than 80 % amino acid similarities in the mature ligand domain [80, 81]. Both BMP2 and BMP4 preferentially bind to BMR1A and BMPR1B, but can also signal through ALK2 [82]. BMP2 and BMP4 share many similar functions, and because of their wide distribution it has been shown that loss of function of either of the genes lead to early embryonic lethality [83, 84].

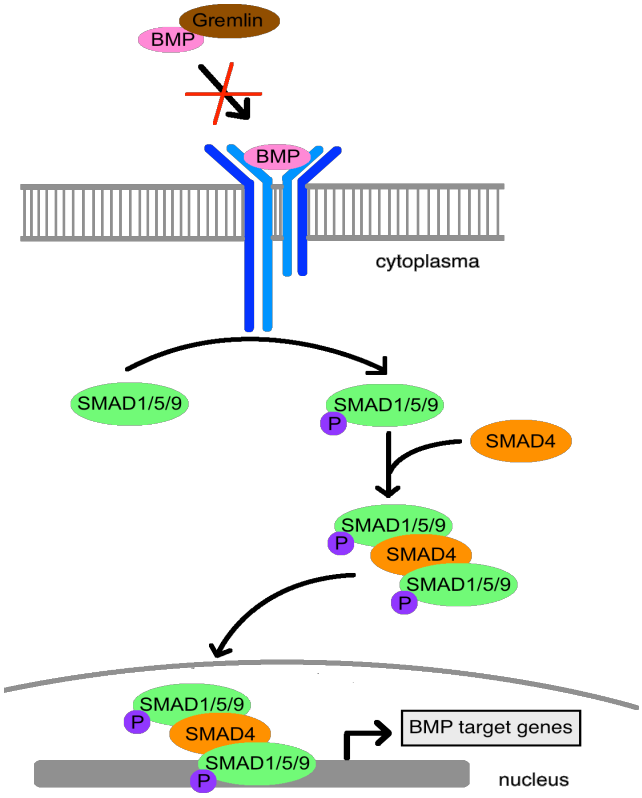


Figure 5 Schematic illustration of the BMP signaling pathway. BMPs and BMP antagonists are secreted into the extracellular environment. BMPs bind to BMP receptors, if not inhibited by BMP antagonists. The signal is transmitted through intracellular SMAD proteins that get phosphorylated and thereby bind SMAD4. The SMAD complex translocates to the nucleus where it acts as a transcription factor for BMP target genes.

1.3.3 BONE MORPHOGENETIC PROTEIN 4

In addition to BMP4s key role in regulating the induction of cartilage, bone, mesoderm and teeth formation, it have been shown to be important in many cancers as well [57, 85-87]. BMP4 is frequently expressed in many cancers and have been found to regulate breast cancer cell functions like proliferation, migration, apoptosis, invasion and epithelial mesenchymal transition (EMT) [88]. BMP4 act both tumor suppressing and promoting. Guo et al. found that BMP4 was overexpressed in highly metastatic cell lines compared to minimally invasive cell lines [88]. This study also found that overexpression of BMP4 decreased proliferation, but increased migration and invasion, also seen by others [89]. Knockdown of BMP4 increased the proliferation and inhibited migration of the cells. In the same study the BMP antagonist NOGGIN was given to the cells, resulting in decreased migration and invasiveness. This again underlines the effects of BMP antagonists, and might be used for cancer treatment.

1.3.4 ANTAGONISTS OF THE PATHWAY

Because of the important role of BMPs during embryonic development and adult homeostasis, their activity is strictly controlled at different levels. Both extracellular and intracellular processes tightly regulate BMP signaling.

The pseudoreceptor BMP and Activin Membrane Bound Inhibitor (BAMBI) is a receptor present on the cell surface that is similar to BMP type I receptors, but antagonize their function. BAMBI form complexes with BMP type II receptors but do not get activated since it lacks the intracellular serine/ threonine kinase domain [90]. Inhibitory SMAD proteins also regulate the pathway. SMAD6 interferes with the phosphorylation of SMAD1 and SMAD5, making them unable to bind to SMAD4 to form an active complex [91]. SMAD7 can bind Smad ubiquitin regulatory factor 1 or 2 (SMURF1/2) and compete with SMAD1/5/8 complex to get activated by the receptors [92]. SMURF1 interacts with SMAD1 and SMAD5 and mediates their degradation, as well as binding SMAD6 and exported out of the nucleus to the type I receptors, targeting them for degradation [93, 94]. The BMP signaling pathway is also regulated by secreted extracellular proteins that directly bind to BMPs preventing them from transmitting the signal through the receptors. Some of the antagonists of BMPs are NOGGIN, GREMLIN 1, DAND5, CHORDIN, DAN and CERBERUS [95, 96]. GREMLIN 1 (GREM1) is a potent inhibitor of BMP4 and can also interact with BMP4 intracellularly. Both BMP4 and GREM1 have importance in cancer development [97], suggesting an aberrant regulation compared to normal tissue.

1.3.5 GREMLIN 1

GREM1 was discovered as a protein that induced secondary axis in *Xenopus laevis* embryos [98]. GREM1 is a member of the CAN (CERBERUS and DAN) subfamily of the BMP antagonists, and is recognized by a C-terminal eight-membered cysteine ring that forms a knot motif similar to the one found in BMPs [99]. GREM1 is essential for development of lung and kidney, limb outgrowth and patterning, and a $GREM^{-/-}$ deletion in mice lead to great developmental abnormalities and lethality [100-102]. GREM1 inhibits the BMP pathway extracellularly by binding BMP2, BMP4 and BMP7 with high affinity (Figure 6). However, GREM1 also regulates BMP signaling intracellularly by interacting with BMP4 precursor protein, thus inhibiting maturation and secretion of active BMP4 [103].

Interestingly, it has recently become apparent that GREM1 is involved in other intrinsic signaling independent of BMP antagonism [104] (Figure 6). GREM1 is shown to have a pro-

angiogenic property by binding to VEGF receptor 2 (VEGFR2), and induce angiogenesis *in vitro* and *in vivo* in the same manner as VEGF [105]. So far in studies of GREM1 in cancer it has been suggested that GREM1 neutralizes BMPs regulatory role in cell proliferation. It is still not known if GREM1 can enhance tumor growth by directly regulating tumor angiogenesis. Kim et al. showed that GREM1 interacts directly with cancer cells to induce cell proliferation, migration and invasion in a BMP- and VEGFR2-independent way [106].

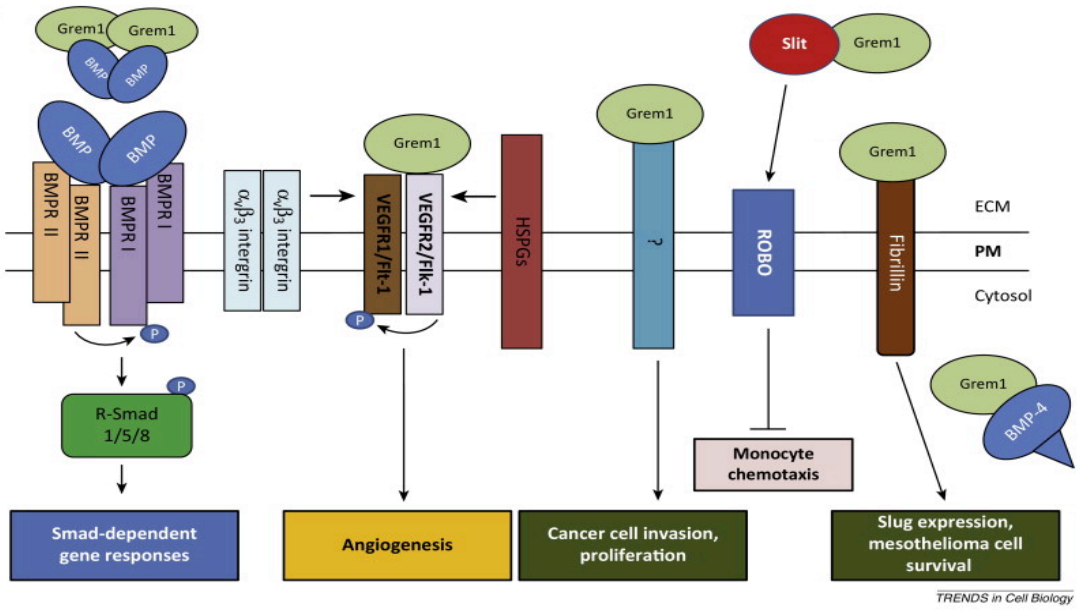


Figure 6 Schematic illustration of the signaling capability of GREM1 GREM1 is well characterized as an antagonist of BMP4, but GREM1 also have other signaling capabilities independent of BMP4 antagonism. It can bind to VEGFR2 and induce angiogenesis, it has been shown to bind to SLIT proteins and thus block monocyte chemotaxis, as well as binding fibrillin to stimulate survival of mesothelioma cells [104]

1.3.6 BMP AND BMP ANTAGONISTS SIGNALLING IN DISEASE

The important regulatory role of BMPs and their antagonists in differentiation and proliferation has been highlighted by all the research done of mice lacking either BMPs or BMP antagonists. An imbalance between their activities may be an underlying cause of pathologies like skeletal disorders, cancer and fibrosis of the eye, heart, lung, and liver [104]. Cancer development is tightly connected to deregulation of differentiation and proliferation. Tumors are heterogeneous and a possible theory states that a small subpopulation of cells termed cancer stem cells (CSC), are capable of initiating and sustaining tumorigenesis [107]. Since BMPs regulate processes during embryonic and adult development by their maintenance of stemness, as well as differentiation of specific cell lineages and involvement

in cell fate decisions, it might be that BMPs play a similar role with CSC (Figure 7). Studies have shown that BMPs are able to initiate CSC differentiation, suggesting a tumor-suppressing role [108]. However, Yan et al. showed that in glioblastomas, CSC seemed to escape BMP-mediated differentiation due to high GREM1 expression that inhibited the BMP pathway [109]. Cells with overexpressed GREM1 showed higher ability of tumor growth, and by targeting GREM1 the growth and self-renewal of CSC decreased, suggesting an oncogenic role of GREM1 [109]. GREM1 is overexpressed in a number of tumors including carcinomas of the lung, breast, colon, and sarcomas compared to normal tissue samples [110]. Another study showed upregulated GREM1 in stroma cells of different carcinomas [111]. It becomes clear, that aberrations in the BMP signaling is an important factor in the development of cancer, however GREM1s role in carcinogenesis is still largely unknown.

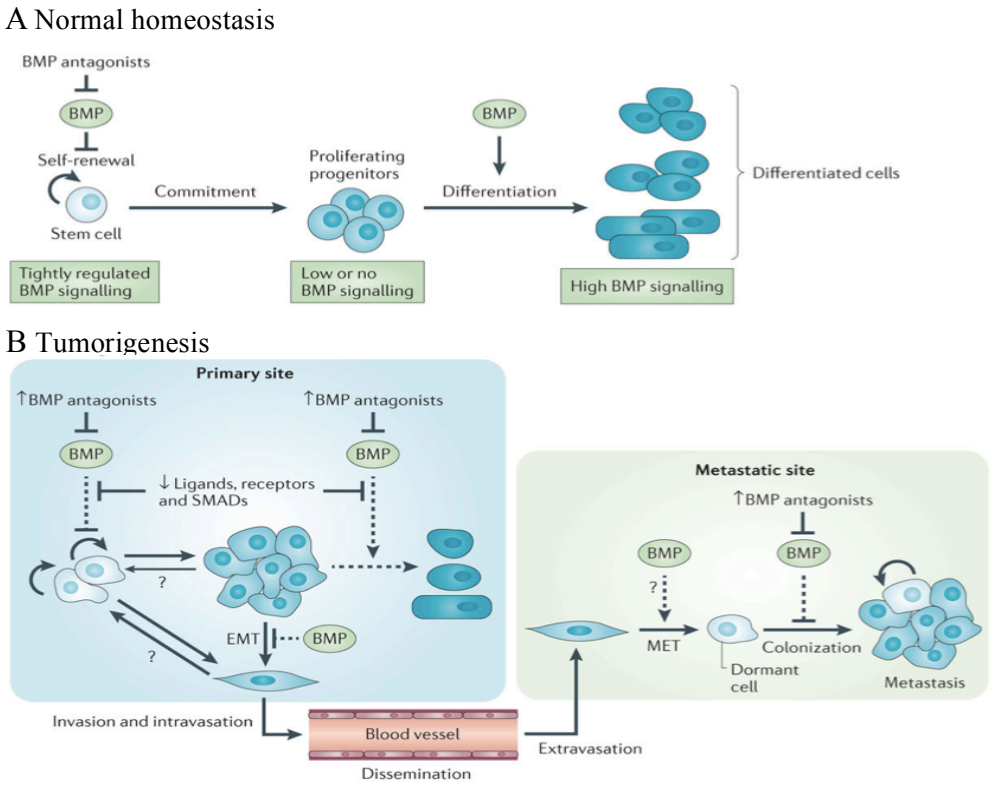
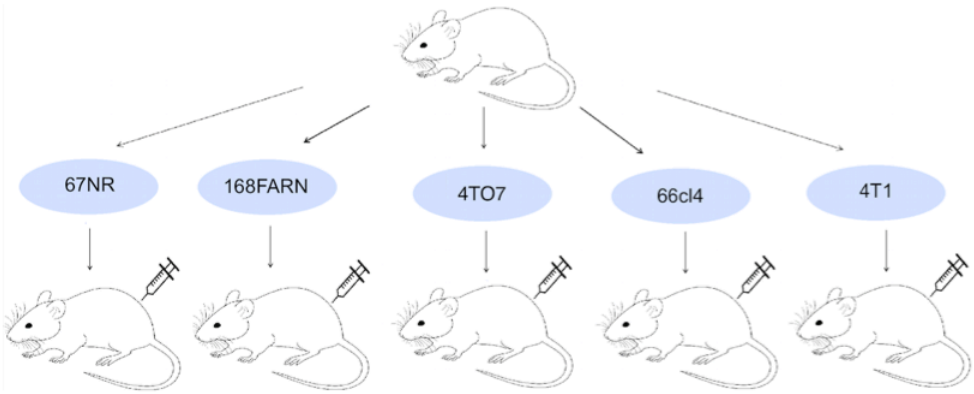


Figure 7 The role of BMPs in normal homeostasis and tumorigenesis. a) Role of BMPs in normal homeostasis in adults. b) BMP signaling in tumorigenesis. At primary site, aberrant BMP signaling results in hyperactive self-renewal pathways and inhibited differentiation. Cells undergo epithelial-mesenchymal transition (EMT) leading to higher motility and invasiveness. By disseminating through the circulation, tumor cells might settle down in distant tissue. Here BMPs might promote tumor cells to go through mesenchymal-epithelial transition (MET). BMPs help the cells stay dormant, but high levels of BMP antagonists block this, making the tumor cells able to colonize and form metastasis. [97]

1.4 MOUSE MODEL

In this study the 4T1 syngeneic BALB/cfC_3H breast cancer mouse model has been utilized. This breast cancer mouse model consists of five cellines; 67NR, 168FARN, 4TO7, 66cl4 and 4T1 that come from the same spontaneous arising BALB/cfC_3H mouse mammary tumor [112, 113]. These mice have a functioning immune system, thus enabling studies of tumor associated immune cells like macrophages and fibroblast. Interestingly, when cells from the five cell lines are implanted back into the fat pad of BALB/c mice they are all able to form primary tumors, but they have different ability to metastasize (Figure 8) [114, 115]. Based on this information, the non-metastatic 67NR and the metastatic 66cl4 were chosen to study the communication with macrophages.



Primary tumor	Yes	Yes	Yes	Yes	Yes
Metastatic propensity	No	Micrometastatic lymph node	Micrometastatic lung, blood, lymph node	Predominantly lung	Lung, bone, lymph node, blood, liver, brain, abdomen

Figure 8 BALB/cfC3H breast cancer mouse model All the five cell lines, 67NR, 168FARN, 4TO7, 66cl4 and 4T1, form primary tumors when they are implanted back into Balb/c mice. They have different metastatic propensity, 67NR never leaves the primary site while 168FARN and 4TO7 form micrometastatic tumor. 66cl4 metastasize to the lung, and 4T1 to lung, bone, lymph node, liver, brain and abdomen.

1.5 AIM

Previous studies have shown that tumor cells interact with cells of the surrounding environment, including macrophages. Some tumor cells have the ability of reprogramming these cells to enhance primary tumor growth and metastasis through the secretion of different compounds. Why some tumor cells are more successful in attracting and polarizing stroma cells is still not fully understood.

The main aim of this thesis was to initiate studies to better understand the communication between tumor cells and macrophages. To study this we utilized the five different tumor cell lines of the 4T1 breast cancer mouse model. The initial aim was divided into the following working hypotheses:

- Investigate if the macrophages in metastasizing primary tumors are different compared to non-metastasizing primary tumors
- Identify tumor-derived signaling compounds that can cause such differences in the macrophages
- Analyze the clinical relevance of such tumor-derived signaling compounds
- Try to mimic the communication between tumor cells and macrophages *in vitro* by cultivating RAW 264.7 macrophages and bone marrow derived macrophages together with the tumor cells in transwell cell culture inserts and by using conditioned medium of the tumor cells and the macrophages

2 MATERIALS AND METHODS

2.1 TRANSCRIPTOME DATA

RNA-sequencing, or whole transcriptome shotgun sequencing, is a method that utilizes next generation sequencing to reveal the presence and quantity of RNA in biological samples. For this thesis, sequencing was performed on RNA isolated from three replicates of the 66cl4 cell line, seven primary tumors and four metastasis samples from 66cl4, as well as three replicates of the 67NR cell line and four primary tumors of 67NR. The transcriptome sequencing was performed by the Genomics Core Facility at Norwegian University of Technology and Science (NTNU), and the data was analyzed by Bjarne Johannessen and Sen Zao, from Prof. Rolf I. Skotheims group at the Institute for Cancer Research at Oslo University Hospital. Gene expression levels of 23 965 genes for each sample was measured in fragments per kilobase of mRNA million mapped reads (FPKM), calculated and filtered.

