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Heterogeneity within the 66cl4 cell line from the 4T1 model of metastatic breast cancer

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ABSTRACT

Breast cancer is one of the most common form of cancer among women globally and metastasis is the primary cause of death in breast cancer patients. In the past decades it has been increasingly known that tumors are heterogenous masses of different cells that can be highly diverse. The study of tumor heterogeneity is important as it might how it might disturb therapeutic resistance as what might work for some cells in the tumor might have no effect on other cells. Tumor heterogeneity can be divided into two categories inter-tumoral heterogeneity and intra-tumoral heterogeneity. In addition, the cancer cells within the tumor may also acquire different phenotypes as the tumor grows and the cells are expanding from the first single fully transformed cell. Tumor cells are surrounded by the tumor microenvironment that comprise of many different cells and a variety of components that can promote tumor progression and metastasis. The 66cl4 cell line which is one of the cell line in the 4T1 breast cancer mouse model was used to obtain sub-clones and identify the heterogeneity of several genes that might play a key part metastasis of tumor cells.

We investigated the heterogeneity of cell line 66cl4 sub-clones and examine the sub-clone's phenotype. Markers from BMP4 and NFE2L2 signaling pathway as well as the 66cl4 sub-clones ability to proliferate and colony formation were analyzed. Immunoblots and qPCR showed that the sub-clones did not differ in their level of the BMP4 and pSMAD but did indicated sign of heterogeneity with respect to GREM1 protein and mRNA level. No correlation between GREM1 and CD24 level were observed. In addition, all the sub-clones had the same CUL3 mutation and NFE2L2 protein and mRNA level. NFE2L2 regulated genes, HMOX1 and NQO1, varied between the sub-clones at both protein and mRNA level. The phenotype of the 66cl4 sub-clones did not demonstrate differences with respect to proliferation rate and ability to form colonies in soft agar. However, a reduced ability to form colonies was observed in all the sub-clones when compared to the 66cl4 mother cell line.

Further research is however needed to see if the 66cl4 sub-clones are heterogenous based on the chosen markers analyzed in this thesis.

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ABBERVATION

ACTB	Beta-actin
AJCC	American Joint Committee on Cancer
ARE	Antioxidant response elements
BAMBI	BMP and Activin Membrane Bound Inhibitor
BMP	Bone morphogenetic protein
BMP4	Bone morphogenetic protein 4
BTB	Board complex/tramtrack/bric-a-brac
bZIP	Basic leucine zipper protein
CAF	Cancer associated fibroblasts
CAN	CERBERUS and DAN
CNC	cap ‘n’ collar subfamily
CRL	Cullin-RING-ligase
CSC	Cancer stem cell
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
GDF	Growth differentiation factor
GREM1	Gremlin 1
GST	Glutathione S-transferase
HER2	Human epidermal growth factor-2 receptor
HMOX1	Heme oxygenase 1
I-Smads	Smads inhibitor
KEAP1	Kelch-like ECH-associated protein1
KLHL	Kelch like protein
NFE2L2	Nuclear factor erythroid 2 like 2
NQO1	NAD(P)H:quinone oxidoreductase 1
pSMAD1/5/9	Phosphorylated SMAD1/5/9
PR	Progesterone receptor
PTI	Protein transport inhibitor
rBMP4	Recombinant BMP4
rGREM1	Recombinant GREM1
RNA	Ribonucleic acid

ROS	Reactive oxygen species
SMAD 1/5/8	SMAD1, SMAD5 and SMAD8
TGF-β	Transforming growth factor-β
TME	Tumor microenvironment
TMN	Tumor, Node and Metastasis system
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor-2

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

There were reported 14.1 million new cases of cancer worldwide in 2012, where breast cancer stood for 1.67 million of all cancer incidents [1]. Breast cancer is one of the most common form of cancer among women globally. Furthermore, in developed areas it is the second most common cause of cancer death in women, whereas it is the fifth most common causes of death from cancer overall in less developed areas. Due to better treatment strategies and early detection of breast cancer better survival rate in developed countries like eastern Asia compared to western Africa can be seen [1].

In 2016 a total of 3 636 out of 32 827 new breast cancer incidences were reported among women in Norway, and the occurrence has doubled since 1957 [2]. The incidence rate from five-year survival in 2012-2016 (89.7%) is gradually increasing compared to 1977-1981 (72.5%), and about nine out of ten women survive. In Norway women between 50-69 years old are offered to take part in a screening program every other year. This screening program is one of the most reliable methods to discover breast cancer early. In addition, by taking part in this program patients can receive treatment rapidly after the discovery which gives a higher success rate. Further, by knowing some of the common signs and symptoms of breast cancer, women can themselves also contribute to early discovery of the cancer. One of the most common symptom are a new lump or mass on the breast and it is often hard or has irregular edges. Other signs and symptoms include swelling or thickening of the breast, nipple retraction, irritations, redness or pain on the nipple or part of the breast. Although these symptoms are common in breast cancer, they can also occur by other reasons than breast cancer [2, 3].

Mutations in the tumor suppressor genes such as *BRCA1* and *BRCA2* can be inherited and greatly increase the risk of breast cancer [2, 3]. Some lifestyle-related factors might increase the risk of getting breast cancer. That includes alcohol consumption, diet, being obese or overweight and little to no physical activity. On the other hand, having children at an early age and breastfeeding might prevent and decrease the risk of getting breast cancer

1.1.2 Breast cancer classification

There are different methods used to classify breast cancer. By combining these methods, it can give each patient a better prognosis and the most suitable treatment. The most common classifications of breast cancer can be divided into four major molecular subtypes. This include luminal A, luminal B, human epidermal growth factor receptor 2 (HER2+)-enriched and basal like also called the triple negative type [4-7]. The molecular subtypes classification is based on whether the subtypes are hormone receptor positive or negative with regards to estrogen-receptor (ER), progesterone-receptor (PR) and HER2. Luminal A express both ER and PR but not HER2 while luminal B express all three receptors. HER2+-enriched express only HER2 and the triple negative type does not express any of the receptors [4-6]. ER is expressed in approximately 70% of the invasive breast cancer. The majority of cancer in subgroups luminal A and luminal B also express PR. Luminal A and luminal B are the most common types of breast cancer and give a good prognosis between 80% - 85% of five years survival rate. The major differences between the two subgroups are their expression status of proliferation-associated genes. In comparison to luminal A and luminal B, HER2+-enriched and triple negative type are lesser common. Furthermore, they are difficult to treat and are in general considered to be associated with poorer prognosis between 50% - 60% of five yeas survival rate [4-6]. The receptors ER and HER2 are used as treatment strategy for patients and these receptors are also one of the main targets for developing drugs. However, the developed drugs are ineffective for the triple negative breast cancer type as they do not express the receptors and might be one of the reasons why this subtype is difficult to treat.

Grading is another method used to classify the tumors in breast cancer patients. It is based on separation of the tumors into different grades depending on morphologic characteristics such as how rapidly the tumor spread and by comparing tumors to normal cells [7]. The grading system ranges from grade 1 to 3 where grade 1 gives the best prognosis and grade 3 gives the poorest prognosis. Grade 1 means that the cancer has a low possibility of spreading, the cancer cells look similar to normal cells and are dividing slowly. Grade 2, the cancer cells are moderately differentiated, and the cancer cells phenotype is between grade 1 and 3. Grade 3 reflects the highest possibility of cancer cells spreading, the tumor is growing fast, and the cancer cells looks abnormal.