The transcriptome data has been analyzed for markers specific for macrophages (including M1 and M2 macrophage), macrophage-recruiting factors and T-cells, as well as for TGF-β superfamily members and for the antagonists of BMP. The expression level of the transcripts has been normalized against the total amount of transcripts in the data set. The selected data is presented in tables in this thesis. 66cl4 and 67NR columns represent the average expression values of replicates from the cell lines or primary tumors. log2 indicates the differential expression between cell lines or primary tumors of 66cl4 and 67NR. Positive value indicates higher expression in 66cl4, e.g. a value of 3 indicates a 8-fold higher expression in 66cl4 than 67NR. p-value is from a t-test performed of the differential expression between 66cl4 and 67NR. The 66cl4 and 67NR average expression values are shown as whole numbers, log2 values are shown with two decimals, and p-values are shown with the lowest possible number of decimals needed to simplify. This is the reason why log2 and p-values are positive, negative while the 66cl4 and 67NR column values are 0.

2.2 KAPLAN-MEIER PLOTTER AND BREASTMARK ONLINE SURVIVAL ANALYSES

Kaplan-Meier plotter and BreastMark are two online databases that enable prognostic analysis of large data sets of cancer patient samples. Kaplan-Meier estimator is a non-parametric statistical analysis that estimates patient survival under different conditions. The Kaplan-Meier plot is a series of declining horizontal steps, with ticks that symbolize the death

of a patient. The hazard ratio is the relationship between the instantaneous hazards in the two groups, and the log rank test is used to assess the significance of the differences in the two groups with a confidence of 95%. To have a significant result ($p < 0.05$) that is clinically relevant there have to be at least 20 % difference in the probability expression correlation to poor prognosis. Therefore a cut-off of $HR > 1.2$ for high expression equals poor prognosis, or $HR < 0.83$ for high expression equals good prognosis was used. In this thesis we wanted to analyze the clinical significance of high or low expression of selected proteins.

2.2.1 KAPLAN-MEIER PLOTTER

Kaplan-Meier Plotter is an easy accessible online database that can assess the effect of expression level of around 55 000 transcripts on survival by using samples from approximately 10 000 cancer patients [116]. That includes samples from 4142 breast cancer patients, as well as samples from ovarian cancer patients, lung cancer patients and gastric cancer patients. The prognostic value of genes are analyzed by splitting the patient samples into two cohorts based on expression of different sets of biomarkers, that again are compared in a survival plot. Hazard ratio with 95 % confidence and logrank P values are also calculated.

2.2.2 BREASTMARK

BreastMark is an online database consisting of about 5 000 breast cancer patient samples [117]. The database utilizes an algorithm that easily identifies subsets of genes that are associated with disease progression or prognosis. This algorithm also integrates survival data from many data sets and gene expression.

2.3 CELL CULTURE

Cell cultures of tumor cells and macrophages were used as an *in vitro* model to study the hypotheses of this study. To facilitate co-culture studies, all cells have been cultured in the same medium: Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, BioWhittaker, BE12-604F) supplemented with 10% fetal bovine serum (FBS) (Thermo Fischer Scientific, #10270-106), 1 % L-Glutamine (Lonza Group, De-17-605E) and 1% Penicillin/Streptomycin (Thermo Fischer Scientific, Gibco, #15070-063).

2.3.1 CELL LINES

RAW 264.7 macrophages

RAW 264.7 macrophages are mouse-derived macrophages established from an Abelson murine leukemia virus-induced tumor (ATCC® TIB-71™).

67NR, 168FARN, 4TO7, 66cl4 AND 4T1

67NR, 168FARN, 4TO7, 66cl4 and 4T1 are BALB/c mouse cell lines derived from a spontaneous tumor. The 67NR and 66cl4 cell lines were obtained from Barbara Ann Karmanos Cancer Institute, while the 168FARN, 4TO7 and4T1 cell lines were a gift from Dr. Tonje S. Steigedal.

2.3.2 SUBCULTIVATION OF CELLS

Cryotubes containing cells were transferred from the liquid nitrogen container and thawed in water bath at 37 °C. The cells were then transferred to a 25 cm^2 flask with medium and placed in a humidified incubator at 37°C and 5% CO_2. After the cells had attached the medium was changed to remove the dimethyl sulfoxide (DMSO) (Sigma Aldrich, D2650) from the cells. After reaching a confluence of 80-90% the cells were subcultivated. After removal of old medium, washing with PBS and detachment of the cells, they were counted with Z2 Beckman counter by taking 20 µl of cell suspension and adding it to 10 ml of isoton II diluent (Beckman coulter, #8546719). The cells were seeded in a six-well plate (9.5 cm^2) at a cell number that is sufficient for the experiments, and stimulated with relevant compounds at appropriate time points.

Subcultivation of macrophages was done by removing the medium, washing once with Dulbeccos Phosphate Buffered Saline (PBS) (Sigma Aldrich, D8537), and then adding PBS with 0.02% Ethylenediaminetetraacetic acid (EDTA) (VWR Chemicals, #20296.291). The cells were incubated with PBS with EDTA for about 5 minutes at 37°C, then either seeded out for experiments or split in an appropriate ratio.

Subcultivation of the BALB/c mouse mammary cell lines 67NR, 168FARN, 4T07, 66cl4 and 4T1 was done by removing the medium, washing once with PBS and then adding trypsin (Lonza, BE17-161E) to detach the adherent cells. The cells were incubated with trypsin for 1-3 minutes at 37°C, then added medium and either seeded out for experiments or split in an appropriate ratio (approximately 1:20 – 1:30 from Monday to Friday, and 1:15 – 1:25 from

Friday to Monday). 67NR, 168FARN, 4TO7 and 4T1 cell lines were subcultivated for approximately 30 passages before they were discarded. 66cl4 cell line was subcultivated to approximately 40 passages.

2.3.3 ISOLATION OF PRIMARY MACROPHAGES FROM BONE MARROW OF BALB/C MICE

The mice were anesthetized by isoflurane, and euthanized according to the regulations. The skin was opened and removed before femur, tibia and humurus bones were harvested. The bones were put in HANKS Balanced Salt solution (Sigma Aldrich, #H9269) and placed on ice. In the cell lab the bones were disinfected with 96% ethanol and washed with HANKS, each for a minute. The flesh was carefully removed from the bones before the ends of the bones were cut. Using a small needle, each bone was flushed with 2-3 ml of HANKS and the cells were collected in a 50 ml tube and centrifuged at 1500 rpm for 6 minutes. The supernatant was discarded and the cells were resuspended in 5 ml red blood cell (RBC) lysis buffer. 30 ml of DMEM containing 10% FBS was added to stop the RBC lysis, and the cells were again centrifuged at 1500 rpm for 6 minutes. The cell pellet was resuspended in 1 ml DMEM containing 10% FBS, 20% L929 that contains GM-CSF needed for differentiation, 1% Penicillin/Streptomycin and 1% L-Glutamine. 10 ml of the medium was added to a bacterial petri dish and the cells were seeded out. The bone marrow-derived macrophages (BMDMs) were allowed to differentiate for 5 days before seeded out for experiments. The BMDMs were seeded out by first taking of the old medium, then washed once with PBS and incubated for approximately 5 minutes at 37°C in PBS with EDTA, before they were seeded out.

2.3.4 TREATMENT OF CELLS

Mouse recombinant BMP4

Mouse recombinant BMP4 (rmBMP4) (R&D systems, #5020-BP) is a ligand that binds to BMP receptors and was given to RAW 264.6 cells, the bone marrow-derived macrophages (BMDM) and the five tumor cell lines to stimulate the Bmp signaling pathway. rmBMP4 was given at different concentrations (1, 5, 10, 15, 20 and 100 ng/ml) to see the effect of SMAD1/5/9 phosphorylation downstream of ligand binding.

DMH1

Dorsomorphin homologue 1 (DMH1) (Sigma Aldrich, D8946) is an analogue of dorsomorphin and selectively blocks the BMP pathway by binding to the intracellular kinase domain of the BMP type 1 receptor. In this study, RAW 264.7 macrophages and BMDMs were incubated with 2 μM DMH1 for 2 hours. It is indicated in the specific experiments if the incubation happened before rmBMP4 stimulation

Mouse recombinant GREM1

Mouse recombinant GREM1 (rmGREM1) (R&D systems, #956-GR) is an antagonist of BMP4 and was added to RAW 264.7 cells and BMDMs to look at the effects on phosphorylation of SMAD1/5/9 in the signaling pathway. rmGREM1 was given to the cells in different concentrations (50, 100, 250, 500 and 1000 ng/ml) and time points (0.5, 1, 6 hours and overnight) as indicated in the experiments, to determine the optimal concentration and incubation time.

Protein Transport Inhibitor

Protein Transport Inhibitor (PTI) (500x, eBiosciences, #00-4980) is a cocktail of Brefeldin A and Monensin, that inhibits transport of proteins to the extracellular space. Secreted proteins will thus accumulate in endoplasmic reticulum (ER) and Golgi apparatus, and be easier to detect by western blotting or intracellular staining. For western blotting, PTI was diluted to a concentration of 1x or 2x and given to the cells for 3, 6 or 18 hours.

Conditioned medium

Cells from 67NR, 168FARN, 4TO7, 66cl4 and 4T1, as well as RAW 264.7 macrophages were grown for 3 days until they were 80-100% confluent. Conditioned medium was taken from the cells and filtered through a 0.2 μm filter before storing it at -20 °C.

2.4 WESTERN BLOT ANALYSIS

Western blot technique, or immunoblotting, is a method that separates proteins in a gel based on the proteins size and charge. The proteins are added a reducing agent that breaks disulfide bonds as well as protease and phosphatase inhibitors that protects them from degradation before the proteins are separated by gel electrophoresis. The proteins are transferred to a membrane that is blocked and stained with antibodies to detect specific proteins.

2.4.1 PROTEIN ISOLATION

Protein isolation

Cell extracts were made by removing the medium from the wells, washing once with PBS and then using cell scrapes to detach the cells from the surface after adding 40µl lysis buffer to the well. The lysis buffer was made of 8 M urea (Merck Millipore, 1084870500), 0.5% Triton-X (Sigma, T8787), 0.1 M dithiothreitol (DTT) (Sigma Aldrich, #43816), phosphatase inhibitor cocktail (PIC) 2 and 3 (Sigma-Aldrich, P5726 and P0044), complete (25x) (Roche Diagnostics, #11873580001). The cell lysates were transferred to eppendorf tubes and vortexed 3 x 15 seconds with 30 seconds on ice in between, before they were centrifuged at 13000 rpm for 15 minutes and the supernatant transferred to new tubes. The cell lysates were stored at -80°C.

Protein concentration in the lysates was measured by using a Biorad protein assay. The Protein Assay Dye Reagent Concentrate (Biorad laboratories, #500-0006) was diluted 1:5 in MilliQ-water. Protein samples were diluted 1:1000 in this solution and incubated for 10 minutes protected from light. To blank the instrument, lysis buffer was diluted 1:1000 in the Biorad solution. Absorbance of the samples was measured on a spectrophotometer (Termo Scientific, Termo Spectronic Genesys 20, #4404-02) at 595 nm, and the concentration was calculated by using this equation (factor 0.019 was obtained from a standard curve made from serial dilutions of a known concentration of purified bovine serum albumin):

Protein concentration [µg/µl] = OD595 mean value x 0.019 x dilution factor

Isolation of membrane proteins

Isolation of membrane proteins was performed to see if GREM1 also could be attached to the cell surface of the tumor cells. 67NR, 168FARN and 66cl4 were seeded out in three 15 cm petri dishes each, respectively 1.7 million cells/plate of 67NR, 0.8 million cells/plate of 168FARN and 1.5 million cells/plate of 66cl4. After two days of incubation at 37 °C the cells were washed once in PBS, before they were scraped in PBS with 0.02 % EDTA. The cells were transferred to 50 ml tubes and washed twice with PBS. During the second washing the cells were counted using the Z2 Beckman counter. After the second wash the cell pellets were resuspended in 3.5 ml PBS and aliquoted in three tubes (one eppendorf tube and two 15 ml tubes) each for different treatments. The eppendorf tubes were centrifuged at 1500 rpm for 5 minutes, the supernatant discarded and the cell pellet resuspended in twice as much urea lysis buffer. It was centrifuged at 12 000 rpm for 15 minutes before the supernatant was transferred to new eppendorf tubes and stored at -80°C.

The eppendorf tubes were centrifuged at 1500 rpm for 5 minutes. In one set of eppendorf tubes the supernatant was discarded and added 1 ml trypsin and incubated for 5 minutes at 37°C. After the incubation, 10 ml of medium was added before the cell suspensions were centrifuged at 1500 rpm for 5 minutes and washed twice with PBS. The supernatant from all the tubes were discarded and added 1 ml of homogenization buffer (end concentration of 250 nM sucrose, 1mM EDTA and 10 mM Tris-HCl). The samples were sonicated at (Branson Digital Sonifier, #100-132-889R and #100-214-283R) at 10% amplitude for 10 seconds with 10 pulses with breaks between. After sonification the samples were centrifuged two times at 1000g for 5 minutes, and the supernatant was collected. The supernatant was further centrifuged at the Optima L-80 XP ultracentrifuge (Beckman Coulter, #392049) at 50000 rpm at 4°C for 60 minutes. The supernatant was collected to be used as a control, while the pellet was dissolved in approximately 100 µl urea buffer and placed at a shaker at 4°C for 60 minutes before it was stored at -80°C. Since the samples did not have any kind of protection, e.g. PIC 2 and 3, it was preceded directly to western blotting. However, these samples were not diluted in Tris-HCl, just directly added lithium dodecyl sulfate (LDS) (4x) sample buffer and DTT.

Isolation of protein from conditioned medium

To isolate proteins from conditioned medium from the five cell lines, 67NR, 168FARN, 4T07, 66cl4 and 4T1, Amicon® Ultra-4 Centrifugal Filter Devices (Merck Millipore, #UFC800324) were utilized. These tubes allow for a fast ultrafiltration of biological samples to concentrate proteins, nucleic acids, antibodies, antigens or microorganisms.

Before the conditioned medium samples were concentrated the tubes were rinsed with distilled H_2O and centrifuged for 45 minutes at 4000g. Then 4 ml medium from the five cell lines were transferred to the tubes and centrifuged for 45 minutes at 4000g. This process allowed the conditioned media to get concentrated to approximately 120 µl. The tubes were then centrifuged with water once to rinse, before the concentrated solution was transferred to eppendorf tubes. The samples were added lysis buffer (8 M urea, 0.5% Triton-X, 0.1 M DTT, PIC 2 and 3, and complete 25x), vortexed and centrifuged at 13000 rpm for 5 minutes at 4 °C. The supernatant was collected and stored at -80°C. After isolation, the protein samples were first diluted 1:10 in lysis buffer before they were added LDS and DTT, for a final concentration of 1x LDS and 0.1M DTT.

2.4.2 GEL ELECTROPHORESIS

To create an equal protein concentration, the protein samples were diluted with 10 mM Tris-HCl (pH 8.0) and added LDS (4x) sample buffer (NuPage, Life Technologies, NP0007) and 1M DTT, to give a final concentration of 1x LDS and 0.1M DTT. The samples were heated on a heating block at 80 °C for 10 minutes. The ladder was made by diluting Odyssey two-color protein molecular weight marker (IR dye 4000, LI-COR Biosciences, 928-40000) in 1x LDS (4x) sample buffer and 10mM Tris-HCl (see supplementary figure in appendix 7.1).

The proteins were separated by using premade gel electrophoresis cassettes with 4-12% or 10% polyacrylamide (Tris-Bis NuPage, Life Technologies, NP0321BOX, NP0322BOX, NP0315BOX). The cassettes were placed in an Xcell Surelock Mini-Cell (NuPage, Life technologies, EI0001) and added 1x MOPS SDS running buffer (20x) (NuPage, Life Technologies, NP0001-02). The proteins were separated at 200 volts (V) for 60 minutes.

After the separation of proteins was completed, the proteins were transferred to a nitro-cellulose blotting membrane (GE Healthcare Life Sciences, Altham, #10600016) that had been soaked in 1x Transfer buffer (20x) (NuPage, Life Technologies, NP0006-1) with 10%

methanol, or a PVDF membrane (EMD Millipore, #IPVH304F0) that had been soaked in methanol for 1 minute to get activated. In Xcell II Blot Module (NuPage, Life Technologies, EI0002) the membrane was put on top of the gel and surrounded by filter papers (Whatman International, #3030917) and blotting pads, and added transfer buffer. Blotting was done by applying 30V for 90 minutes.

2.4.3 MEMBRANE BLOCKING AND IMMUNOSTAINING

The membrane was blocked in a 1:1 dilution of Odyssey Blocking buffer TBS (LI-COR Biosciences, #927-50000) and TBST, Tris buffered saline with 0.1% (v/v) Tween-20 (Sigma Aldrich, P1379). For all the staining steps the membrane was carefully placed in a 50 ml tube with the specific antibody and placed on a roller plate at 55 rpm. The blocking buffer was also used to dilute the primary (Table 1) and secondary antibodies (Table 2). The staining of primary antibodies was done at a roller plate over night at 4°C. After staining, the membrane was washed 3 x 10 minutes with TBST, and then stained with secondary antibody for 1 hour at room temperature. The membrane was washed 3 x 10 minutes in TBS, before it was left for drying. The Odyssey Infrared Imaging System (LI-COR Biosciences) was used to detect the proteins on the membrane.