Another method used to categorize cancer is the staging system. The most frequently utilized system is the American Joint Committee on Cancer (AJCC) Tumor, Node and Metastasis (TNM) system [7]. This system is based on both pathological stage and clinical stage that includes the size of the tumor, spreading to nearby lymph node or distant sites, different

hormone receptor expression and grading of the tumor. After this is determined, each individual will be divided into different stages. Patients that are categorized in the same stage tends to be given similar treatment. The stages are ranged from 1 to 4 where lower number equals better prognosis.

1.1.3 Tumor heterogeneity in breast cancer

Previously it was thought that cells within a tumor have similar genomic and phenotypic characteristics to each other at any given stage of cancer. However, in the past decades it has been increasingly known that tumors are heterogenous masses of different cells that can be highly diverse. Tumor heterogeneity can be divided into two forms; intertumoral heterogeneity or intratumoral heterogeneity (figure 1) [8, 9]. Intertumoral heterogeneity is the variation between patients with tumors that histologically have the same ancestry cells of the tumor. They differ in expression of molecular subtypes (figure 1A) [8, 9]. The genetic aberrations cancer cells acquire can be the consequence of internal- or external factors that leads to mutations. The mutations might result in oncogenes or tumor suppressor genes getting either a gain- or loss-of-function [10]. Together these mutations lead to variations seen between patients.

Intratumoral heterogeneity is the variation between primary and metastatic sites within the same patient (figure 1B) as well as the sub-clonal diversity that occurs in different sections of the same tumor where the cells within the tumor have different phenotype [8, 9]. The sub-clones can either be intermingled or spatially separated where they show different morphology and staining behavior (figure 1C). The cause of intratumoral heterogeneity is mainly due to genetic alterations such as somatic mutations, chromosomal imbalance and epigenetic heterogeneity. Some of the hallmarks suggested by Hanahan and Weinberg can explain how cancer cells obtain these properties [10].

There are several possible models proposed to explain how intratumoral heterogeneity occurs and this includes clonal evolution model, cancer stem cell model and mutator phenotype model [11]. The clonal evolution model suggests that the total tumor mass that is formed comes from monoclonal or polyclonal subpopulation proliferation and expansion. All the clones have the potential of continuous proliferation and selection. Contrary to this is the cancer stem cell model which suggest that precursor cells give rise to different subpopulations within the same tumor [11]. The mutator phenotype model suggest an enormous degree of diversity in the tumor mass rather than sub-clonal populations. The diversity is caused by gradual and random accumulation of mutations in the tumor as it evolves [11].

A study in breast cancer utilizing 4T1 mouse model of breast tumor has recently demonstrated the heterogeneity of different sub-clones. When co-implanting the same amount of two 4T1 sub-clones they displayed differences in their metastatic potential [12].

Tumor heterogeneity can be one among many challenges that arise when finding the most suitable treatment for patients. For instance, when performing a biopsy only a small part of the tumor is taken as sample and analyzed. This might cause a loss of relevant lesions that could be of therapeutic importance [13]. However, by understanding the cause and characterization of tumor heterogeneity it may lead to better knowledge and progression of therapeutic therapy, hence giving a better possibility to overcome the challenges of personalized cancer treatment [9].

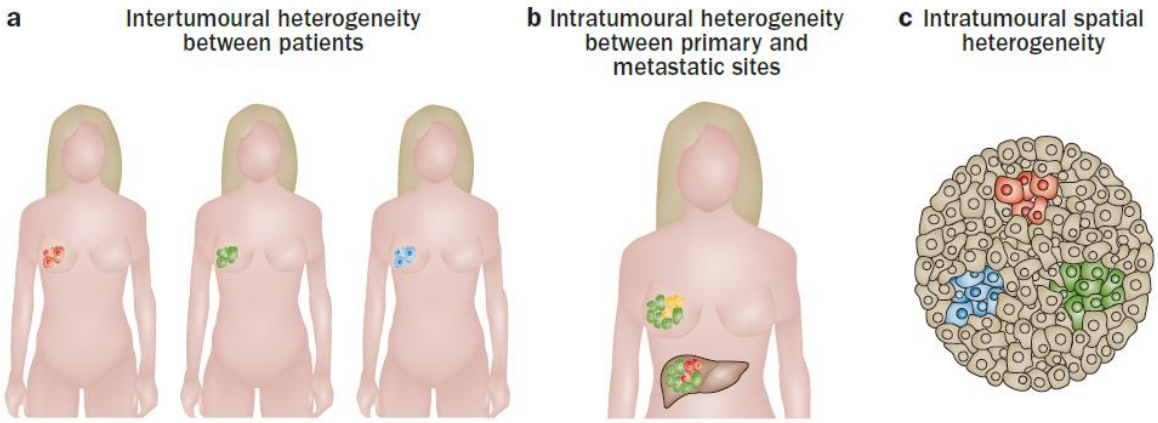


Figure 1: Tumor heterogeneity. A. Intertumoral heterogeneity between patients with histologically have the same tumor but may differ in their genetic properties. B. Intratumoral heterogeneity seen between the primary site and metastatic site due to a combination of different genetic mutations. C. Intratumoral spatial heterogeneity due to different subpopulations present in different sections of the tumor. Adapted from [9]

1.1.4 Tumor microenvironment

Tumor cells have the ability to disturb the normal homeostasis in a healthy tissue in favor of their proliferation, survival and migration [14, 15] . These changes can be influenced among other factors by the tumor microenvironment (TME). Tumor cells can be found in the TME as well as other components such as extracellular matrix (ECM), blood vessel cells, inflammatory cells, cancer-associated fibroblasts (CAF) and immune cells (figure 2). These cells secret chemokines, cytokines and growth factors that are important for the tumor growth and survival [14, 15].

In normal healthy tissue fibroblasts typically suppress tumor formation. However, during tumorigenesis fibroblast are activated and obtain a modified phenotype that gives them a unique cancer-promoting property [16-18]. The activated fibroblasts are often called CAFs [19]. CAFs have abilities to remodel the ECM and secrete cytokines as well as stimulate angiogenesis. A recent study demonstrates CAFs role in enhancing tumorigenesis in prostate cancer [20]. Similarly, Orimo et al. demonstrated that in human breast carcinoma CAFs turned out to be more competent than normal fibroblasts in promoting growth of cancer cells and angiogenesis partly through secretion of cytokine stromal-derived factor1 [21]. Thus, the bipolar role of fibroblast in cancer depends on the differentiation state of fibroblasts and how far the tumor has developed.

In addition to CAFs, immune cells also promote tumor cells development [22]. The immune system comprises of cells such as macrophages, natural killer cells, T-cells, B-cells and dendritic cells. Some of the immune system's function is to monitor and protecting cells against invading and infectious microbes as well as eliminate damaged cells. The immune cells can produce proinflammatory cytokines and chemokines which recruits and attracts more immune cell to the inflammatory site. Together the cells work to eliminate cell damage. The same process will also happen to cancer cells as the immune system will recognize it as foreign and try to eradicate the cancerous cells. Cancer cells must escape this immune detection and if they succeed they produce compounds that will affect immune cells. Chemokines, matrix-degrading enzymes and angiogenic growth factors that immune cells secrete will contribute to support the development and invasion of tumors. The immune cells contribution to cancer is often seen in cancers associated with chronic inflammation where the immune cells, particularly cells from the myeloid lineage have the ability to produce these compounds [18, 19, 22].