Table 1 Primary antibodies used for Western Blot staining

Antibody	Species	Molecular Weight	Dilution	Manufacturer
GREM1	Goat IgG	21 kDa	1:500	R&D systems (AF956)
BMP4	Mouse IgG	47 kDa	1:1000	Abcam (ab93939)
p-SMAD1/5/9	Rabbit IgG	60 kDa	1:1000	Cell Signaling Technology (#4086)
p-SMAD3	Rabbit IgG	48 kDa	1:2000	Abcam (ab52903)
ARG1	Rabbit IgG	38 kDa	1:1000	Merck Millipore (ABS535)
p-IRF3	Rabbit IgG	45-55 kDa	1:1000	Cell Signaling Technology (#3661)
p-STAT1	Rabbit IgG	84 + 91 kDa	1:1000	Cell Signaling Technology (#6772)
p-p65	Rabbit IgG	65 kDa	1:1000	Cell Signaling Technology (#5970)
ACTB	Mouse IgG	42 kDa	1:5000	Abcam (ab6276)
ERK1/2	Mouse IgG	42 + 44 kDa	1:2000	Cell Signaling Technology (#9107S)

Table 2 Secondary antibodies used for Western Blot staining

Antibody	Dilution	Manufacturer
Donkey anti-chicken IgG – IR Dye 800CW	1:5000	LI-COR Biosciences, #926-32218
Goat anti-rabbit IgG – IR Dye 800CW	1:5000	LI-COR Biosciences, #926-32211
Donkey anti-goat IgG – IR Dye 800CW	1:5000	LI-COR Biosciences, #926-32214
Goat anti-mouse IgG – IR Dye 800CW	1:5000	LI-COR Biosciences, #926-32210
Donkey anti-goat IgG– IR Dye 680RD	1:5000	LI-COR Biosciences, #926-68074
Goat anti- rabbit IgG – IR Dye 680RD	1:5000	LI-COR Biosciences, #926-68071
Goat anti-mouse IgG – IR Dye 680RD	1:5000	LI-COR Biosciences, #926-68070

2.5 GREM1 ANALYSIS IN CONDITIONED MEDIUM AND BLOOD SERUM USING ELISA

Enzyme-linked immunosorbent assay (ELISA) is a method that utilizes antibodies to detect cytokines, proteins or peptides in liquid samples. The cytokine of interest is immobilized by the use of capture antibody. The detection of the cytokine is then made possible by the use of detection antibody, which then binds to HRP conjugated to streptavidin. Substrate oxidation is then detected by absorbance using spectrophotometer. In this thesis we used the DuoSet ELISA Development system (R&D Systems, #DY956) to analyze conditioned medium from the five tumor cell lines and from blood serum of Balb/C mice for GREM1.

2.5.1 SAMPLE PREPARATION

Conditioned medium from the five tumor cell lines, 67NR, 168FARN, 4TO7, 66cl4 and 4T1, was taken from cells grown for 3 days (80-100% confluence) and filtered through a 0.2 μm filter before storing at -20 °C.

From mice injected with tumor cells of cell line 67NR, 168FARN and 66cl4, blood was drawn directly from the heart while the mice were anaesthetized. The blood was left for 30 minutes at room temperature before it was centrifuged at 1800xg for 10 minutes. The serum was collected and stored at -20°C until the experiments were conducted.

2.5.2 ASSAY

First the Capture Antibody was diluted to a working concentration of 4 µg/ml in PBS without carrier protein. A 96-well microplate was coated with 100 µl diluted Capture Antibody per well, sealed and incubated overnight in room temperature. The next day, the plate was aspirated three times with Wash Buffer (0.05% Tween in PBS) by using Hydrospeed™ plate washer (Tecan, Bergman Diagnostics). After washing the liquid was completely removed by inverting the plate and blotting it against paper towels. The plates were blocked by adding 300 µl per well of Reagent Diluent, and incubated at room temperature for 60 minutes. Reagent diluent was prepared by diluting bovine serum albumin (BSA) (Sigma Aldrich, #A7030) to a working concentration of 1% in PBS. After blocking, the plate was washed three times and added 100 µl of standards diluted in Reagent Diluent or samples. The plate was covered and incubated overnight. The next day, the plate was again washed three times before added 100 µl to each well of Detection Antibody diluted in Reagent Diluent and incubated for two hours at room temperature. After the incubation was done, the plate was washed three times, and Streptavidin-HRP was diluted to working concentration with Reagent Diluent and 100 µl of it was added to each well for 20 minutes protected from light. The plate was again aspirated three times. Substrate solution was made by making a 1:1 mixture of Color Reagent A and Color Reagent B, and 100 µl of the mix was added to each well and incubated for 20 minutes at room temperature protected from light. Then 50 µl of Stop Solution was added to the wells. Immediately after adding Stop Solution the optical density was determined by using iMark Microplate Absorbance Reader (BIO-RAD, #16692) and the Microplate Manager® Software.

2.6 MIGRATION ANALYSIS OF BMDMS USING XCELLIGENCE

The xCELLigence system (Roche, Switzerland) is a method that enables monitoring of live cells in the culture environment, using differences in impedance as principle of detection. The system monitors cell processes like cell growth, proliferation, adhesion, cell viability as well as cell morphology. The Cell Invasion and Migration plate (CIM) consists of two chambers. In the lower chamber the chemoattractant is added, while the cells to be analyzed are placed in the upper chamber. The bottom of the upper chambers is a microporous membrane that contains gold microelectrode sensors. Cells migrate through these pores and when they come in contact with the gold particles the impedance changes. These changes reflect the sum of

cell number and their adherence, thus the number of migrated cells (cell index) (see supplementary figure in appendix 7.2).

In this thesis, the xCELLigence Real-Time Cell Analyzed (RTCA) was used to study the migration of BMDMs in response to conditioned medium from BMDMs, 67NR, 168FARN and 66cl4, or in response to the tumor cells or BMDMs themselves. Tumor cells or conditioned medium was added to the lower chambers of the plates. The tumor cells (10 000 cells/well) and BMDMs (100 000 cells/well) were seeded in DMEM containing 2% FBS, while conditioned medium from the BMDMs and the tumor cells was diluted 1:5 with DMEM before transferring 160 µl to the chambers. To validate the system DMEM supplemented without or with FBS (10%) was used as positive and negative control. The chamber was closed before the upper chamber was added 50 µl of DMEM with 2% FBS. The plate was placed in the RTCA DP instrument and left for 30 minutes for the cells to attach and equilibrium to be reached. Background measurements were done. After 30 minutes had passed, 100 µl of BMDMs in suspension (100 000 cells/well) was added to the upper chambers. The chamber was closed and left in room temperature for 30 minutes for the cells to settle, before it was placed in the RTCA DP instrument. Cell index was measured every 10 minutes for the total length of time

2.7 STATISTICS

Statistical test have been performed on the most crucial experiments in this thesis, which have been done at least three times. One-way ANOVA test and one sample t-test have been utilized to analyze the significance of the findings. Statistical significance of the findings is marked with stars. Standard deviations are presented as error bars if suitable to show the variations around the mean value.

3 RESULTS

The aim of this thesis was to investigate the communication between tumor cells and macrophages. By utilizing RNA sequencing data the different abilities of 66cl4 and 67NR primary tumors to polarize macrophages was assessed, as well as analysis of different tumor-secreted compounds that could cause this. The clinical relevance of the putatively secreted compounds was analyzed by online databases, and *in vitro* experiments were performed to try to mimic the communication between tumor cells and macrophages *in vitro*.

3.1 TUMOR CHARACTERIZATION BY RNA-SEQUENCING

3.1.1 66CL4 PRIMARY TUMOR CONTAINS MORE M2 MACROPHAGES THAN 67NR

To investigate the heterogeneity of primary tumors, a method was developed to quantify the amount of tumor DNA and host DNA in the tumors by looking at the difference between wild type allele and mutated allele. It was estimated that approximately 30-40% of the DNA in both tumors of 67NR and 66cl4 was host DNA (unpublished data, Ulrike Neckmann). Preliminary FACS results also suggest that approximately 10% of the cells in primary tumors of 67NR and 66cl4 are macrophages (unpublished data, Jennifer Mildenberger). Several studies have shown that the amount of macrophages in a tumor correlates to poor prognosis in patients. The polarization of macrophages into M1 or M2 macrophages also affects the tumors, based on their different anti- or pro-tumor properties.

In Bjørkøys group there has previously been performed RNA sequencing of cell lines and primary tumors of 66cl4 and 67NR, as well as of lung metastasis from 66cl4. These cell lines come from the same spontaneous arising BALB/cfC_3H mouse mammary tumor, and while 66cl4 predominantly metastasize to the lungs, 67NR do not leave the primary site. This transcriptome data represents the amount of RNA transcripts present in the cell lines and primary tumors. Over the past decades it has been shown that different cells can be recognized by the expression of specific markers, and by searching the transcriptome data for these cell-specific markers we can identify different cell types in the tumor. The transcriptome data set was searched for five established general markers of macrophages, Cd68, Itgam, Emr1, Csf1r and Spi1 [40, 118, 119], and found that for four out of five markers the mRNA expression is almost doubled in 66cl4 primary tumor compared to 67NR tumor (Table 3). The markers are not expressed in the cell lines of 66cl4 or 67NR, suggesting that they do come from macrophages.

Table 3 Four out of five general macrophage markers are almost doubled in primary tumors of 66cl4 compared to 67NR 66cl4 and 67NR; Average expression values from cell line or primary tumor. log2; indication of the differential expression between cell lines or primary tumors of 66cl4 or 67NR. Positive value indicates higher expression in 66cl4. p-value; from t-test of the differential expression between 66cl4 and 67NR.

	Cell culture				Primary tumors			
Gene	**66cl4**	**67NR**	**log2**	**p-value**	**66cl4**	**67NR**	**log2**	**p-value**
Emr1	0	0	0,32	1	61	68	-0,15	0,3501
Cd68	1	5	-1,89	0,0001	182	97	0,91	0,0002
Itgam	0	0	-0,38	1	86	44	0,98	0,0002
Csf1r	0	0	-0,72	1	164	121	0,45	0,0058
Spi1	0	0	1,05	1	58	37	0,66	0,0002

Since the mRNA expression of four out of five macrophage markers was higher in 66cl4 primary tumors than 67NR primary tumors it might suggest that there is a difference in the two tumors ability to recruit macrophages. The transcriptome data set was searched for specific factors that has been shown to recruit macrophages into tumors; Csf1, Vegfa, Ccl5, Ccl4, Ccl3 and Ccl8 [47] (Table 4). Both 66cl4 and 67NR primary tumors have high expression of Csf1, Ccl5 and Ccl8. The macrophage-recruiting factors Csf1, Vegfa and Ccl5 are also expressed by cell lines of 66cl4 and 67NR.

Table 4 Macrophage-recruiting factors are expressed in cell lines and primary tumors of 66cl4 and 67NR

	Cell culture				Primary tumors			
Gene	**66cl4**	**67NR**	**log2**	**p-value**	**66cl4**	**67NR**	**log2**	**p-value**
Csf1	249	131	0,93	0,0001	154	65	1,24	0,0002
Vegfa	14	19	-0,41	0,0016	17	25	-0,58	0,0010
Ccl5	46	1	5,11	0,0001	29	187	-2,67	0,0002
Ccl4	0	0	3,48	0,3078	10	16	-0,67	0,0241
Ccl3	0	0	0,00	1	7	9	-0,25	0,4771
Ccl8	0	0	-0,15	1	410	1197	-1,54	0,0002

Based on the transcriptome data, there are signs of macrophages being recruited to the tumors, as well as signs of macrophages being present in the tumors. However, from the transcriptome data it cannot be discriminated if the high mRNA levels of the macrophage markers correlate with the number of macrophages, or just high expression of those markers in few macrophages. Macrophages can be polarized into M1 and M2 macrophages and thus have different functions, therefore it is not only important to look at the number of macrophages or the amount of general macrophage markers, but also markers for M1 and M2

macrophages [42]. To estimate different functions of macrophages in 67NR and 66cl4 tumors from the RNA sequencing data, the transcriptome data set was searched for the expression patterns of markers that characterize M1 (Table 5) and M2 macrophages (Table 6).

Table 5 Expression of M1 macrophage markers in 66cl4 and 67NR primary tumors M1 macrophage markers are divided into groups based on their expression levels in 66cl4 compared to 67NR. p-value < 0.05

M1 macrophage marker	Genes / Primary tumor
66cl4 > 67NR	Il12b, Cd86, Tlr2, Fcgr3, Fcgr2b, Il6ra, Il1a, Ifnar1, Ifnar2, Ifngr2, Cxcl12
66cl4 < 67NR	Cd74, Ifng, Cxcl9, Cxcl10, Cxcl11, Ccl4, Ccl5, Ccl19, Ccl25
66cl4 = 67NR	Nos2, Tnf, Cd80, Tlr4, Fcgr1, Il6, Il12a, Il1b, Ifngr1, Ccl2, Ccl3, Ccl21a, Ccl21b, Ccl21c, Ccr7 Marco

Table 6 Expression of M1 macrophage markers in 66cl4 and 67NR primary tumors M2 macrophage markers are divided into groups based on their expression levels in 66cl4 compared to 67NR. p-value < 0.05

M2 macrophage markers	Genes / Primary tumor
66cl4 > 67NR	Msr1, Mrc1, Arg1, Cd163, Cd14, Fcer1g, Il1rn, Ccl17, Ccl24, Cxcr2
66cl4 < 67NR	-
66cl4 = 67NR	Scarb1, Il10, Ccl22, Ccr2, Cxcr1

M1 macrophage markers were found expressed in primary tumors of both 66cl4 and 67NR. M2 macrophage markers were highly expressed in 66cl4 tumors, but not in 67NR. M2 macrophages have been related to tissue repair, tumor promotion and angiogenesis, and it might be that they contribute to 66cl4 primary tumors ability to metastasize.

The findings from the transcriptome data show that macrophages seem to be recruited and present in primary tumors of 66cl4 and 67NR. However, 66cl4 primary tumors express higher amounts of M2 macrophage markers, suggesting different polarization of the macrophages that is due to stimulation of different compounds.

3.1.2 TGF-B SUPERFAMILY MEMBER BMP4 IS EXPRESSED BY 66CL4 CELL LINE AND PRIMARY TUMORS

Based on the indication that macrophages are recruited and present in both primary tumors, and that the expression of several M2 macrophages markers are higher in tumors of 66cl4, the tumor cells seem to influence macrophages differently. Polarization is a differentiation process that can be affected by many classes of signaling compounds. One of the families of

signaling compounds that is known for its involvement in differentiation of many cell types, including macrophages, is the transforming growth factor-β (TGF-β) superfamily [120]. Given that 66cl4 tumor cells might have better abilities to recruit or polarize macrophages, the transcriptome data set was searched for signs of different mRNA expression of TGF-β superfamily members (Table 7).

Table 7 Transcriptome data analysis of Tgf-β superfamily members

	Cell culture				Primary tumors			
Gene	**66cl4**	**67NR**	**log2**	**p-value**	**66cl4**	**67NR**	**log2**	**p-value**
Tgfb1	19	3	2,57	0,00012	45	25	0,86	0,00020
Tgfb2	0	0	2,63	0,00012	1	0	2,99	0,00020
Tgfb3	2	7	-1,78	0,00012	27	13	1,08	0,00020
Bmp1	7	8	-0,17	0,2194	23	25	-0,12	0,4549
Bmp2	0	0	0	1	2	1	1,52	0,0002
Bmp3	0	0	-1,16	1	0	0	0,01	1
Bmp4	47	0	10,75	0,0039	39	0	6,58	0,0002
Bmp5	0	0	3,36	1	0	0	2,39	1
Bmp6	0	0	1,41	1	0	0	0,69	1
Bmp7	0	0	1,32	1	0	0	2,13	1
Bmp8a	0	0	1,74	1	0	0	1,61	1
Bmp8b	0	0	-0,1	1	0	0	-1,27	1
Bmp10	0	0	-1,04	1	0	0	-2,34	1
Bmp15	0	0	0	1	0	0	-1,44	1
Inhba	4	2	0,95	0,00012	4	1	1,38	0,00055
Inhbb	0	0	-0,13	1	5	8	-0,61	0,00055
Inhbc	0	0	-1,72	1	0	0	-2,27	1
Inhbe	0	0	0,76	1	0	0	1,25	1
Inha	0	0	1,81	1	0	0	4,22	1
Gdf1	0	0	-2,23	1	0	0	2,56	1
Gdf2	0	0	1,12	1	0	0	1,99	1
Gdf3	0	0	0	1	0	0	0,61	0,30422
Gdf5	1	0	7,2	0,30783	0	0	2,96	1
Gdf6	0	0	0	1	0	0	-0,49	1
Gdf7	0	0	0,39	1	0	0	3,79	1
Gdf9	0	0	-2,02	1	0	0	-0,06	0,94014
Gdf10	0	0	0	1	0	0	1,19	1
Gdf11	11	8	0,4	0,00549	6	7	-0,16	0,55172
Gdf15	0	1	-4,77	0,00482	1	3	-2,34	0,00020
Mstn	0	0	0	1	0	0	-1,04	1

Tgf-β itself seems to be significantly upregulated in 66cl4 compared to 67NR. Tgf-β3 is also upregulated in 66cl4, but the most striking difference is found for Bmp4. Bmp4 mRNA is expressed at high levels in 66cl4 but not expressed in 67NR.

To assess the protein levels of BMP4, we cultured the five cell lines and treated them with protein transport inhibitor (PTI) for 6 hours prior to harvesting to prevent the secretion of BMP4 and thus facilitate detection in cell lysates. Immunoblotting for BMP4 showed high protein levels of BMP4 in the metastatic 66cl4 cell line compared to the non-metastatic 67NR cell line (Figure 9).

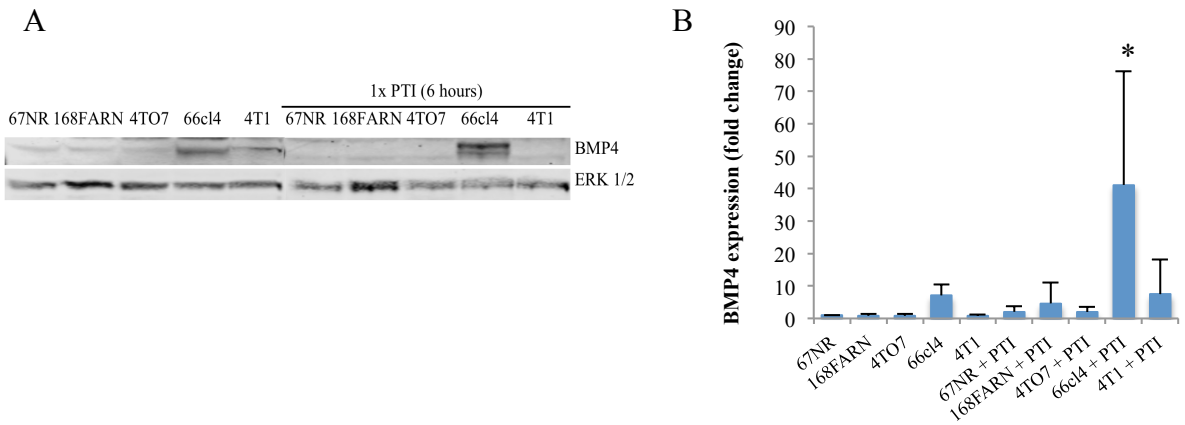


Figure 9 Protein expression of BMP4 in the five different tumor cell lines A. Immunoblotting for BMP4 in the five different tumor cell lines, both untreated and after 1x PTI treatment for 6 hours. ERK 1/2 was used as a loading control B. Quantification of the average BMP4 protein expression levels of 3 experiments A one-way ANOVA was performed on the PTI stimulated BMP4 expression levels.

Expression of BMP4 increased after PTI stimulation, suggesting that BMP4 is differentially expressed also at protein level and that the protein is secreted. Conditioned medium from the five tumor cell lines was analyzed by ELISA as well as western blot analysis. Initial experiments using these two different methods did not detect BMP4. This might suggest that none of these approaches were sensitive or specific enough for BMP4 detection. However, studies have shown that BMP4 is secreted [104]. Thus, further experiments in this thesis were done based on the findings that BMP4 accumulate in 66cl4 cells upon protein transport inhibitor and the assumption that BMP4 is secreted from these cells.

3.1.3 BMP4 EXPRESSION IS NOT CORRELATED TO PATIENT PROGNOSIS

Before we moved on, we wanted to check the clinical significance of BMP4 expression in breast cancer. In breast cancer, mortality is mainly caused by metastasis. Thus, online databases like KM-plotter and BreastMark, showing prognosis in relation to differences of mRNA expression, were used to test for a putative role of elevated BMP4 for metastasis.

The KM-plotter database of 3554 breast cancer patients was divided into two cohorts of either high or low expression of BMP4. To have a significant result ($p < 0.05$) that is clinically relevant, there have to be at least 20 % difference in the probability expression correlation to poor prognosis. Thus, a cut-off of HR > 1.2 for high expression equals poor prognosis, or HR < 0.83 for high expression equals good prognosis was used. BMP4 expression is thus not clinically significant (Figure 10A). The Breastmark online database was also used to confirm the findings. The BreastMark database of around 5000 breast cancer patient samples was divided into two cohorts based on high or low expression of BMP4. The plot from BreastMark (Figure 10B) supports the data from KM-plotter. The BMP4 expression alone does not make a difference in the patients. However, the effect of BMP4 is strongly regulated by inhibitors, which also might affect bioactivity of ligand and possibly the ability of the tumor cells to metastasize and cause poor prognosis.

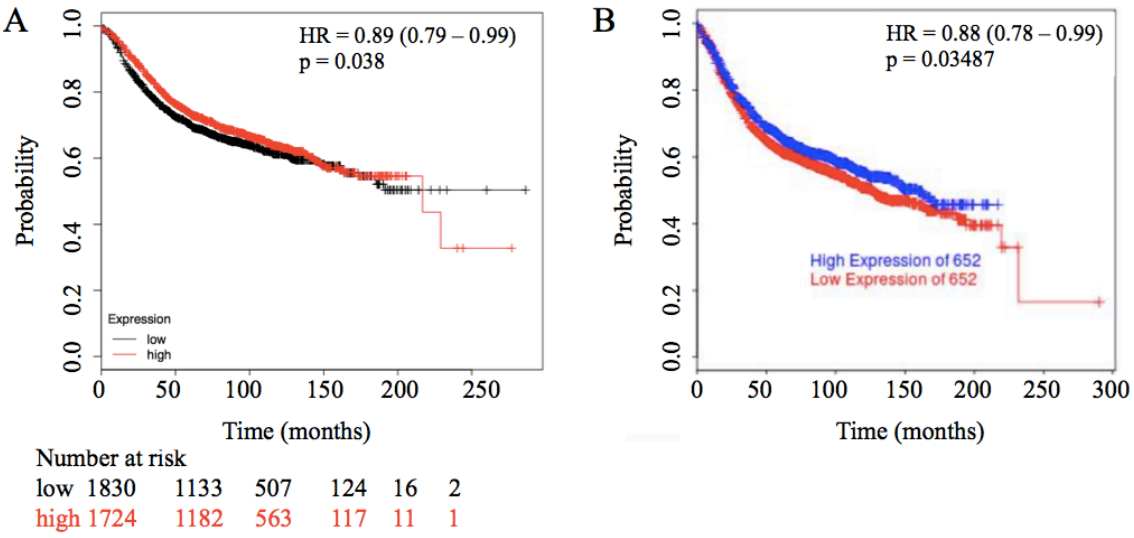


Figure 10 Survival analysis of BMP4 expression x-axis; timeline after study start. y-axis; estimated survival probabilities. HR; relationship between the hazards in the two groups. p= significance of the differences in the two groups with a confidence of 95%. A. KM-Plotter B. BreastMark

3.1.4 GREM1 IS PRODUCED AND SECRETED BY 66CL4 CELL LINE AND PRIMARY TUMORS

BMP family members are important regulators of differentiation processes in both embryogenesis and adult life. These signaling compounds are therefore tightly regulated. BMPs bind to BMP receptors that get activated and transmit the signal inside the cell and upregulate transcription of target genes that control differentiation, polarization and activation of cells. Since BMP4 expression level did not show a clinical significance, RNA expression of 27 BMP antagonists was analyzed in the RNA-sequencing data [95, 96] (Table 8).

Table 8 mRNA expressions of BMP antagonists in 66cl4 and 67NR cell culture and primary tumor

	Cell culture				Primary tumors			
Gene	**66cl4**	**67NR**	**log2**	**p-value**	**66cl4**	**67NR**	**log2**	**p-value**
Bambi	1	0	5,77	0,00012	1	0	1,52	0,0002
Bmper	14	8	0,86	0,00012	6	2	1,86	0,0002
Cer1	0	0	0	1	0	0	-2,93	1
Chrd	0	0	-3,4	1	0	0	-2,96	0,06445
Crim1	21	16	0,37	0,05801	13	13	0	0,99088
Dand5	2	4	-0,74	0,00012	4	3	0,07	0,8716
Dcn	0	0	-0,62	1	11	35	-1,61	0,0002
Fst	2	0	5,01	0,00012	5	0	4,33	0,0002
Fstl1	5	0	6,3	0,00012	51	10	2,39	0,0002
Fstl3	1	2	-0,73	0,7434	0	0	0	1
Fstl4	0	0	1,34	1	0	0	0,23	1
Fstl5	0	0	0,61	1	0	0	3,37	1
Grem1	15	0	11,91	0,19687	6	0	9,05	0,09583
Grem2	0	0	-0,2	1	0	0	-1,3	0,067
Kcp	0	0	2,76	1	0	0	0,22	1
Nbl1	0	0	-0,72	0,06666	3	2	0,67	0,0736
Nog	0	0	0	1	0	0	-0,41	1
Rgmb	8	12	-0,56	0,00012	39	19	1,05	0,0002
Sost	0	0	0	1	0	0	0	1
Sostdc1	0	0	0	1	0	0	-2,03	1
Tbx1	0	0	0	1	0	0	1,15	1
Tdgf1	0	0	0	1	0	0	0,04	1
Tll1	0	0	2,87	1	1	0	0,71	0,08821
Tob1	11	24	-1,06	0,00012	13	22	-0,8	0,0002
Tsku	9	7	0,44	0,00104	8	7	0,06	0,7947
Twsg1	18	26	-0,55	0,00012	35	48	-0,45	0,00161
Wisp3	0	0	-0,15	1	0	0	1,62	1

Eleven out of 27 BMP antagonists were expressed in both cell lines and primary tumors of 66cl4 and 67NR, these are Bambi, Bmper, Crim1, Dand5, Fst, Fstl1, Grem1, Rgmb, Tobl, Tsku and Twsg1. These BMP antagonists were further analyzed for clinical significance using the KM-plotter, only the BMP antagonists that significantly correlates to poor prognosis is shown (Figure 11). High expression levels of Crim1, Dand5, Fst and Rgmb correlate to good prognosis in patients, while high expression of Grem1 is correlated to poor prognosis in breast cancer patients. By additionally using the BreastMark database, it was confirmed that high expression of GREM1 correlates with poor prognosis (see supplementary figure in appendix 7.3).

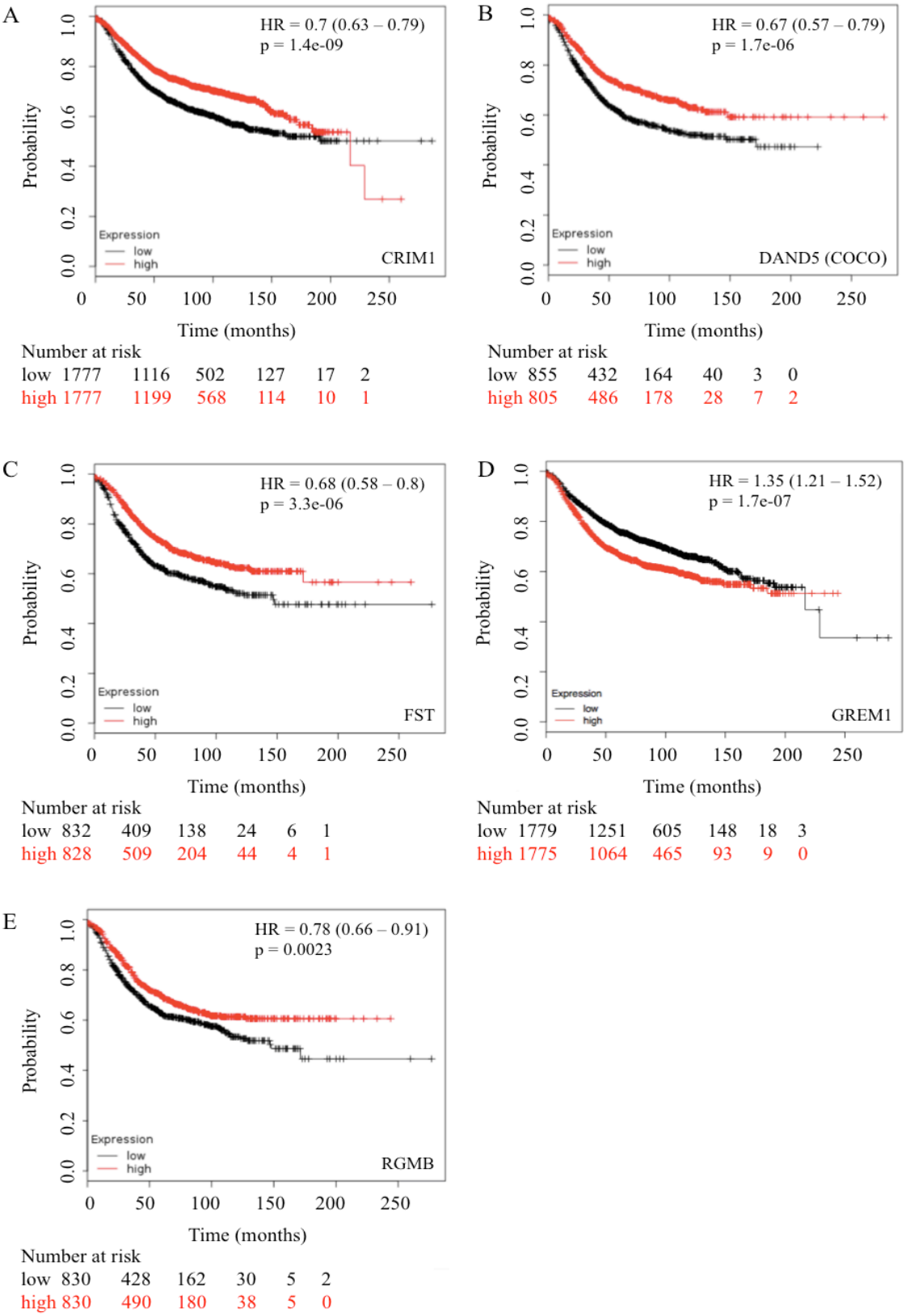


Figure 11 Kaplan-Meier plots of the clinically relevant BMP antagonist expressed in 66c4 and 67NR A. CRIM1 B. COCO C. FST D. GREM1 E. RGMB

The upregulation of GREM1 in 66cl4 cell lines and primary tumor is the only one of the BMP antagonists that shows correlation between high expression and poor prognosis. However, in the transcriptome data we see a high log fold change but a poor p-value. Therefore, GREM1 protein expression had to be assessed.

The five tumor cell lines were cultured and supplemented with PTI for 6 hours before harvest. GREM1 expression was highly expressed by 168FARN and 66cl4 cell lines (Figure 12A and B). Both increased in response to PTI. As the GREM1 protein expression increased with PTI stimulation, the conditioned medium from the five tumor cell lines was analyzed by ELISA to check if GREM1 was secreted. The conditioned medium from 168FARN and 66cl4 contained high levels of GREM1 (Figure 12C). Additionally, the conditioned medium was concentrated and a western blot analysis was performed. This confirmed that GREM1 was found in the conditioned medium from 168FARN and 66cl4 (Figure 12D).

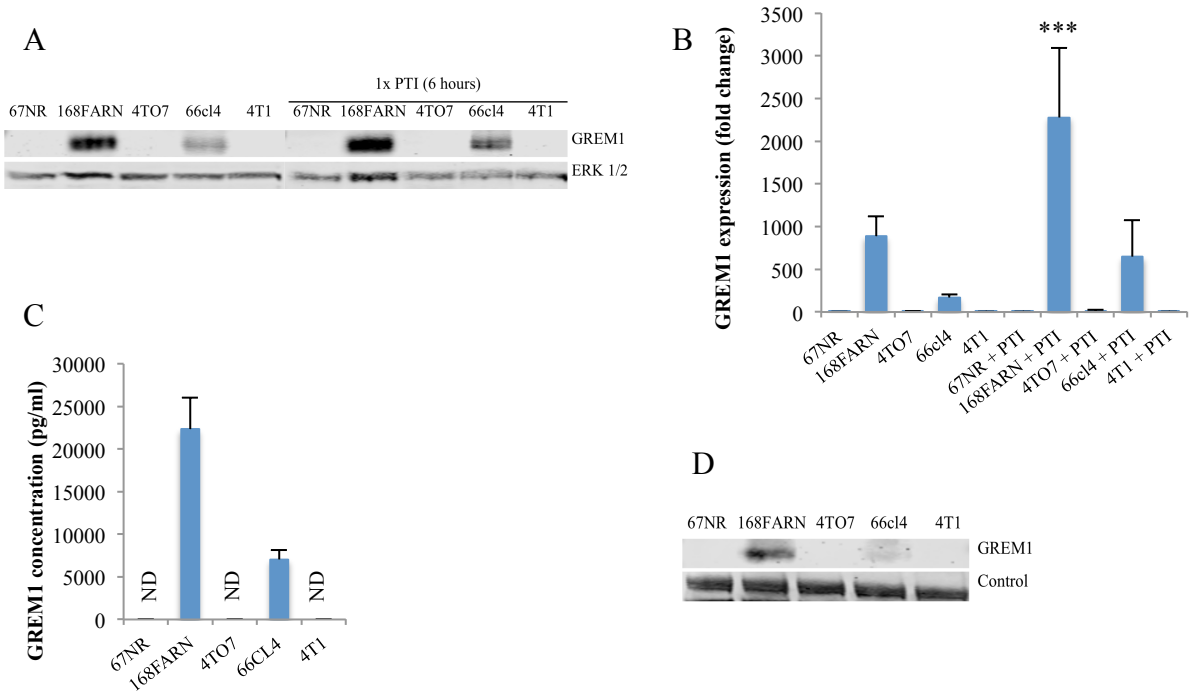


Figure 12 GREM1 protein levels and secretion from the 168FARN and 66cl4 tumor cell lines A. Immunoblotting for GREM1 in the five different tumor cell lines, both untreated and after 1x PTI treatment for 6 hours B. Quantification of the average GREM1 protein expression levels (n = 3). A one-way ANOVA test was performed on the PTI stimulated GREM1 expression levels C. ELISA of the conditioned medium D. Western blot for GREM1 in concentrated conditioned medium samples

GREM1 has also shown to be cell surface-associated [121]. By utilizing a method described by Gao et al. [122], membrane proteins of 67NR, 168FARN and 66cl4 were isolated and analyzed by Western blot (Figure 13). Consistent with previous results, we find highest GREM1 protein levels in the membrane sample of 168FARN.