Furthermore, the vascular network is crucial for tumorigeneses as it can restrict the tumor growth by limiting oxygen and nutrients supply. Vessel formation involves modification of existing vessels and formation of new vessels which may also form an escape route for tumor cells at the primary site [23]. Here, endothelial cells and pericytes growth and migration are important components. Pericytes support endothelial cells and provide survival signals in the process of vasculature maturation. Another important factor in the spread of metastasis is lymph vessel recruitment as lymphangiogenic can create another escape route for cancer cells in addition to blood vessels [23-25].

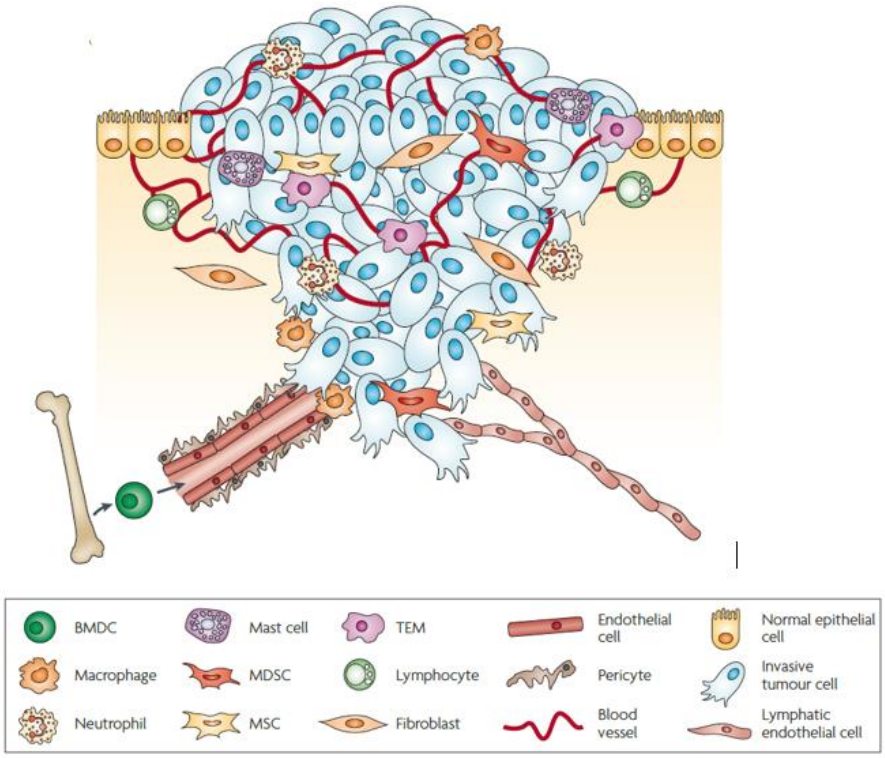


Figure 2. Tumor microenvironment. Tumor cells and other cells can be found in the tumor microenvironment which support the development of tumor. These cells include fibroblasts, immune cells, bone-marrow derived cells and cells from the lymphatic and angiogenic cells. Adapted from [14]

1.1.5 Metastasis

Metastasis is the primary cause of death in breast cancer patients worldwide. It is characterized by the spread of malignant tumor cells from its initial site as a primary tumor to other distant organs. Metastases can occur years later after the first clinical diagnosis and if the tumor is discovered after it has metastasized the clinical outcome is much poorer [24, 25]. Some type of cancer will metastasize to specific organs showing a repeated pattern which leads to the introduction of the "seed-and-soil" theory. This theory proposed that the seed (the cancer cells) are dependent of the soil (the secondary organ) which results in organ specific metastases [26]. This is also observed in breast cancer patients who are in their last stage of breast cancer. These patients have metastases seen in specific tissues such as bone, brain, draining lymph node, liver and lung [25].

The development of a secondary tumor is formed through a metastatic cascade. The primary tumor ability to create angiogenesis is an important initiation step in the metastatic cascade which is also one of the hallmarks of cancer suggested by Hanahan and Weinberg [10]. Angiogenesis is one of six essential physiological changes in cells that regulates malignant

growth [10]. The tumor cells must detach from the primary site, penetrate and survive in the ECM and blood vessels (intravasation), migrate, extravasate the blood vessel and invade adjacent tissue all this done while escaping detection of immune cells [25, 27]. The last step in the cascade is for the cells to settle down in the secondary site, create a new microenvironment and start proliferating.

It is known that metastasis is an inefficient process as a large amount of cancer cells can be detected in a cancer patients blood, but only a few of these cells can survive and grow in the new site [28]. The steps in the metastatic process is rate-limited as it was thought that most of the cancer cells that migrated into the circulation were quickly destroyed by immune cells and hemodynamic forces [29, 30]. Later it was suggested the early stages in hematogenous metastasis is completed quite efficiently but were very inefficient in the growth phase. This indicate that the tumor growth after extravasation is the key step in the metastatic cascade [28].

1.2 TRANSFORMING GROWTH FACTOR-β SUPERFAMILY

1.2.1 TGF-β superfamily

The transforming growth factor-β (TGF-β) superfamily includes more than 30 cytokines that are secreted as homodimers and heterodimers and capable of binding specific receptors on target cells [31]. TGF-β superfamily can be divided into several subfamilies and some of them are TGF-β subfamily, activin/inhibin subfamily, bone morphogenetic protein (BMP) subfamily, growth differentiation factor (GDF) subfamily and Nodal proteins subfamily. These cytokines have a key role in the regulation of early vertebrate development and biologic processes. This includes cell proliferation and differentiation, maintenance of tissue homeostasis, regulation of immune responses and wound healing [31]. TGFβ superfamily can also affect tumor development and metastasis, due to a redeployment in early development or a perturbation of their action in tissue homeostasis [32]. A disturbance in their signaling can also lead to other pathologies such as vascular - and immune related diseases, skeletal disease and fibrosis [32].

1.2.2 Bone morphogenetic protein subfamily

The BMP subfamily belong to the TGFβ superfamily and consist of approximately 22 members. This subfamily was first discovered in the 1960s by its role in bone and cartilage formation [33, 34]. Later it was discovered that BMPs could also promote differentiation and limit self-renewal and determine cell fate in stem cells [35]. They are involved in biological processes such as kidney and liver metabolism and formation of muscles tissues [35-37]. It is also shown that diseases including skeletal- and vascular diseases as well as cancer can be

consequences of impairment and/or abnormalities of BMP regulation [38-41]. Overexpression of BMPs in prostate cancer has shown to increase tumorigenesis whereas in gastric cancer concentration of BMPs were low [39]. In addition, experimental studies of breast cancer demonstrated enhanced tumor cell proliferation and invasion abilities when BMP ligands and receptors were upregulated [42, 43]. Bone morphogenetic protein 4 (BMP4) is a member of the BMP subfamily that has shown to be important in many cancers including breast cancer.