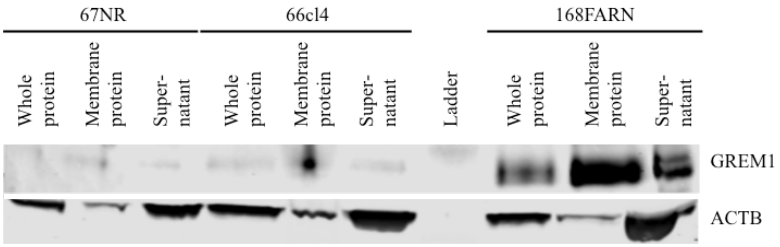


Figure 13 GREM1 membrane protein expression in whole protein samples, membrane protein samples and supernatant of 67NR, 168FARN and 66cl4 cells

Collectively these data suggests that GREM1 is produced in high amounts by 168FARN and 66cl4, it is secreted, it can be associated with the cell surface after secretion. High expression of GREM1 is also correlated to poor prognosis in breast cancer patients, and might be a target for diagnosis or therapy in the clinics.

BMP4 and GREM1 are regulators of differentiation, but it is still not known if they are a part of the differentiation or polarization seen in macrophages in 66cl4 primary tumors. Both BMP4 and Grem1 are therefore interesting candidates for further studies of the communication between tumor cells and macrophages.

3.2 RAW 264.7 MACROPHAGES *IN VITRO* STUDIES

3.2.1 RAW 264.7 MACROPHAGES REACT TO RECOMBINANT BMP4 AND GREM1 STIMULATION

There are macrophages present in primary tumors of both 66cl4 and 67NR. The difference seems to be in the two tumors ability to polarize the macrophages. 66cl4 primary tumors produce and secrete BMP4 and GREM1, which might be part of the differentiation of macrophages. To see if macrophages react upon stimulation with BMP4 and GREM1 at all, RAW 264.7 macrophages were cultured and stimulated them with 5 or 50 ng/ml recombinant mouse rmBMP4, 1 μg/ml recombinant mouse rmGREM1 as well as the chemical inhibitor of BMP signaling DMH1 (2μM) for 30 min (figure 14A). rmBMP4 stimulation induced

signaling in RAW 264.7 macrophages as seen by upregulated phosphorylation of SMAD1/5/9 (downstream of BMP receptors). DMH1, which targets the intracellular kinase domain of BMPR1, effectively reduced phosphorylation of SMAD1/5/9 both in unstimulated and rmBMP4-stimulated macrophages, suggesting a basal level of signaling through the SMAD pathway in RAW 264.7 macrophages. rmGREM1 supplementation did not down regulate basal SMAD1/5/9 phosphorylation in unstimulated RAW 264.7 macrophages, however it decreased the phosphorylation in response to rmBMP4 stimulation. The following experiments were all done once as initial experiments to optimize rmGREM1s antagonistic effects of the signaling. rmGREM1 (1 µg/ml) was added to the RAW 264.7 macrophages for 0.5, 1, 6 hour and overnight (Figure 14B). rmGREM1 and rmBMP4 was added to the RAW 264.7 macrophages in different combinations (rmGREM1 first and the rmBMP4, both simultaneously, both premixed in medium, and rmBMP4 first and then rmGREM1) (Figure 14C). Lastly, rmGREM1 was added in different concentrations (50, 100, 250 and 500 ng/ml) to investigate its inhibiting effect on rmBMP4-stimulated RAW 264.7 macrophages (Figure 14D).

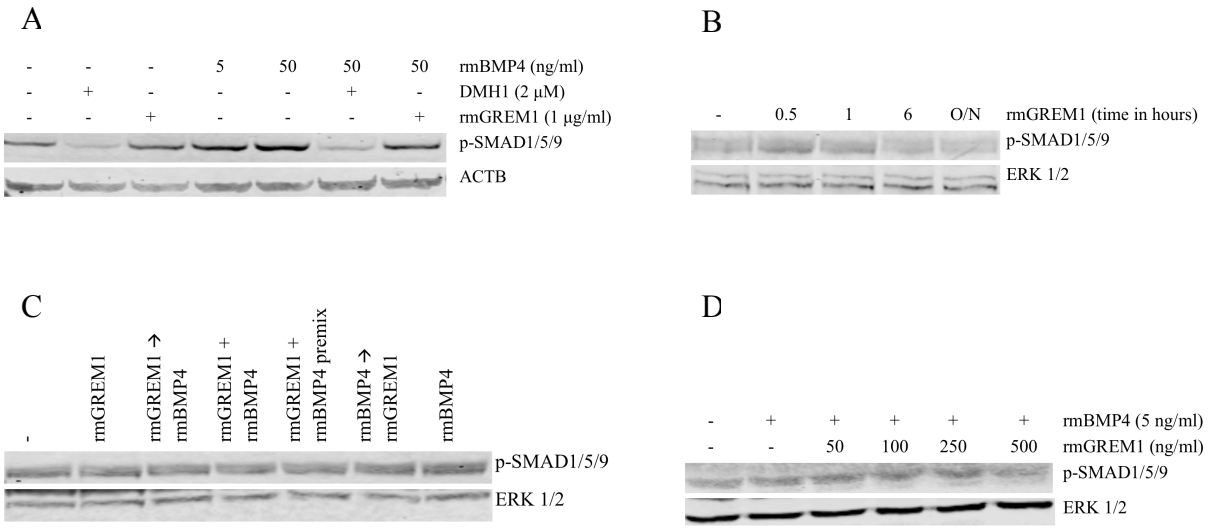


Figure 14 RAW 264.7 macrophages stimulated with rmBM4 and rmGREM1 A. Western blot of p-SMAD1/5/9 expression after stimulation with recombinant BMP4, GREM1 and DMH1. B.Western blot of p-SMAD1/5/9 expression after GREM1 timeseries. C. Western blot of p-SMAD1/5/9 expression after different combinations of rmGREM1 and rmBMP4 stimulation. D. Western blot of p-SMAD1/5/9 expression after r stimulation with different rmGREM1 concentrations

These initial experiments show that rmBMP4 stimulation increase the phosphorylation of SMAD1/5/9 phosphorylation at concentrations as small as 5 ng/ml. It also seems like the RAW 264.7 macrophages have basal SMAD1/5/9 phosphorylation that is not particularly affected by rmGREM1. BMP4 and GREM1 were found to be produced by some of the five cell lines, and they might affect the SMAD pathway in a similar way as the recombinant proteins.

3.2.2 RAW 264.7 MACROPHAGE SIGNALING IS ALTERED UPON ADDING CONDITIONED MEDIUM FROM THE FIVE TUMOR CELL LINES

Recombinant mouse rmBMP4 and rmGREM1 do have an effect on RAW 264.7 macrophages by altering phosphorylation of SMAD1/5/9. Based on the transcriptome data and the initial experiments of the five cell lines, 168FARN tumor cells and 66cl4 tumor cells produce and secrete GREM1, and 66cl4 produce and secrete BMP4 as well. To see if the conditioned medium from the tumor cells could alter basal SMAD1/5/9 phosphorylation, RAW 264.7 macrophages were stimulated for two hours with conditioned medium from all the five cell lines that had been cultured for three days. Conditioned medium from all the five tumor cell lines were able to inhibit SMAD 1/5/9 signaling to a varying degree (Figure 15A and B). Conditioned medium from 168FARN and 4T1 was the most potent conditioned medium causing a significant down regulation of SMAD1/5/9 phosphorylation. However, all five tumor cell lines do produce BMP antagonists to some extent, thus all might inhibit SMAD signaling. 168FARN and 66cl4 have high amounts of GREM1 that can down regulate the phosphorylation, and 4T1 have high amount of the BMP antagonist DAND5 (COCO) [122].

Since rmBMP4 increased expression of p-SMAD1/5/9, it would be interesting to see if conditioned medium from the five tumor cell lines were able to inhibit SMAD signaling also after rmBMP4 stimulation. RAW 264.7 macrophages were cultured and stimulated for two hours with conditioned medium from the five cell lines. When two hours had passed 1 ng/ml or 5 ng/ml rmBMP4 was added for 30 minutes. After stimulation with rmBMP4 there is a clear down regulation of SMAD1/5/9 expression in the RAW 264.7 macrophages treated with conditioned medium from 168FARN, 66cl4 and 4T1 (Figure 15C, D, E and F). The down regulation is stronger in the cells that only got 1 ng/ml rmBMP4. This is probably due to the high amount of GREM1 produced by 168FARN and 66cl4, as well as DAND5 in 4T1.

In all experiments with both intrinsic and rmBMP4 stimulated SMAD signaling, conditioned medium from 168FARN, 66cl4 and 4T1 are the most potent media to down regulate phosphorylation of SMAD1/5/9.

Stimulating RAW 264.7 macrophages with conditioned medium from tumor cells is a simplified method to see what tumor cell secreted substances can do. However, it does not allow for a two-way communication between the two cell types and secretion of substances from the tumor cells might be initiated or enhanced in the presence of other cells. Tumor cells and RAW 264.7 macrophages was therefore cultured in a transwell for 48 fours, and 30 minutes before harvesting the cells were treated with 1 ng/ml rmBMP4 (see supplementary figure in appendix 7.4).

There was no difference in RAW 264.7 macrophages in the phosphorylation of SMAD1/5/9 after they were grown in transwell with the tumor cells (Figure 15G). However, the SMAD1/5/9 activation is altered in the different tumor cells after stimulation with rmBMP4. 168FARN and 66cl4 tumor cells upregulate the signaling through SMAD1/5/9 after rmBMP4 stimulation. However, one experiment is not enough to conclude about the effect of tumor cells and macrophage communication in transwell.

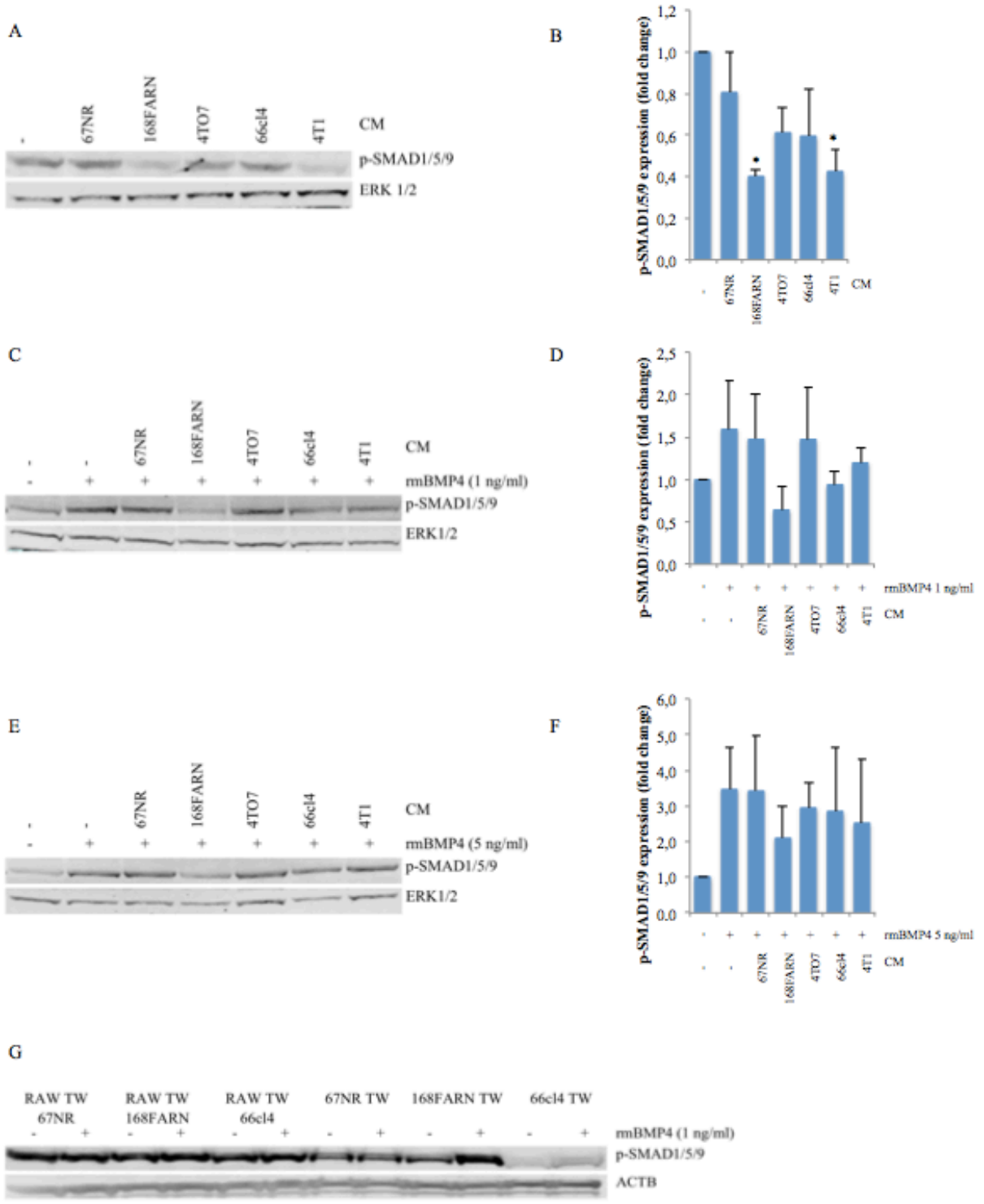


Figure 15 Conditioned medium (CM) from the five cell lines and transwell with tumor cells affect SMAD1/5/9 phosphorylation in RAW 264.7 macrophages A. Western blot of basal SMAD1/5/9 phosphorylation in the RAW 264.7 macrophages. B. Quantification of the average values from 3 replicate experiments. One-way ANOVA test was performed. C. Western blot of 1 ng/ml rmBMP4 stimulated SMAD1/5/9 phosphorylation in the RAW 264.7 macrophages. D. Quantification of the average values from 3 replicate experiments. One-way ANOVA test was performed. E. Western blot of 5 ng/ml rmBMP4 stimulated SMAD1/5/9 phosphorylation in the RAW 264.7 macrophages. F. Quantification of the average values from 3 replicate experiments. One-way ANOVA test was performed. G. Western blot of p-SMAD1/5/9 in RAW 264.7 macrophages after transwell experiment with tumor cells

Stimulating the RAW 264.7 macrophages with conditioned medium from the tumor cells lines did affect the cells, but the cultivation of macrophages and tumor cells in transwell did not affect the RAW 264.7 macrophages. However, the tumor cells seemed to be affected by the presence of macrophages. To see if this was due to the two-way communication between tumor cells and macrophages, the conditioned medium from RAW 264.7 macrophages alone had to be checked.

3.2.3 RAW 264.7 MACROPHAGES CONDITIONED MEDIUM AFFECT SMAD1/5/9 PHOSPHORYLATION IN THE TUMOR CELLS

The presence of RAW 264.7 macrophages in transwell seemed to have an effect in the tumor cells. To see if the same effect on the SMAD signaling was seen with RAW 264.7 macrophage conditioned medium, tumor cells was cultured and stimulated with RAW 264.7 macrophage conditioned medium for two hours. Also conditioned medium from the RAW 264.7 macrophages have an effect on the different tumor cell lines, but the variations are too big do draw any conclusions of it (Figure 16). However, conditioned medium form RAW 264.7 macrophages is really potent at inhibiting SMAD1/5/9 phosphorylation in 66cl4.

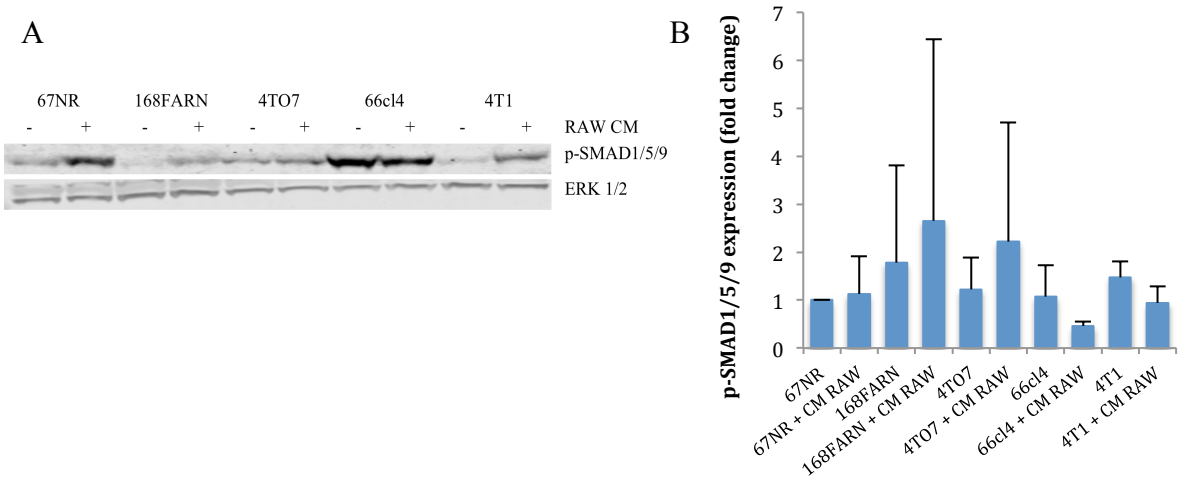


Figure 16 Conditioned medium from RAW 264.7 macrophages affect SMAD activation differentially in the five tumor cell lines A. Western blot analysis of SMAD1/5/9 phosphorylation pathway after stimulation with conditioned medium from RAW 264.7 macrophages. B. Quantitative analysis of average p-SMAD1/5/9 expression levels of 3 replicate experiments against ERK 1/2 loading control

Conditioned medium from RAW 264.7 macrophages does affect the SMAD pathway in tumor cells, but with great variations. To see if these effects were due to BMP4 and GREM1, RAW 264.6 macrophages was analyzed for the expression of BMP4 and GREM1.