1.2.3 Bone morphogenetic protein 4 and signal transduction

Like other members of the BMP subfamily BMP4 play a dual role in both being tumor suppressor and tumor promoter. BMP4 has a key role in many aspects of embryonic development such as teeth formation, cartilage and bone formation and is broadly expressed in embryos and adult tissues [34, 44-46]. A loss of function of the BMP4 can have a severe effect and lead to early embryonic lethality [47] . It is also suggested that depending on the secreted BMP4 concentration, it may lead to different biological responses. A high expression of BMP4 results in ventral fate while low expression leads to dorsal neural and muscular tissue formation [48]. BMP4 has also been linked to increased migration and invasion in gastric, melanoma, colorectal and ovarian cancer cells and is important in a many other cancers [49-51]. In breast cancer BMP4 has been found to regulate processes like proliferation, migration, invasion, apoptosis and epithelial-to-mesenchymal transition (EMT) [52]. An experimental study showed that highly metastatic cell line had an overexpression of BMP4 compared to cell lines that had little invasive properties. The overexpression of BMP4 was found to increase migration and invasion and decrease proliferation of cancer cells [52]. Another study of the BMP subfamily showed that BMP4 and BMP7 was found to be the most frequent expressed BMP and having the highest expression level in breast cancer [42].

BMP4 signaling is initiated when BMP4 is secreted to the ECM (figure3). Activated BMP4 form mature homodimers that transmits signals through transmembrane BMP receptor I and II [53, 54]. When BMP receptors is activated, an intracellular signal phosphorylate and activate Smad1, Smad5 and Smad8 (Smad1/5/8) proteins. Smad1/5/8 together forms a heteromeric complex with Smad4 and translocate to the nucleus. This results in nuclear accumulation of the complex and binding to sequence-specific DNA which leads to activation of targeted BMP4 genes [55, 56].

The regulation of BMP4 signaling pathway depends on Smad4, but Smad1/5/8 can also regulate the signaling pathway [56]. Furthermore, BMP4 antagonists inhibit signaling both on the plasma membrane and in the cytosol of the cell. In the extracellular space secreted

antagonists such as Gremlin1 (GREM1) and Noggin proteins are known to bind specific BMP4 and prevent the initiation step in the signaling pathway [55]. On the plasma membrane BMP and Activin Membrane Bound Inhibitor (BAMBI) competing with BMP receptor I to bind to BMP receptor II [53, 54]. Smads inhibitory (I-Smads) proteins, Smad6 and Smad7 are also known to regulate the BMP4 signaling pathways [52, 53]. The BMP4 signaling pathway is therefore not only depend on BMP4 but also on the concentration and activity of their antagonists [32, 53].

Genetic or epigenetic changes that leads to overexpression of BMP4 inhibitors, loss of BMP receptors or ligands and a loss of Smad4 are some of the reasons that can cause downregulation of BMP4 signaling. While low or suppressed BMP4 signaling can contribute to tumor formation, high BMP4 signaling can prevent tumor progression and metastasis by providing a natural barrier to EMT [32]. In addition, it can prevent the colonization of tumor cells by enforcing a dormant state thus blocking tumor invasion [32]. EMT is a cell-biological event and are important in tissue remodeling, wound healing, fibroses and tumor progression. Cancer cells can disrupt the epithelial homeostasis that can leads to carcinogenesis by obtaining mesenchymal traits important in invasion and extravasation or by facilitating epithelial traits that gives the cells ability to colonize and form macroscopic metastasis [57, 58].

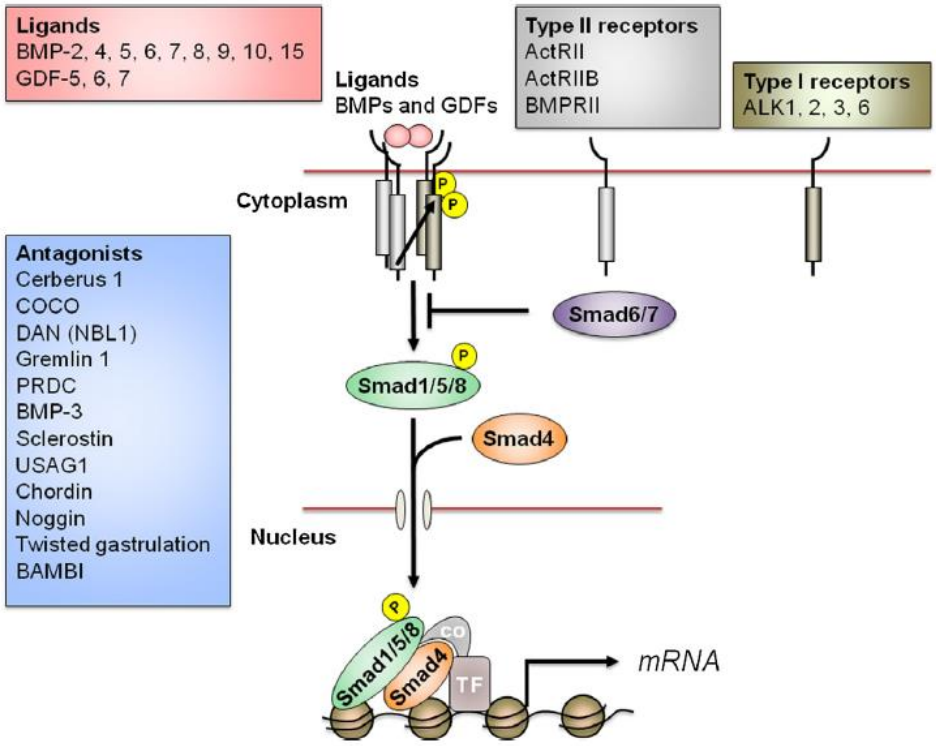


Figure 3. BMP4 signaling pathway. When secreted BMP4 binds to its receptor an intracellular signal is transmitted leading to phosphorylation and activation of Smad1/5/8. Activated Smad1/5/8 forms a complex with Smad4 and together are translocated to the nucleus. This complex and acts as a transcription factor and activate transcription of targeted genes. Inhibitors such as Grem1, BAMBI and Smad6/7 can prevent different steps in this signaling pathway. Adapted from [39]

1.2.5 Gremlin1

GREM1 is one among many BMPs antagonists that are important for controlling the BMP signaling pathway. GREM1 was originally discovered as a protein capable of inducing secondary axis in *Xenopus* embryos and is localized on region 15q13-q15 in the human chromosome [59, 60]. GREM1 is shown to be important during limb outgrowth and pattering, kidney and lung development [61, 62]. During the organogenetic processes GREM1 acts as an inhibitor and regulate BMPs action in a signaling feedback loop. The importance of GREM1 has been shown in knockout studies of GREM1 (GREM1-/-) in mice where GREM1 was associated with abnormal skeletal pattern, developmental abnormalities and neonatal lethality [61, 62].

GREM1 belongs to the CAN (CERBERUS and DAN) subfamily of BMP antagonists and it contains a cysteine rich region and an eight-membered ring with a C-terminal cysteine knot motif that is structurally similar to many members of TGFβ superfamily [63, 64]. GREM1 acts as an antagonist of BMP2, BMP4 and BMP7 by preventing their activation and signaling [55].

In addition, GREM1 bind BMP4 intracellularly by interacting with BMP4 precursor protein. This will prevent the production and secretion of mature BMP and thus downregulating BMP signaling intracellularly [48]. GREM1 is also capable of signaling independently of its role as a BMP antagonist (figure 4). Vascular endothelial growth factor (VEGF) and GREM1 are proangiogenic factors that belong to the same cysteine-knot family, suggesting that they may have similar functions and structures. This has been shown in a study where GREM1 interacted with vascular endothelial growth factor receptor-2 (VEGFR2) function as an agonist and initiate VEGF-mediated angiogenic response both *in vivo* and *in vitro* [65]. It has also been demonstrated in a study by Kim et al. that human lung adenocarcinoma cells (A549 cells) with transfected GREM1 could interact with cancer cells in a BMP and VEGFR2 independent manner [66]. This lead to an increase in cancer cells migration, invasion and proliferation [66]. Furthermore, GREM1 can also inhibit monocyte chemotaxis by binding to secreted Slit proteins [67]. Thus, suggesting GREM1 signaling capabilities are independent of BMP signaling and might suggest a more complex role in cancer process.

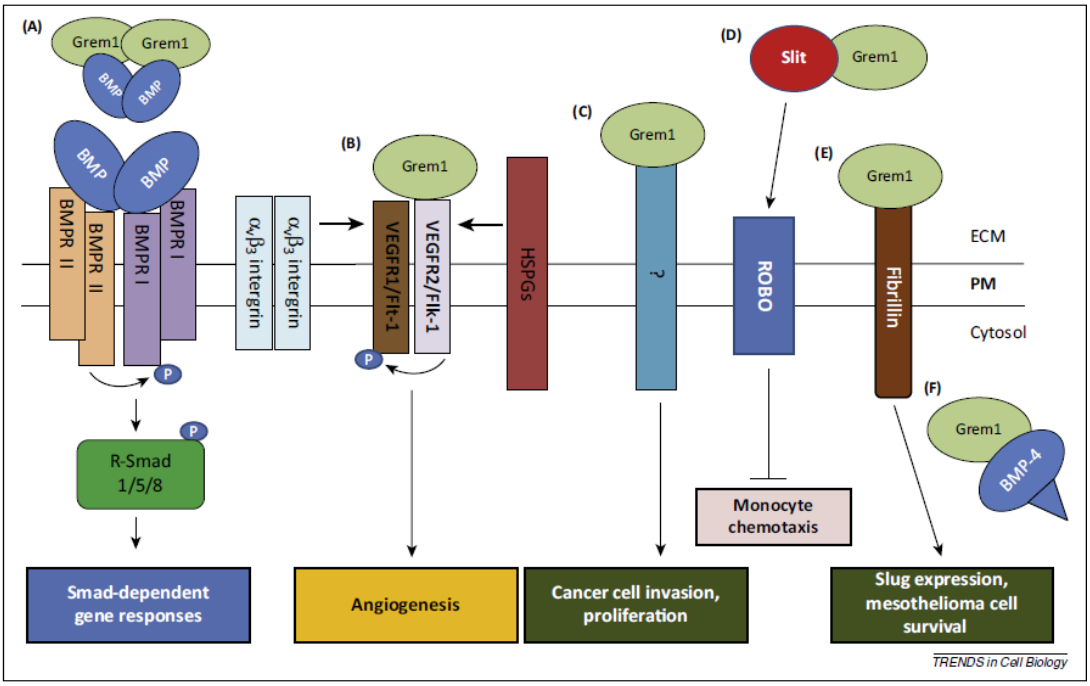


Figure 4. GREM1 signaling mechanism. **A)** GREM1 dimers binding to BMP dimers and preventing it from binding to receptors and inhibiting BMP signaling. **B)** GREM1 promotes angiogenesis by binding to VEGFR2 in endothelial cells. **C)** Using an unidentified mechanism GREM1 can promote invasion and proliferation in cancer cells. **D)** GREM1 can bind to slit1/2 and inhibit monocyte chemotaxis. **E)** GREM1 associates with fibrillin microfibrils leading to expression of slug which results in EMT and survival of mesothelioma cell. **F)** GREM1 binding to precursor protein BMP4 in the cytosol and preventing secretion of mature BMP4. Adapted from [55]

Overexpression of GREM1 has shown to be linked with oncogenic roles in several human cancer such as kidney, colon, breast, lung, cervix and sarcomas in comparison to normal tissue samples [68]. An upregulation of GREM1 has also proven to enhanced cancer cells survival and expansion in cancer associated stroma cells of different carcinomas [69]. The role of GREM1 in carcinogenesis is still largely unknown, nevertheless it is significant in regulation of BMPs.

1.2.6 BMP and GREM1 potential role in cancer stem cell differentiation

The role of cancer stem cells in carcinogenesis has been debated for several years. A possible hypothesis is that a small sub-clonal population of cells called cancer stem cells (CSC) have characteristics that are similar to normal stem cells [70, 71]. CSC have the properties to initiate, drive tumorigenesis and can contributes to tumor heterogeneity caused by cell differentiation [70, 71]. In addition, CSC display resistant to chemotherapies and radiotherapies [72]. The involvement of BMPs in embryonic and adult development by promoting differentiation and limit stem cells self-renewal, it is speculated that BMPs might also have similar role in CSC. BMPs are highly expressed by glioblastomas and studies showed that BMPs could induce differentiation of CSC [73, 74]. Later it was discovered that CSC were able to escape the BMP-induced differentiation due to the high expression of GREM1 that inhibited the signaling pathway. Thus, preventing differentiation of CSC and loss of CSC within the tumor. Cells that had high GREM1 expression had enhanced tumor growth. In this way GREM1 might contribute to CSC proliferation. A decrease in growth and self-renewal of CSC was seen by targeting GREM1 [75]. The identification of CSC markers is important as it might provide a better understanding of CSCs role in tumorigenesis.

1.3 CD24 CANCER STEM CELL MARKER

CD24 is an anchored cell surface glycoprotein and is a ligand for p-selectin that can be found on platelets or endothelial cells. Their interaction enhances tumorigeneses by facilitating passage of tumor cells in the circulation during metastasis [76, 77]. Within the same tumor the cells can show a functional heterogeneity, for instance only a limited proportion of cancer cells have self-renewal and tumor-initiating abilities showing differences in malignancy [78]. A study in breast cancer showed that these cells have a specific phenotype, CD44+/CD24^{low}Linage- [78]. Thus, CD24 was suggested as a marker to distinguish between CSC and nontumorigenic cells [78]. Following this, another study showed that a subpopulation of cells that is enriched for CSC properties, defined by CD24 expression, had high tumorigenic

properties and the CSC was highly metastatic [79]. In vivo CD24 cancer cells could differentiate and thus, creating intra-tumor heterogeneity [79]. Also, high level of CD24 in breast cancer is correlated to poor prognosis [80]. In ovarian cancer a study showed that the CD24+ subpopulation was more resistant to chemotherapy compared to CD24- subpopulation [81].

1.4 OXIDATIVE STRESS

Reactive oxygen species (ROS) are chemical species that contains oxygen and is naturally produced by cells under normal or exposure to toxic conditions. Overproduction of ROS where the cell cannot compensate for the detoxification of the reactive intermediates can cause disturbance and lead to cellular damage. The consequence is DNA damage which is an important cause for mutations and can give rise to tumor growth. By understanding some of the cellular mechanism that leads to ROS it can give an insight to better therapeutic targets.