3.2.4 RAW 264.7 MACROPHAGES PRODUCE BMP4 BUT NOT GREM1

Since the presence of RAW 264.7 macrophages in transwell and the stimulation with RAW 264.7 conditioned medium could have an effect on the tumor cells, RAW 264.7 macrophages were seeded out and treated with 1x PTI for 3 or 6 hours and analyzed for GREM1 and BMP4 expression. RAW 264.7 macrophages do produce BMP4, and the expression slightly increases after PTI treatment (Figure 17A and B). However, RAW 264.7 macrophages do not produce GREM1 (Figure 17C). A similar experiment was done on fibroblast by another master student in the group, and fibroblasts do express high amounts of both GREM1 and BMP4. As the transcriptome data show, the primary tumors express high amount of GREM1 that might be produced by tumor cells, but also by other cells in the primary tumor. Studies have shown that GREM1 is produced also by the fibroblasts that are an abundant cell type in tumors.

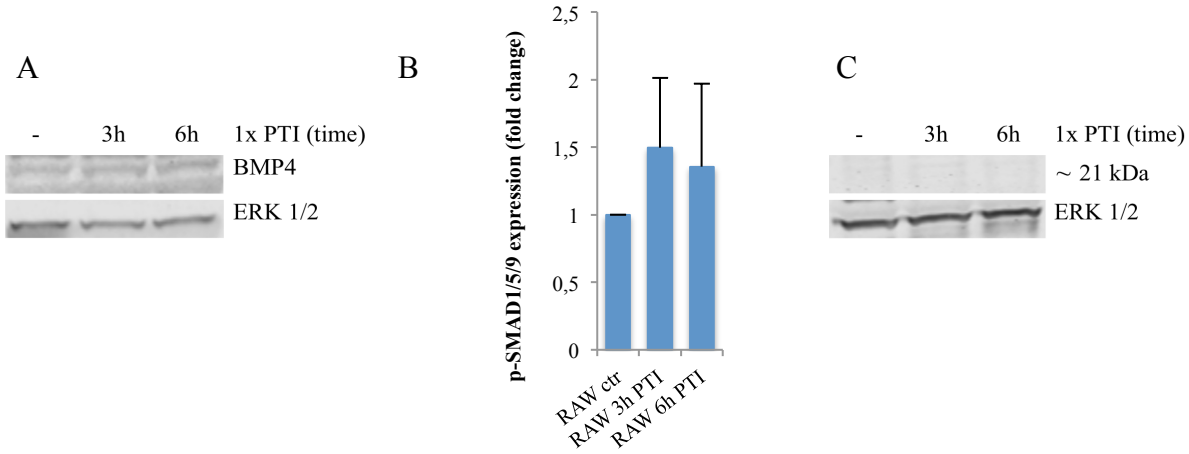


Figure 17 RAW 264.7 macrophages produce BMP4 but not GREM1 A. Western blot of BMP4 RAW 264.7 macrophages treated with or without 1xPTI for 3 or 6 hours. BMP4 is expressed by the RAW 264.7 macrophages and it slightly increases after 3 hours PTI stimulation B. Quantification of average BMP4 expression of 3 replicate experiments. A one-simple t-test was performed on the values, however the small increase in BMP4 expression is not significant C. Western blot of RAW 264.7 macrophages treated with or without PTI. RAW 264.7 macrophages do not produce GREM1.

Even though the RAW 264.7 macrophage cell line is a good way to study communication with tumor cells, it is not ideal and culture cells might react differently than TAMs. To better mimic the situation in a tumor, bone marrow-derived macrophages, which are a macrophage type that is more similar to macrophages that could be recruited in a tumor, was studied.

We therefore decided to use primary bone marrow-derived macrophages (BMDMs) of BALB/c mice to reproduce our results in a macrophage type that is more similar to macrophages that could be recruited in a tumor.

3.3 BONE MARROW-DERIVED MACROPHAGES *IN VITRO* STUDIES

3.3.1 BMDMS REACT UPON STIMULATION WITH RECOMBINANT BMP4 AND GREM1 AND CONDITIONED MEDIUM FROM THE TUMOR CELLS

BMDMs from Balb/C mice are more similar to macrophages that are recruited to tumors than RAW 264.7 macrophages. Since RAW 264.7 macrophages were affected by stimulation with rmBMP4, rmGREM1 and conditioned medium from the tumor cells, similar experiments was done with BMDMs to see if the data from RAW 264.7 macrophages could be reproduced. BMDMs were seeded out and stimulated with different concentrations of rmBMP4 (20, 100 and 200 ng/ml) as well as 1 μg/ml rmGREM1, the chemical inhibitor of SMAD signaling DMH1 (1 μM) and conditioned medium from 67NR, 168FARN and 66cl4. The chemical inhibitor DMH1 is able to inhibit signaling down the SMAD pathway in BMDMs as well as it did in RAW 264.7 macrophages (Figure 18A). .rmBMP4 stimulation at 200 ng/ml slightly increased the phosphorylation of SMAD1/5/9 thus having less effect than in RAW 264.7 macrophages (Figure 18B). rmGREM1 is more potent at inhibiting the signaling in BMDMs than in RAW 264.7 macrophages both with and without Bmp4 stimulation. However, the effects are small making it difficult to interpret. Conditioned medium from 67NR, 168FARN and 66cl4 do not affect the SMAD pathway in BMDMs.

To see if BMDMs were affected by the presence of tumor cells, BMDMs were seeded in transwell with 67NR, 168FARN and 66cl4 tumor cells, and SMAD1/5/9 phosphorylation seemed to be upregulated in BMDMs grown together with all the tumor cells (Figure 18C).

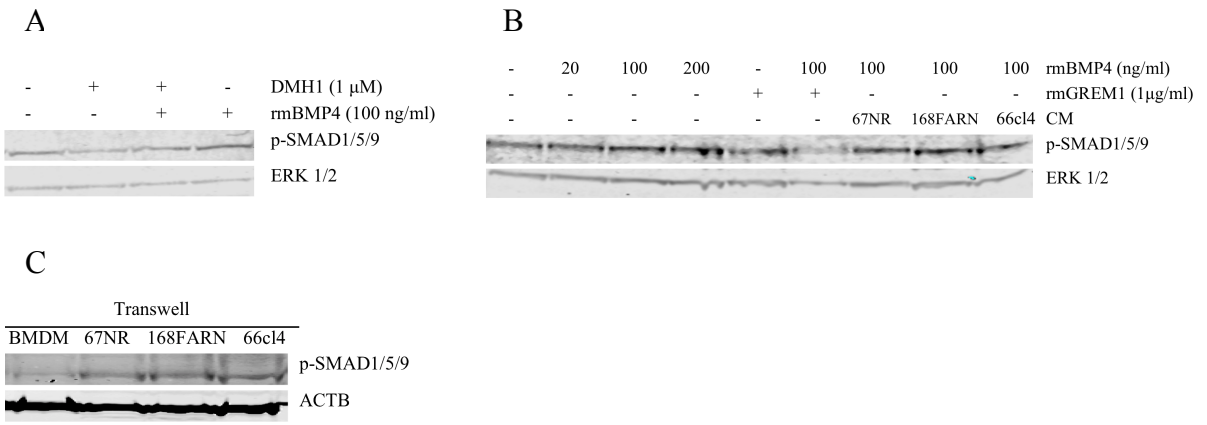


Figure 18 Bone marrow-derived macrophages alter the expression of p-SMAD 1/5/9 upon stimulation with recombinant proteins, tumor cells or conditioned medium from the tumor cells A. Western blot of p-SMAD1/5/9 in BMDMs after DMH1 inhibition B. Immunoblotting of p-SMAD1/5/9 in BMDMs after stimulated with rmBMP4, rmGREM1 and conditioned medium from the tumor cells. C. Immunoblotting of p-SMAD1/5/9 in BMDMS in transwell with the tumor cells

Conditioned medium from the tumor cells and cultivation in transwell with the tumor cells lines showed varying effects of the SMAD pathway. SMAD signaling and its regulation by BMP4 and GREM1 is important for differentiation and/or polarization of cells. Therefore, BMDMS was analyzed for morphological changes in the presence of tumor cells or their conditioned medium.

3.3.2 CONDITIONED MEDIUM FROM THE TUMOR CELL LINES CAUSE MORPHOLOGICAL CHANGES IN BMDMS

To see if conditioned medium of the different tumor cells, or the presence of tumor cells changes the morphology of the BMDMs, they were seeded out and stimulated with conditioned medium for two days, or seeded out with tumor cells in transwells and analyzed by light microscopy with 20x magnification (Figure 19). BMDMs stimulated with conditioned medium from 67NR, 168FARN and 66cl4 looked more confluent and differentiated, while the BMDMs that hade been stimulated with conditioned medium from 4TO7, 4T1 or their own conditioned medium were more round and less confluent. Similar to the effects by conditioned medium, BMDMs grown in transwell with BMDMs, 4TO7 and 4T1 had a rounder appearance, while BMDMs grown in transwell with 67NR, 168FARN and 66cl4 looked more differentiated.

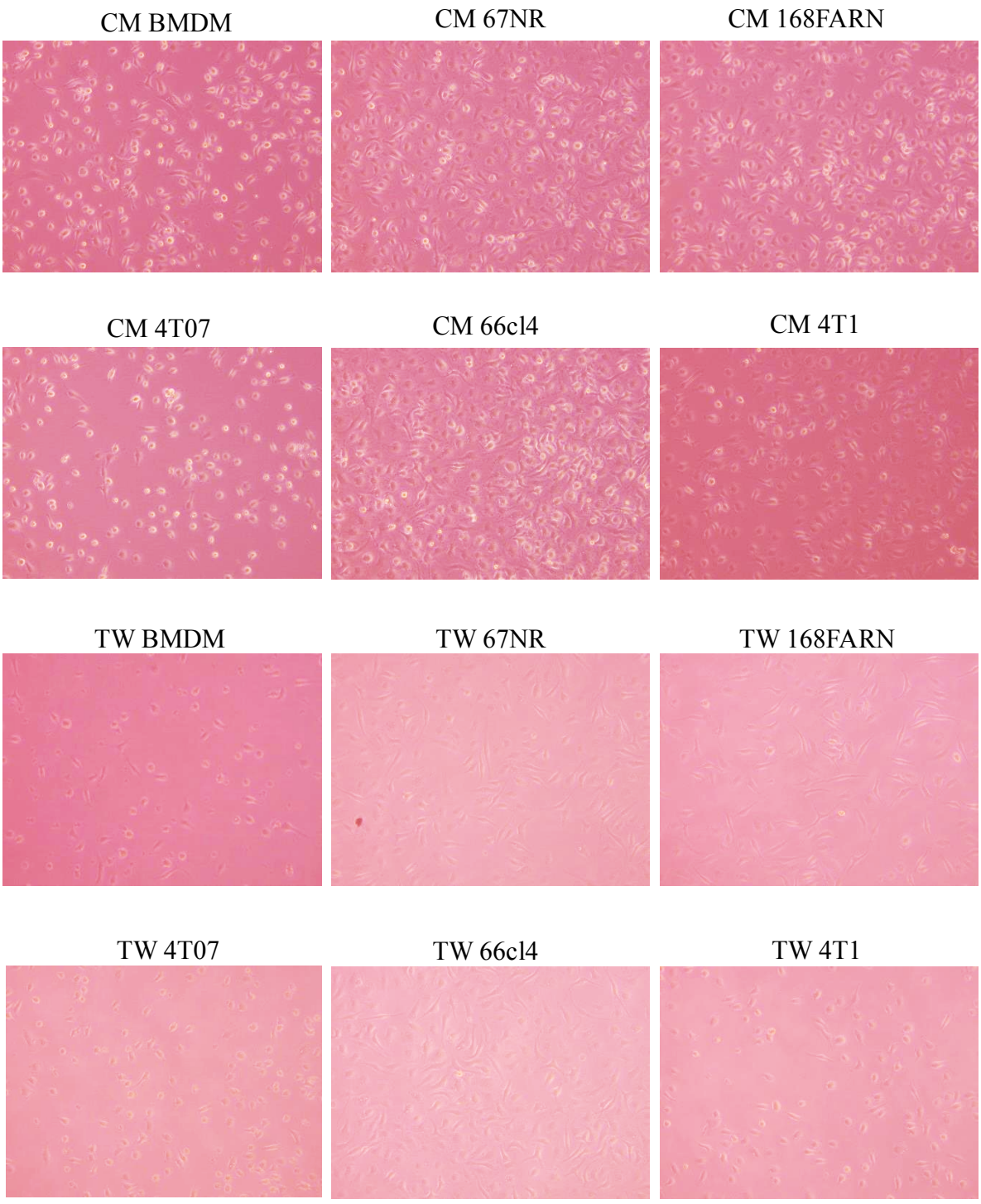


Figure 19 Bone marrow-derived macrophages change their morphology in response to conditioned medium from the tumor cells or BMDMs or the presence of tumor cells or BMDMs in transwell

The differences in morphological changes in BMDMs upon stimulation with conditioned medium from tumor cells or from the presence of tumor cells are due to differentiation processes in the cells. Based on the transcriptome data, 66cl4 have a higher expression of M2 macrophage markers and might be better equipped at differentiating macrophages. To assess if BMDMs were being differentiated, BMDMs were analyzed for expression of the M2 macrophage marker ARG1.

3.3.3 M2 MACROPHAGE MARKER ARG1 IS UPREGULATED IN BMDMS IN RESPONSE TO TRANSWELL WITH THE TUMOR CELLS

In cooperation with Jennifer Mildenberger, a western blot analysis was performed to look at the expression of the M2 macrophage marker ARG1 in BMDMs after cultivation with tumor cells in transwell. BMDMs grown together with 168FARN and 66cl4 showed an upregulation of ARG1, in comparison to BMDM grown together with BMDMs (Figure 20). The higher expression of ARG1 might mean that 168FARN and 66cl4 are able to differentiate the BMDMs into a more M2-like state.

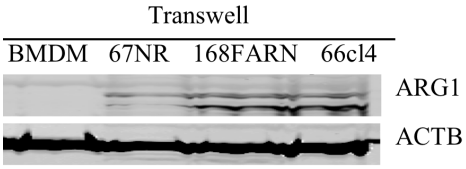


Figure 20 BMDMs grown in a transwell with 67NR, 168FARN and 66cl4 upregulates the expression of M2 macrophage marker ARG1

Since BMDMs seemed to be affected by conditioned medium from the tumor cells, and the cultivation of BMDMs together with the tumor cells in transwell seemed to affect both polarization of macrophages as well as the SMAD pathway, it might suggest that the tumor cells are differently equipped to attract and polarize macrophages. Transcriptome data showed that both primary tumors of 67NR and 66cl4 expressed macrophages recruiting factors, however their ability to attract macrophages had to be assessed.