1.3.1 Nuclear factor erythroid 2 like 2 and signaling pathway

Reactive oxygen and nitrogen species can lead to oxidative stress that impairs normal cellular function and may contribute to the development of cancer, chronic diseases and toxicity. Oxidative stress can activate many transcription factors and one of them is Nuclear factor erythroid 2 like 2 (NFE2L2). NFE2L2 was originally identified to bind to the NF-E2/AP1 motif in the beta-goblin protein [82]. It is localized in region 2q31 on the human chromosome and is a member of cap ‘n’ collar (CNC) subfamily of basic leucine zipper protein (bZIP) transcription factors [82, 83]. NFE2L2 has an important part in xenobiotic response and disease processes, predominantly processes involving electrophilic and oxidative stress [84]. NFE2L2 can therefore directly affect the homeostasis of reactive oxidative- and electrophilic stress by regulating phase II detoxifying enzymes that carries an antioxidant response elements (ARE) in their genes. Such enzymes includes glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) and antioxidative proteins such as heme oxygenase 1 (HMOX1) [84].

One of the largest classes of ubiquitine E3 ligase is the Cullin-RING-ligase (CRL). CRL is constructed around a Cullin family scaffold protein, and each Cullin domain bind substrate-specific adaptors. Cullin-3-based CRLs binds specific to the board complex/tramtrack/bric-a-brac (BTB). Kelch like (KLHL) protein family represent the largest group of BTB, and the most well-known member of this family is Kelch-like ECH-associated protein1 (KEAP1) [84, 85]. Normally NFE2L2 is kept in the cytoplasm where it is rapidly tagged for ubiquitination and proteasome degradation by KEAP1-CUL3-E3 complex. However, upon oxidative- or

electrophilic stress the complex is destabilized and allows the cells to release NFE2L2 [84, 86]. NFE2L2 then accumulate in the cytoplasm and translocate to the nucleus where it binds to ARE upstream to the promoter region. The transcription of NFE2L2 cellular defense genes, such as NQO1 or HMOX1 will be activated (figure 5). Thus, KEAP1-CUL3-E3 complex can regulate the oxidative stress response by regulating NFE2L2 transcription factor and by this contribute to defense against several pathologies.

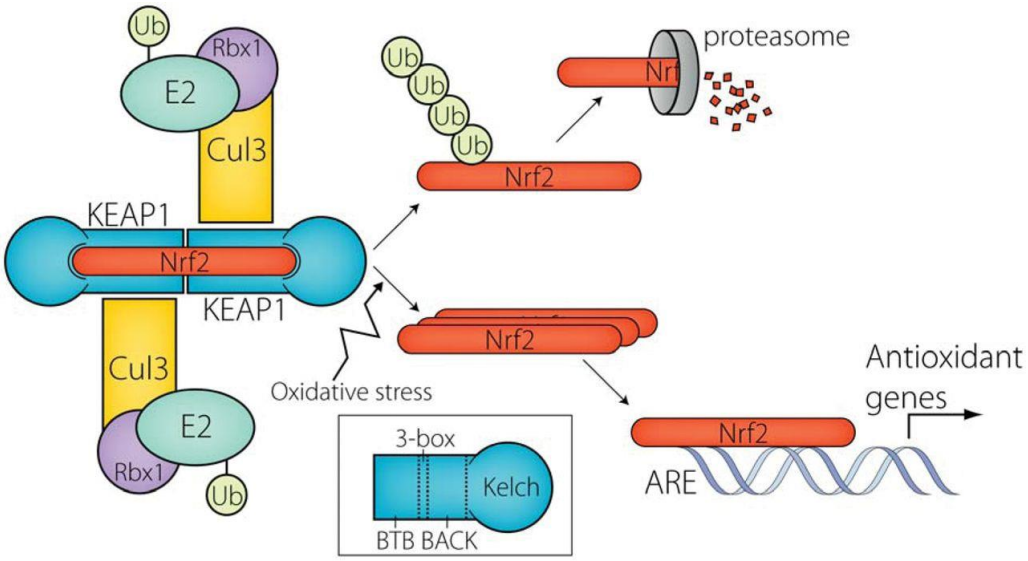


Figure 5 NFE2L2 signaling pathway. KEAP1-CUL3-E3 complex regulates the oxidative stress response by regulating NFE2L2 (also known as Nrf2) concentration in the cells. NFE2L2 is targeted for degradation rapidly but during cellular stress the KEAP1 complex is destabilized leading to accumulation of NFE2L2. NFE2L2 will translocate to the nucleus and bind to ARE and activate targeted cellular defense genes. Adapted from [85]

A lack of NFE2L2 in mice showed a markedly decrease of induction and expression of GST and NQO1 thus establishing NFE2L2 role in regulating ARE-dependent phase II enzymes [87, 88]. Similarly, the important role of NFE2L2 has also been shown where a lack of NFE2L2 increases chemical induced carcinogenic in cells and it is associated with conditions with oxidative pathology [89-92]. The temporary activation of NFE2L2 showed tumor suppression abilities from toxins and carcinogens. However, a continued activation of NFE2L2 resulted in transactivation of its downstream genes. This contributed to provide a potential better environment for the cancer cells to proliferate [93, 94]. An upregulation of NFE2L2 can also be seen by reduced expression or loss of function of Keap1 which is considered a "gatekeeper" of antioxidant defense genes by regulating NFE2L2 [95-97]. The effect of NFE2L2 on its targeted genes such as NQO1 and HMOX1 may contribute for therapeutic drugs development.

1.3.2 NAD(P)H: quinone oxidoreductase

NAD(P)H: quinone oxidoreductase 1 (NQO1) was first discovered by Ernster and Navazio in 1958 and was later revealed to be a ubiquitous cytosolic flavoenzyme that catalyzes the reduction of quinone substrates [98]. NQO1 reduces quinones to hydroquinones in one single step by using NADH or NADPH as substrates. Thus, preventing the one electron quinones which result in production of cell damaging radical species. NQO1 gene contain an ARE promotor that is regulated by NFE2L2 transcription factor, both in normal condition and during oxidative stress conditions [99-101]. Furthermore, it has recently been discovered that NQO1 might play a part in preventing cancer progression regardless of its enzymatic activities by binding to tumor suppressor p53 thus preventing p53 from being targeted for proteasome degradation [102, 103]. This is especially important under conditions of cellular stress when expression of NQO1 is elevated.

Although NQO1 has various roles in protecting from cancer it is also found that a high level of NQO1 can lead to tumor development. Upregulation of NQO1 is found in several cancers such as breast, colon, pancreatic, lung and melanoma [104-106]. In breast and cervical cancer overexpression of NQO1 shows poor differentiation and enhance the ability of the tumor to metastasize to the lymph node. This was associated to a lower disease-free survival and five-year overall survival rate [107, 108]. In response to cell damage such as antioxidant, ionizing radiation, heat shock, hypoxia and electrophilic attack NQO1 together with other detoxifying enzyme genes, such as HMOX1 and GST, will be activated [87] .

1.3.3 Heme oxygenase 1

Heme oxygenase (HMOX) enzymatic properties was first described in 1968 by Tenhuen et al. and was shown to be a rate-limited enzyme that catabolizes heme into three biological active products. These products are carbon monoxide (CO), biliverdin and free iron (Fe^{2+}) [109, 110]. Heme oxygenase exists in three isoforms HMOX1, HMOX2 and HMOX3 [111]. Upon induction of cellular stress agents such as ultraviolet irradiation, endotoxin and hypoxia HMOX1 will be activated. The common feature of these agents is that they can generate ROS [112-114]. When this happens HMOX1 are elevated and will protect cells from inflammatory damage, inhibit cell apoptosis, reducing oxidative injury and regulate cell proliferation. The exact mechanism of this is unclear, but it is thought to be through one or more of its byproducts [115-118].

The significant role of HMOX1 has been demonstrated in studies using mice with HMOX1 null mutation (HMOX1 -/-). These studies showed that the majority of the animals did not

survive to term and those who survived did not live beyond one year of birth [119, 120]. Mice that survived exhibit conditions such as growth retardation and microcytic anemia showed evidence of iron deposition and chronic inflammation. In addition, when induced by endotoxin the cells from these mice was more susceptible to oxidative stress [119, 120]. Similar results were seen in a patient with HMOX1 deficiency. The patient exhibited comparable phenotypic alteration as those observed in the experimental mice [121]. These observations support the key role of HMOX1 in iron homeostasis and oxidation stress response. Although HMOX1 have cytoprotective role it can also promote tumor formation. HMOX1 is upregulated in many cancers and it is indicated that it might promote cancer cells proliferation and invasion [122-125]. The exact effect of HMOX1 in cancer progression can be tissue specific but overall HMOX1 is thought to protect cancer cells from apoptosis induced by chemotoxic drugs [126]

1.5 BALB/cfC3H 4T1 BREAST CANCER MOUSE MODEL

The BALB/cfC3H-Mouse 4T1 breast cancer model contains five cell lines; 67NR, 168FARN, 4TO7, 66cl4 and 4T1, that are isolated from the same spontaneously arising primary tumor (figure 6) [127]. The tumor cells are injected into the fat pad of the BALB/c mice and all five sub-clones form primary tumors in the mammary gland. However, the sub-clones differ in their ability to metastasize to distant sites including lymph node, bone, blood, brain and bone [127]. The cell line 4T1 metastasize to multiple organs. 66cl4 metastasize and form secondary tumors in lungs. 4TO7 micrometastasizes to lymph node, lungs and circulation. 168FARN micrometastasizes to lymph node. Micrometastases are when the tumor cells can leave the primary site but cannot complete the metastatic cascade that involves processes from invasion to transportation and end with growth of tumor cells at the new site. The cell line 67NR is non-metastatic [128].

The BALB/cfC3H-Mouse 4T1 breast cancer model has a fully functional immune system and by utilizing this model it can represent a clinically relevance as an experimental animal model [129]. The immune system is an important component for anti-tumor defense. Using immuno-deficient mice as host for human cancer cells can lead to loss of important information regarding tumor-host interactions.

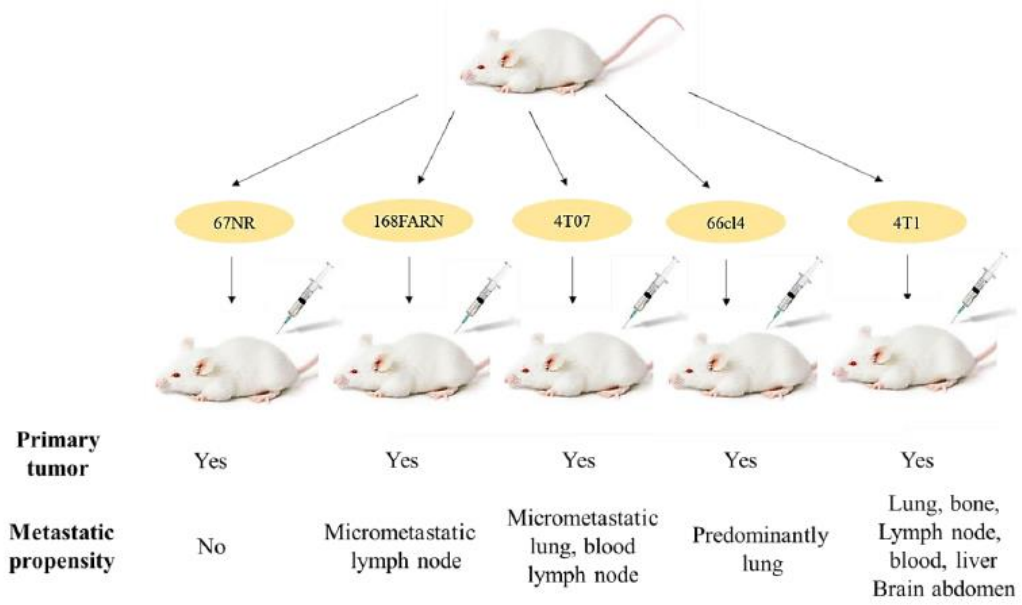


Figure 6 BALB/cfC3H mouse model. The breast cancer mouse model contains five cell lines; 67NR, 168FARN, 4T07, 66cl4 and 4T1, that are isolated from the same spontaneously arising primary tumor showing different metastatic propensity.

2 AIMS OF THE STUDY

Earlier studies in the group where knockout of GREM1 and NQO1 by CRISPR/Cas9 gene editing was carried out, and analyses of the clones both *in vivo* and *in vitro* indicated that the individual clones behaved very differently when compared to each other and to the original 66cl4 cell line (unpublished data, Ulrike Neckmann and Camilla Wolowczyk). From this, it was hypothesized that the 66cl4 cell line is heterogeneous with respect to features important for primary and secondary formation. If so, such information is important to obtain in order to better evaluate experimental results from studies based on single cells clones using this model. Thus, the heterogeneity of sub-clones derived from 66cl4 culture were investigated based on important markers related to other ongoing projects in the group.

The overall hypotheses are divided into several working hypotheses:

- Make sub-clones from single cells of 66cl4 cell line
- Sub-clones of 66cl4 display varying mRNA and protein expression levels of BMP4, phosphorylated SMAD1/5/9 and GREM1
- The already identified mutation in the CUL3 gene in 66cl4 is present in only some of the sub-clones and correspond to the clones demonstrating elevated expression level of NFE2L2, NQO1 and HMOX1
- Sub-clones of 66cl4 differ with respect to proliferation rate and ability to form colonies in soft-agar

3 MATERIAL AND METHODS

3.1 CELL CULTURE

Cell culture from cell line 66cl4 were used for *in vitro* studies in this thesis to test the hypothesis. 66cl4 is among five cell lines from the BALB/c mouse that arise form a spontaneous tumor. The 66cl4 cell line was obtained from Barbara Ann Karmanos Cancer Institute. In order study the heterogeneity of tumor cells in this thesis 66cl4 cells were sorted on a FACS Aria Fusion (BD Biosciences) in 96 well plates to obtain single cells. The single cells were studied under the microscope to directly sort out viable single cells. The single cells were further expanded, and 20 new sub-clone cell lines were obtained. When cells reached 80-90% confluence the sub-clones were split in duplicates or triplicates in 24 well plates to get identical copies of each sub-clone. The sub-clones were then stored in smaller volumes in cryotubes at -80°C for backup and ensure low passage number. Together with 66cl4 mother cell line, 10 clones were randomly chosen and analyzed. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, BioWhittaker, BE12-604F) supplemented with 10% fetal bovine serum (FBS) (Thermo Fischer Scientific, #10270-106), 1% Penicillin/Streptomycin (ThermoFischer Scientific, Gibco, #15070-063) and 1% L-Glutamine (Lonza Group, De-17-605E).