3.3.4 TUMOR CELLS AND CONDITIONED MEDIUM OF TUMOR CELLS ATTRACT BMDMS

To test the tumor cells and the conditioned medium from tumor cells ability to recruit BMDMs, a migration assay was performed. Conditioned medium Positive control was BMDMs grown in normal DMEM, negative control was BMDMs grown in DMEM without serum (Figure 21A). Conditioned medium of BMDMs, 67NR, 168FARN and 66cl4 are all able to recruit the BMDMs, 66cl4 conditioned medium being the most potent one (Figure 21B). Conditioned medium from BMDMs seem to recruit BMDMs to a certain point before the recruitment decreases. All tumor cells are able to recruit macrophages (Figure 21C). 168FARN and 66cl4 cells are better than 67NR cells at recruiting macrophages. However, the controls did not work, so the experiments have to be optimized for a conclusion to be reached

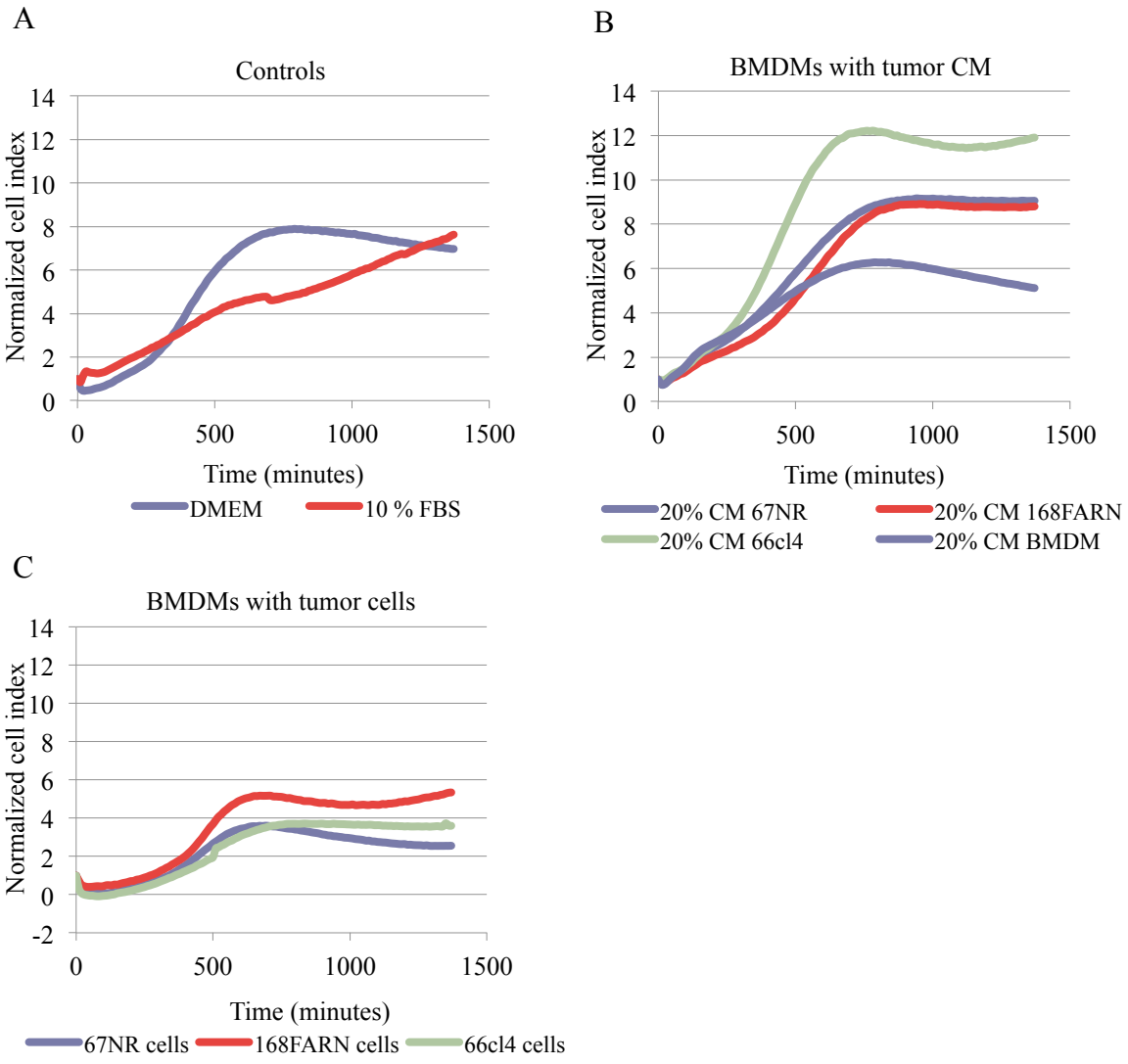


Figure 21 Migration assay analysis showing the tumor cells and tumor cells conditioned medium ability to recruit macrophages A. Conditioned medium from BMDMs, 67NR, 168FARN and 66cl4 are all able to recruit BMDMs but to varying extent. B. 67NR, 168FARN and 66cl4 tumor cells are all able to recruit macrophages

3.4 GREM1 COULD NOT BE FOUND IN THE BLOOD OF BALB/C MICE INJECTED WITH TUMOR CELLS

Some studies have shown that patients with cardiovascular disease have elevated levels of GREM1 in their blood [123]. We therefore collected blood from healthy BALB/c mice, as well as BALB/c mice injected with 67NR, 168FARN and 66cl4 tumor cells and performed both ELISA and Western blot analysis. However, we did not immediately get a clear result, suggesting that optimization is required before we can identify GREM1 in the blood.

3.5 INFLAMMATORY SIGNALING IS UPREGULATED IN RAW 264.7 MACROPHAGES WHEN TREATED WITH 66CL4 CONDITIONED MEDIUM

BMP4 and GREM1s regulation of the SMAD pathway is only a very small percentage of pathways that are altered due to the communication between macrophages and tumor cells. Inflammatory signaling might be switched on during recruitment of macrophages into tumors. Also, there is a higher expression of markers for M2 macrophages in the 66cl4 primary tumor compared to that of 67NR primary tumors, suggesting less pro-inflammatory signalling in 66cl4. The phosphorylation of some central inflammation-related transcription factors was therefore examined in RAW 264.7 macrophages stimulated with 100% conditioned medium from 66cl4 and 67NR for 30 minutes and 2 hours, and with 50% conditioned medium for 24 hours. STAT1 activation is seen in the cells after 100% CM stimulation for 30 minutes and 2 hours (Figure 22A). However, STAT1 activation is only seen in RAW 264.7 macrophages stimulated with conditioned medium from 66cl4 after 50% CM stimulation for 24 hours (Figure 22B). IRF3 and p65 did not seem to be activated.

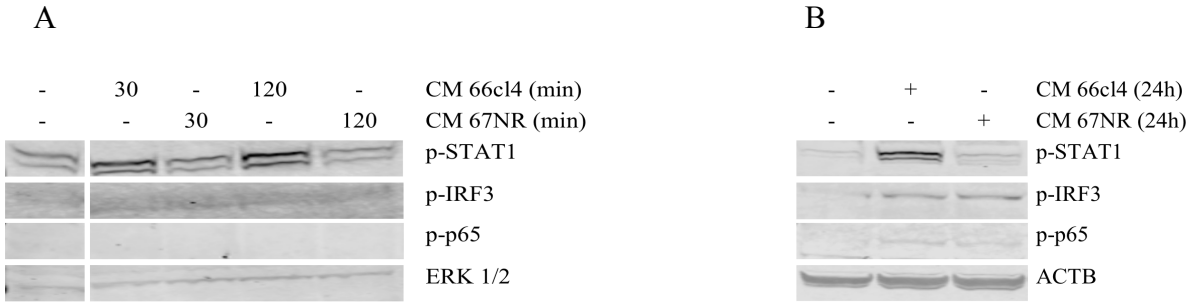


Figure 22 Conditioned medium from 66cl4 stimulates activation of STAT1, whilst conditioned medium from 67NR does not A. Western blot of p-STAT1, p-IRF3 and p-p65 expression after conditioned medium stimulation for 24 hours B. Western blot of p-STAT1, p-IRF3 and p-p65 expression after conditioned medium stimulation for 30 minutes or 2 hours

Based on the transcriptome data shown in table 5, more M2 macrophages in 66cl4 tumors was expected. This would suggest that there are more immune cells in 67NR that fight the tumor. *In vitro* experiments showed however activation of pro-inflammatory STAT1 signaling when RAW 264.7 macrophages were treated with conditioned medium of 66cl4.

To assess the infiltration of immune cells and especially T-cells into 66cl4 and 67NR tumors, the transcriptome data was searched for specific T-cell markers [124] (Table 9). All of the T-cell markers, Cd3d, Cd3e, Cd3g, Cd4, Cd8a and Cd8b1 are higher expressed in 67NR primary tumors. The markers are not expressed in cell lines of 67NR and 66cl4, suggesting that they do come from infiltrated T-cells in the primary tumors. These findings might suggest that the M2 macrophages in 66cl4 primary tumors could dampen the immune reaction in those tumors.

Table 9 mRNA expression of specific T-cell markers in cell lines and primary tumors of 67NR and 66cl4
67NR primary tumors have a much higher expression of T-cell specific markers than that of 66cl4. Cell lines of 67NR and 66cl4 do not express any of the markers, thus suggesting that the markers are actually expressed by T-cells in the primary tumors [124].

	Cell culture				Primary tumors			
Gene	**66cl4**	**67NR**	**log2**	**p-value**	**66cl4**	**67NR**	**log2**	**p-value**
Cd3d	0	0	1,26	1	2	4	-1,32	0,00189
Cd3e	0	0	-0,13	1	1	5	-1,91	0,00020
Cd3g	0	0	1,48	1	4	14	-1,65	0,00020
Cd4	0	0	-1,42	1	2	7	-2,26	0,00020
Cd8a	0	0	0	1	1	5	-2,70	0,00020
Cd8b1	0	0	0	1	1	4	-2,39	0,00020

4 DISCUSSION

The thesis aimed to study the communication between tumor cells and macrophages. By utilizing the 4T1 breast cancer mouse model, a higher expression of markers of alternatively activated M2 macrophages was found in primary tumors of the metastasizing 66cl4 compared to primary tumors of the non-metastasizing 67NR. We hypothesize that this contributes to 66cl4 tumor cells ability to form metastasis. The communication between tumor cells and macrophages was studied by focusing on TGF-β superfamily member BMP4 and its antagonist GREM1, as they are important for differentiation and proliferation of cells. Of the five tumor cell lines only 66cl4 produces and secretes BMP4. However, both 168FARN and 66cl4 produce GREM1 that is secreted and also associated to the cell surface.

By treating RAW 264.7 macrophages with conditioned medium from the five different tumor cell lines, it was seen that conditioned medium from 168FARN and 66cl4 can inhibit both basal and rmBMP4-stimulated SMAD1/5/9 phosphorylation. Cultivating tumor cells together with RAW 264.7 macrophages affected the SMAD signaling in the tumor cells. The stimulation of tumor cells with RAW 264.7 macrophages conditioned medium also affected the SMAD signaling differently in the tumor cells. However, the effect of the conditioned medium from the macrophages was varying and gave conflicting results.

In bone marrow-derived macrophages (BMDMs), both conditioned medium from the tumor cell lines and the presence of them in transwell changes their morphology. The SMAD pathway was only affected by the presence of tumor cells. However, both conditioned medium and the presence of 67NR, 168FARN and 66cl4 was able to attract BMDMs.

Lastly, to assess the inflammatory state in the macrophages, central inflammation-related transcription factors were examined. In RAW 264.7 macrophages p-STAT1 was upregulated after stimulation with 66cl4 conditioned medium compared to 67NR conditioned medium.

4.1 66CL4 PRIMARY TUMORS HAVE MORE M2 MACROPHAGES THAN 67NR PRIMARY TUMORS

Several studies have found a positive correlation between the number of macrophages and poor prognosis [53, 54]. According to the data presented in this thesis, there are macrophages in primary tumors of both 67NR and 66cl4. Previously in the group there was performed an analysis of mutated DNA versus host DNA in tumor samples that showed that there is approximately 30-40% normal DNA in the tumor. Preliminary FACS results staining for the macrophage surface markers EMR1 and ITGAM, indicates that there are approximately 10%

macrophages in primary tumors of 67NR and 66cl4. Together with the expression levels of macrophage markers in the transcriptome data, it indicates that a substantial amount of macrophages are present within the tumor. The general macrophage markers, Emr1, Itgam, Csfr1 and Spi1, are not expressed in the tumor cell lines, suggesting that there are macrophages in the tumors. Though, in a tumor, tumor cells might start to express some of these markers but that is unlikely.

However, we do not know if expression of the markers correlates to number of cells or the activity of the cells. There might be the same number of cells in both 67NR and 66cl4 primary tumors, only that the macrophages present in 66cl4 are more active. Therefore, it is important to use other methods like e.g. immunostaining with antibodies to quantify the number of macrophages in the primary tumors. An immunostaining allows not only determination of the number of macrophages but also their location within the tumor. It would be interesting to see if there is a difference in the localization of macrophages in the two different primary tumors.

Based on the fact that there are macrophages in the primary tumors, and that there is a high expression level of macrophage recruiting factors, we can speculate on the importance of macrophages for the tumors. It is well known that macrophages are cells of the innate immune system, that directly fight tumors through phagocytosis of transformed cells, or indirectly by either presenting antigens to the adaptive immune cells, or by producing cytokines and chemokines that recruit more immune cells to the site. However, macrophages have also been shown to be tumor promoting, which is also stated by the fact that a high number of tumor-associated macrophages is correlated to poor prognosis [53, 54]. Macrophages produce a vast pool of growth factors, cytokines and chemokines that can contribute to tumor growth. Macrophages also produce MMPs and other proteases that break down the ECM, facilitating tumor cell escape from the primary site [41].

Even though macrophage markers are expressed in primary tumors of both 67NR and 66cl4, we speculate that the macrophages in the two tumors are different and that the tumor cells differ in their ability to polarize macrophages. Transcriptome data shows expression of typical M1 macrophage markers in both primary tumors of 67NR and 66cl4, but a higher expression of specific M2 macrophages markers in the metastatic 66cl4 tumors than that of 67NR primary tumors. M2 macrophages are macrophages that upon stimulation are polarized in to a direction that facilitates tissue repair, angiogenesis and tumor promotion [46]. These are characteristics that might help tumor cells to grow and eventually leave the primary site and form metastasis [49]. However, in order to prove that M2 macrophages contribute to metastasis, other experiments are needed, e.g. mice lacking macrophages or M2 macrophages

to see if 66cl4 cells are able to form metastasis when injected in such mice. It should also be proven that it is the number of macrophages that contribute to 66cl4 primary tumors metastatic potential. Again, immunohistochemistry of tumor tissue to localize the macrophages, or FACS analysis to find the number of macrophages could be performed.

It would also be interesting to assess the activation of the M2 macrophages that might not correlate with their numbers. Then we can say that there seems to be more active M2 macrophages in 66cl4 than in 67NR. In that case there have to be something present in the tumor microenvironment of 66cl4 tumors that triggers these macrophages to be more active and promoting. It is known that tumors produce high amount of cytokines and chemokines that can recruit and differentiate/ polarize other cells, including TGF-β superfamily members.

One of these members, BMP4, and its antagonist GREM1 was therefore chosen for further studies of tumor-stroma communication.

4.2 HIGH EXPRESSION OF BMP ANTAGONIST GREM1 IS CORRELATED TO POOR PROGNOSIS

The initial studies of this thesis showed that cell lines and primary tumors of 66cl4 produce BMP4 and GREM1. This was confirmed by western blot analysis. The protein expression of both proteins was increased after stimulation with protein transport inhibitor (PTI) for 6 hours, which suggests that the proteins are secreted. GREM1 was detected in the conditioned medium by ELISA and western blot. BMP4 could not be detected, neither by ELISA nor western blot. However, based on findings from the western blot with PTI treatment and previous studies done it the field [104], BMP4 is still believed to be secreted. To be able to detect BMP4 in the medium, the methods should be optimized. A possibility would be to cultivate the cells in medium with reduced or no serum. Serum is constituted of albumin, as well as other lipids, amino acids and sugars that interfere the blot with many unspecific bands especially around 30-70 kDa. Cultivating cells without serum is difficult, because the cells are dependent on its constituents to grow, but it might be possible for a couple of hours. To reduce the amount of unspecific bands, the samples were diluted without any success.

By utilizing the online databases KM plotter and BreastMark, the clinical significance between expression levels of the proteins and prognosis in breast cancer patients was assessed. High expression of BMP4 seems to be favorable for the patients, but it is not significant. However, high expression of the antagonist, GREM1, is significantly correlated to poor prognosis in patients. The expression of BMP4 and GREM1 in the tumor cells, together

with GREM1s correlation to poor prognosis in patients is the reason why the proteins were chosen for further work. Of the BMP antagonists analyzed in the transcriptome data, its seen that some are expressed differently in 66cl4 and 67NR cell lines and primary tumors. However, only GREM1 was significantly correlated to poor prognosis in breast cancer patients.

There have been performed studies on other BMP antagonists as well. Gao et al. found that the BMP antagonist DAND5 (COCO) promotes tumor initiation and metastasis in the 4T1 breast cancer mouse model. DAND5 sustains the expression of stem cell markers (Nanog, Sox2 and Oct4) and it can be used as a predictor of metastatic relapse to the lung in breast cancer patients [122]. All these findings are equivalent to a poor overall survival, however DAND5 is not found clinically relevant when using BreastMark and KM-plotter, as GREM1 is.

In addition to GREM1s antagonizing effects of BMP signaling, it is shown to bind VEGF receptor-2 (VEGFR2), a receptor that upon ligand binding is phosphorylated and transmits a signal that result in endothelial cell proliferation, migration and the formation of angiogenic sprouts [105]. There are no studies that directly link GREM1 to angiogenesis, but it is possible that GREM1 also plays a role in this part of tumorigenesis. Metastases are the major cause of cancer death in patients, and the formation of new blood vessels in the tumor creates an escape route for disseminated cancer cells. When injecting 66cl4 or 67NR tumor cells in BALB/c mice and later harvesting the tumors, 66cl4 tumor are well vascularized and less dense, while tumors of 67NR cells are less vascularized and denser.

To initially study the effect of GREM1 in the communication between tumor cells and macrophages, *in vitro* experiments were performed.

4.3 TUMOR CELLS AND RAW 264.7 MACROPHAGES ARE RECIPROCALLY AFFECTED BY THE OTHERS CONDITIONED MEDIUM AND EACH OTHERS PRESENCE

Since it was seen that BMP4 and GREM1 were produced and secreted by 66cl4 and 168FARN cell lines, their role in the possible communication between the tumor cells and RAW 264.7 macrophages was studied. Initial experiments were performed to see if the RAW 264.7 macrophages responded at all to stimulation with BMP4 and GREM1. After optimizing concentrations and time points, concentrations as low as 5 ng/ml recombinant mouse rmBMP4 was able upregulate the activation of SMAD1/5/9. Recombinant mouse rmGREM1

is needed at a concentration of 500 ng/ml or higher, as well as an incubation time of 6 hours or longer for it to be able to inhibit the signaling. Based on the previous findings from transcriptome data and western blot analysis, RAW 264.7 macrophages were treated with conditioned medium from the tumor cells to see what effect that had on the basal SMAD signaling. The expression of BMP4 did not seem to have an effect on the SMAD signaling, since SMAD1/5/9 phosphorylation was not induced, instead conditioned medium from all the tumor cells was able to reduce the signaling in the SMAD pathway in the macrophages. However, in the three independent replicates there was a great difference especially in the effect of conditioned medium from 67NR. What was clear in the experiments is that conditioned medium from 168FARN and 66cl4 are the most potent at inhibiting signaling through the SMAD pathway. This is thought to be because of the high amount of GREM1 produced and secreted by 168FARN and 66cl4 cells. The inhibiting effect of the other conditioned mediums are most likely due to other BMP antagonists, e.g. DAND5 that is produced in high mounts by the 4T1 cell line. The inhibiting effect of the conditioned medium was also seen when RAW 264.7 was stimulated with rmBMP4.

There is a two-way communication between macrophages and tumor cells in tumors. Therefore it was also important to see how macrophages contribute to this. By utilizing transwell plates, it was also possible to see how the cells reacted to each other when the two-way communication was possible.

4.4 RAW 264.7 MACROPHAGES PRODUCE BMP4 THAT CAN ACT ON THE TUMOR CELLS

Experiments with tumor cells and RAW 264.7 macrophages in transwell, where they share their medium, gave no clear results and were difficult to interpret. Studies show that stroma cells, and especially fibroblast, express GREM1. RAW 264.7 macrophages were therefore analyzed for production and secretion of BMP4 and GREM1. The performance of western blots showed that RAW 264.7 macrophages produce BMP4 but not GREM1. The BMP4 produced by RAW 264.7 macrophages may be secreted and work on the surrounding cells, e.g. the tumor cells to start differentiation processes. However, if the tumor cells upregulate the expression of GREM1, secrete it so that GREM1 can be bound to the tumor cells surface, it might protect themselves from BMP4. In that way tumor cells do not get differentiated, but rather sustain their self-renewal potential. Gao et al. speculated that DAND5 binds to the pericellular matrix and thus obtains a high concentration around the cell [122]. Because of

DAND5s high binding affinity towards BMP proteins they believed that DAND5 could protect the tumor cells by inhibiting BMPs binding to the receptors.

Further, conditioned medium of the RAW 264.7 macrophages to the five tumor cell lines and performed a western blot analysis to check the level of phosphorylated SMAD1/5/9. In 67NR, 168FARN and 4TO7 it seemed like the SMAD signaling was upregulated after stimulation with conditioned medium from the macrophages. In 66cl4 and 4T1 the opposite was seen. Since the RAW 264.7 macrophages do not produce GREM1, this down regulation of SMAD phosphorylation has to be due to other BMP antagonists in the conditioned medium. It can also be down regulated if 66cl4 and 4T1, binds respectively GREM1 and DAND5 on their own cell surface in response to a higher BMP concentration to protect themselves from BMPs activity.

Using RAW 264.7 macrophages was a good starting point in the investigation about communication between macrophages and tumor cells, but they are not ideal when it comes to their similarity to the macrophages that are in the body. Cell lines that have been cultivated for a long time are not normal cells anymore. The conditions are also not similar as in the body, the cells are grown on plastic dishes, they are given nutrition through serum, and grown by themselves or in transwell plates in a monolayer culture. As an attempt to mimic the communication between tumor cells and macrophages in a tumor a little better, macrophages derived from the bone marrow of BALB/c mice was used.

4.5 SMAD SIGNALING IN BONE MARROW-DERIVED MACROPHAGES IS ALSO ALTERED UPON STIMULATION WITH BMP4 AND GREM1

Bone marrow-derived macrophages (BMDMs) were used in similar experiments as RAW 264.7 macrophages to see if they behaved in a similar way. BMDMs did not respond that well to BMP4 stimulation as the RAW 264.7 macrophages did, a concentration of 100 ng/ml was needed to see an effect. However, GREM1 was more potent at inhibiting the signaling through the SMAD pathway in BMDMs compared to RAW 264.7 macrophages. The BMDMs was also stimulated with conditioned medium from 67NR, 168FARN and 66cl4, without showing a significant effect on the phosphorylation of SMAD1/5/9. It might suggest that the BMP4 level in the BMDMs are so high that GREM1 in the conditioned medium is not able to inhibit the signaling. However, this might be different in a tumor, where the stroma is continuously exposed to the secretion from the tumor cells. Another essential

problem with the BMDM experiments was low protein yield due to a limited number of cells harvested from the bone marrow, which also might affect the results.

Further a migration assay of the BMDMs was performed. BMDMs were seeded together with cells of 67NR, 168FARN and 66cl4, or given conditioned medium of the respective tumor cell lines. Unfortunately, no significant difference was seen in the migration of BMDMs in this experiment. This was the first time the migration assay was performed on BMDMs, and the method should be optimized before a conclusion is made.

However, a difference in the BMDMs appearance was seen after stimulation with conditioned medium from the five tumor cell lines, or after cultivation in transwell plates with the tumor cells. The cells appeared more differentiated after stimulation of conditioned medium from 67NR, 168FARN and 66cl4, while they are fewer, more round and undifferentiated after stimulation with conditioned medium from BMDMs, 4TO7 and 4T1. The same tendency is seen after the transwell experiment. This has been seen several times, also in a concentration dependent manner where diluted conditioned medium show reduced effects. However, these effects are not seen in RAW 264.7 macrophages. We know that there are differences in the expression level of cytokines and chemokines released from the tumor cells, which might be the reason behind the different morphology of the BMDMs.

The SMAD signaling is important for the differentiation or polarization of cells, and might be one of the reasons for different metastatic propensity of the tumor cells. However, the communication between tumor cells and macrophages is extensive and includes other pathways important for e.g. the inflammatory state in the tumors, which also might contribute to the aggressiveness of the tumors

4.6 CONDITIONED MEDIUM FROM 66CL4 CELLS UPREGULATES THE INFLAMMATORY SIGNALING IN RAW 264.7 MACROPHAGES

In this thesis the communication between tumor cells and macrophages have been focused on BMP4, GREM1 and their regulation of the SMAD signaling. However, BMP4 and GREM1 are not the only compounds that are secreted in this complex communication. As tumors often develop after a sustained inflammation in tissues, the expression of some inflammatory proteins was analyzed in the RAW 264.7 macrophages. It was seen that 66cl4 conditioned medium upregulated the phosphorylation of STAT1. STAT1 (Signal transducers and activators of transcription 1) is a member of the JAK-STAT pathway that mediates signals from interferons [125]. Many of the IFN-responsive genes are upregulated in 66cl4,

which might suggest that they secrete IFN. After IFNs bind to the receptors, Janus Kinases (JAKs) binds and gets phosphorylated, before STAT1 binds to the JAKs and gets phosphorylated. The phosphorylated STAT1s dissociate from the receptor, translocate to the nucleus and stimulate gene expression of inflammatory genes. However, STAT1 is also a potent activator of apoptosis and it influences the cellular homeostasis,

STAT1 is generally thought of as a tumor suppressor. It inhibits tumor cell growth by activating cell cycle regulators and apoptosis. It can also stimulate the innate and adaptive immune system to act against transformed cells and acts anti-angiogenic [126]. However, there is an increasing amount of evidence of tumor promoting functions of STAT1. STAT1 induces a immune-suppressed microenvironment, it supports the invasion of tumor cells, and it induces therapy resistance [126]. A study showed that high expression levels of STAT1 were correlated to the infiltration of tumor-associated macrophages [127]. Coccia et al. showed that STAT1 is important for the maturation of monocytes into macrophages [128]. In our study we observed that conditioned medium from 66cl4 upregulated the activation of STAT1 in Raw 264.7 macrophages. It might be that this influences TAMs in a way that favors tumor growth.

In the study of Coccia et al., the macrophages were stimulated with TGF-β, which seemed to inhibit the differentiation process of monocytes towards macrophages [127]. Since the number of macrophages in a tumor is correlated to poor prognosis, the inhibition of monocyte differentiation might be beneficial for patients. As BMP4 is a member of the TGF-β super family, it has many of the same properties. If BMP4 also negatively effects maturation of macrophages, simultaneous expression of higher amounts of GREM1 in the tumor cells might prevent disadvantageous effects of BMP4 on the macrophages preventing them from being polarized to M2 macrophages. BMP4 and GREM1 should therefore also be analyzed for any effect on macrophage polarization.

As GREM1 is highly expressed in the metastasizing 66cl4 cell lines, and that high expression of GREM1 correlates to poor prognosis in breast cancer patients, GREM1 could be a potential therapeutic target.

4.7 GREM1 AS A TARGET FOR THERAPY

The online database analysis done of GREM1 in both Kaplan-Meier plotter, as well as BreastMark, showed that high expression levels of GREM1 is correlated to poor prognosis in breast cancer patients. The drawback with these analyses is that it does not tell which cells that express GREM1. Based on the transcriptome data and initial western blot analysis it was found that 168FARN and 66cl4 cells produce GREM1. It was also found that RAW 264.7 macrophages do not express GREM1, while an experiment performed by another master student in the group indicated that fibroblasts produce a high amount of GREM1. However, it is not known if cancer-associated fibroblasts or other cells in the tumor microenvironment produce GREM1 in 66cl4 primary tumors.

On this basis, it would be interesting to knock out GREM1 in either the tumor cells or fibroblasts in mice to see if there is any difference in tumor progression. Another interesting possibility is to use neutralizing GREM1 antibodies. These could be given to patients with tumors with high GREM1 expression. If an anti-GREM1 therapy gets available in the future, the easiest way to diagnose these patients would be by a blood sample. Müller et al. saw that patients with coronary artery disease had an elevated level of GREM1 in the blood compared to healthy controls [123]. This was the reason why blood of the BALB/c mice that had developed tumors was tested. Positive results were not achieved, and there might be several reasons for that. A reason might be that in cardiovascular disease the inflammation is located on the inside of the blood vessels, which is not the case in tumors. Another issue might be that the ELISA was not specific enough, and should therefore be optimized. However, it might also simply suggest that this is not a good method of diagnosing cancer patients. A better method might be to take a biopsy of the tumors that can be used for analyzing mRNA levels of Grem1, or for immunohistochemical staining.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The complexity of the tumor microenvironment has been studied extensively, but much is still unknown. This thesis has focused on the communication between tumor cells and macrophages. By analyzing transcriptome data of primary tumors and cell lines of the non-metastasizing 67NR and the metastatic 66cl4 and performing western blot analysis, it was found that the aggressive tumor cells produce and secrete both BMP4 and its antagonist GREM1. Also the micrometastatic 168FARN cell line produces high amounts of GREM1. To analyze the clinical relevance of BMP4 and GREM1 mRNA expression in breast cancer patients, BreastMark and KM-plotter was used. It was found that high levels of GREM1 correlates to poor prognosis in breast cancer patients. Expression levels of BMP4 did not show a clinical relevance. Since the tumor cells produce and secrete GREM1 and BMP4, conditioned medium from tumor cells were given to macrophages. Conditioned medium from 168FARN, 66cl4 and 4T1 inhibited the SMAD pathway in RAW 264.7 macrophages. In addition, 66cl4 conditioned medium was able to upregulate the activation of STAT1. In bone marrow-derived macrophages (BMDMs), conditioned medium from 67NR, 168FARN and 66cl4, as well as the presence of the tumor cells in transwell changed their morphology. The presence of 67NR, 168FARN and 66cl4 cells also activated the SMAD pathway of the BMDMs, and upregulated the expression of M2 macrophage marker ARG1. Both conditioned medium from, and the presence of 67NR, 168FARN and 66cl4 seemed to attract BMDMs, however these last results will have to be reproduced in order to draw any firm conclusions.

To elucidate this further, it would be interesting to knock out GREM1 in tumor cells, inject them into the fat pad of BALB/c mice and look at the size of the primary tumor, the number of metastases in the lungs and the number/presence of tumor cells in lymph node and blood. By injecting GREM1 knock out tumor cells into the tail vain of BALB/c mice it could also be possible to assess the number of metastases/amount of tumor cells in lungs. It would also be interesting to inject wild type 66cl4 and 67NR tumor cells as well as GREM1 knock outs into BALB/c mice and nude mice (lacking T-cells) to see if there is a difference in primary tumor size, number of tumor cells in blood/lymph nodes or number of metastases in lungs, in the presence or absence of T-cells. From all these mice, TAMS could be isolated from their primary tumors, too see if their functions differ. The interaction between tumor cells and BMDMs should also be investigated more, by sequencing the RNA of BMDMs stimulated with conditioned medium from 67NR and 66cl4, or after cultivation in transwell with tumor cells to see which signaling pathways are changed

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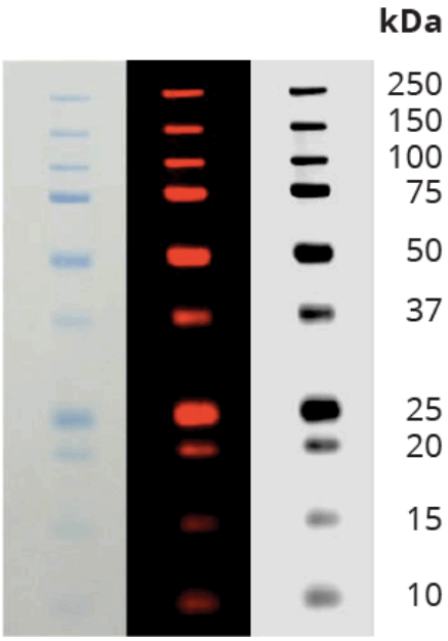
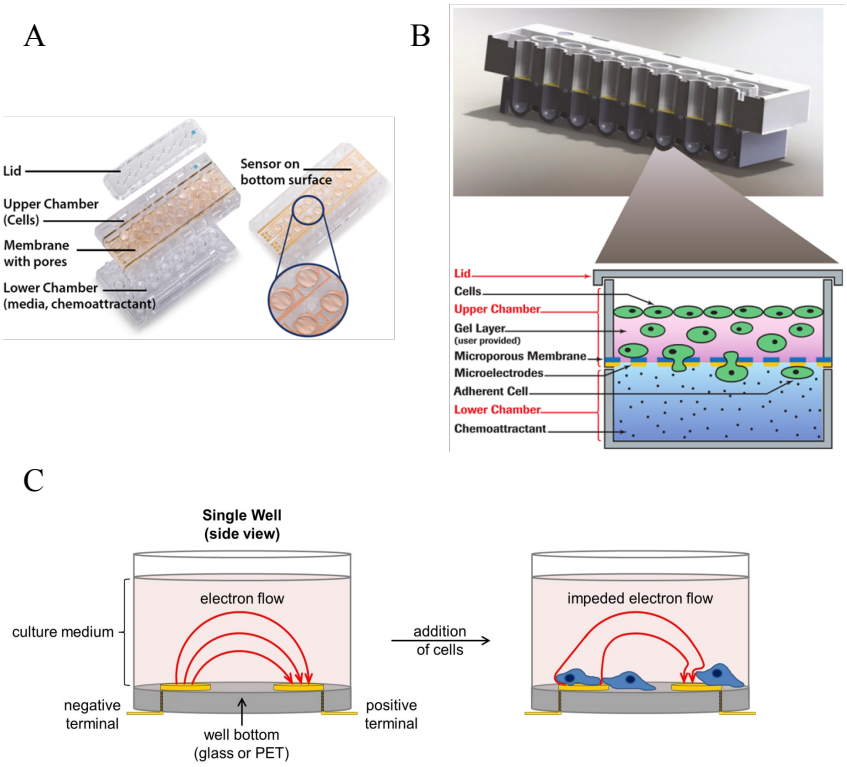
7 APPENDIX

7.1 APPENDIX 1



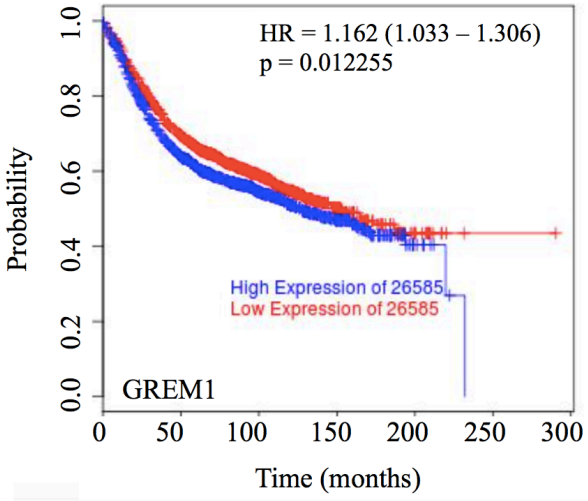
Representative image of Odyssey Protein Molecular Weight marker. Image generated using a digital camera (left) and Odyssey CLx Infrared Imaging System (center, right).

7.2 APPENDIX 2



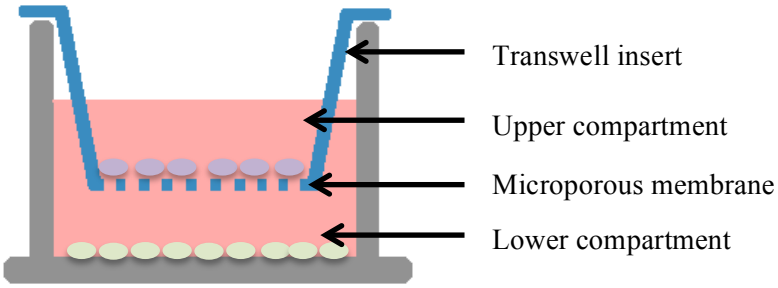
Schematic illustration of the xCELLigence system A and B. The CIM plate has two separable sections. Cells are seeded in the upper chamber and can move through the microporous membrane into the lower chamber that contains a chemoattractant. The cells adhere to the microelectrode sensors that will lead to an increase in impedance, which is measured in real time by the RTCA DP Instrument C. Side view of the upper chamber before and after cells are added, as an illustration on the impedance measurement. The bottom of the chamber is covered in gold microelectrodes which when submerged in solution and added current creates an electric potential across the electrodes. Then electrons will move from the negative terminal to the positive terminal. When cells adhere to the electrodes it will interfere with the electron flow and cause a change in impedance that is dependent on the number of cells that migrate.

7.3 APPENDIX 3



Survival analysis of GREM1 expression from the BreastMark database x-axis; timeline after study start. y-axis; estimated survival probabilities. HR; relationship between the hazards in the two groups. p= significance of the differences in the two groups with a confidence of 95%.

7.4 APPENDIX 4



Schematic illustration of the set-up of a transwell experiment Cells are seeded in both upper and lower compartment. The microporous membrane of the upper transwell allows signals to pass between the compartments, but do not allow cells to pass.