3.1.1 Subcultivation of cells

The 66cl4 single cells were sorted to sub-clones on 96 well plates to reach 70-90% confluence before they were transferred to a 25cm^2 flask, added medium and placed in an incubator at 37°C and 5% CO_2. The cells were left for two to four days to reach a confluence of 80-90% before subcultivated. This was done by removing the old DMEM medium, washing twice with Dulbeccos Phosphate Buffered Saline (PBS) (Sigma Aldrich, #D8537) to ensure that all medium with serum has been removed before trypsin (Lonza, BE17-161E) was added to detach the adherent cells from the bottom of the flask. The cells were then incubated for one to three minutes (min) at 37°C before adding medium the flask to stop the trypsination and pipetted up and down a few times to obtain single cells. The cells were either seeded out for experiments or split into new flask in an appropriate ratio for two to four days. Before seeding cells for experiments we counted the cells using Z2 Beckman counter by adding 20µl of cell suspension to 10ml of isotone II diluent (Beckman coulter, #8546719), then seeded a sufficient cell number in an appropriate container. The cells were placed in an incubator at 37°C and 5% CO_2.

3.1.2 Treatment of cells

Protein transport inhibitor

For immunoblot experiments the cells were treated with Protein Transport Inhibitor (PTI) (500x, eBiosciences, #00-4980-93) for 6 hours (h) before cells were harvested. PTI is a cocktail containing Brefeldin A and Monensin. This cocktail will inhibit the transportation of proteins to the extracellular space and lead to accumulation of secreted protein in the Golgi apparatus and endoplasmic reticulum (ER). This can then be detected by intracellular staining or immunoblotting. PTI was diluted in a ratio 1:500.

Recombinant mouse BMP4 and GREM1

Recombinant mouse BMP4 (rmBMP4) (R&D systems, #5020-BP) or recombinant mouse GREM1 (rmGREM1) (R&D systems, #956-GR) was given to 66cl4 sub-clone WT, A6 and C6. rmBMP4 was given to cells to stimulate the BMP signaling pathway and by this we studied the effect of SMAD1/5/9 phosphorylation and downstream effect upon ligand binding. rmGREM1 on the other hand is an antagonist of BMP4 and by adding it to the cells the effect of phosphorylation of SMAD1/5/9 and downstream signaling was investigated. The cells were stimulated with 100 ng/ml rmBMP4 and 5ng/ml rmGREM1 at 24h, 48h and 72 h.

3.2 IMMUNOBLOT ANALYSES

Western blotting also known as immunoblotting, is a method that can detect specific proteins and separate these proteins by gel electrophoreses, based on their size or charge. The proteins are separated in the gel, then transferred to either nitrocellulose or polyvinylidene fluoride (PVDF) membrane and incubated with a blocking solution to reduce nonspecific protein to bind. After this step the specific protein on the membrane is visualized by incubating it with primary and secondary antibody.

3.2.1 Isolation of protein

Cells were seeded on a 10cm^2 dish to reach approximately 80-90% confluence prior to cell harvesting. Cells were harvested by washing twice with cold sterile PBS and let the dish dry for 30 seconds (s) to make sure all PBS is removed. 90-120µl 8M Urea lysis buffer were added to the dish and a cell scraper were used to detach the cells from the bottom of the dish. The lysis buffer consists of 8M Urea (Merck Millipore, 1084870500), 0.50% Triton-X (Sigma, T8787),) 0.1M dithiothreitol (DTT) (Sigma Aldrich, #43816), phosphatase inhibitor cocktail (PIC) 2 and 3 (5x) (Sigma-Aldrich, P5726 and P0044), complete (25x) (Roche Diagnostics, #11873580001)

and MilliQ water. The cell lysate was then transferred to labeled 1.5ml Eppendorf tubes and stored at -80ºC.

The protein samples were placed on a shaker in a cold room at 4ºC to shake for 2-4 h to completely lysate the cells and obtain as much protein as possible. After this the samples were centrifuged at 13.000 revolution per minutes (rpm) for 15 min at 4ºC and the supernatant was transferred to new labeled 1.5ml Eppendorf tubes. Total protein concentration in the lysate was measured by using BioRad Protein Assay Dye Reagent Concentrate (BioRad laboratories, #500-0006). The BioRad reagent was diluted 1:5 with MilliQ water. The protein sample was then diluted 1:1000 with the BioRad solution in a 1.5ml Eppendorf tube. This mixture was incubated for 10 min and protected from light. The instrument was blanked by using lysis buffer diluted 1:1000 with the BioRad solution. By using a spectrophotometer (Termo Scientific, Termo Spectronic Genesys 20, #4404-02) the absorbance of the samples was measured at 595nm. The concentration of total protein was calculated by using this equation: Protein concentration (µg/µl) = OD595 mean value x 21'

3.2.2 Gel electrophoresis

The protein samples were diluted with 10mM Tris-HCl to get an equal protein concentration of all samples to run on the gel. The samples were then mixed with 4x Lithium Dodecyl Sulfate (LDS) sample buffer (NuPage Life Technologies, NP0007) and 1M DTT in a 5:1 ratio. To denature the proteins in the solution, the samples were incubated at 80ºC on a heating block for 10 min and then placed on ice immediately after taking the samples out. The ladder was prepared by adding Odyssey two color protein molecular weight marker (IR dye 4000, LI-COR Biosciences, 928-40000) to a mixture diluted to 1:4 with 4x LDS and 10mM Tris-HCl.

Premade gel electrophoresis cassettes with 4-12% polyacrylamide (Bis-Tris NuPage, Life Technologies NP0322BOX, WG1403BOX) were used to separate the proteins in the samples. 1x MOPS SDS running buffer were added to an Xcell Surelock Mini-Cell and the cassettes were placed in this box. The proteins were separated at 80 volts (V) for 15 min and 180V for 1 h.

A 0.40µm nitrocellulose membrane (GE Healthcare Life Sciences, Altham, #10600016) were soaked in a buffer that contained 1x Transfer buffer (NuPage Life Technologies, NP0006-1) and 10% methanol for the membrane to get activated. In addition, filter papers and blotting pads were also soaked in the same buffer. After the gel electrophoresis were completed, the proteins were transferred to the pre-soaked nitrocellulose blotting membrane. The blotting was

done by placing blotting pads, filter paper, gel, membrane, filter paper and blotting pads in this order in an XCell II Blot Module (NuPage Life Technologies, EI9051) container and transfer buffer were added to the box. Blotting was done on 4ºC on 30V for 2 h (Invitrogen, PowerErase 500).

3.2.3 Blocking membrane and immunostaining

The membrane was blocked by mixing Odyssey Blocking buffer TBS (LI-COR Biosciences, # 927-50000) with Tris buffered saline (TBS) (Sigma Aldrich, P1379) in a ratio 1:1 for 1h at room temperature on a roller plate. The membrane was placed in a tube with lid on and added the specific primary and secondary antibody according to table 1 and 2. To dilute the antibodies TBS with 0.1% (v/v) Tween-20 (TBST) was mixed with Odyssey Blocking buffer TBS in a ratio 1:1. The staining of primary antibody was done by incubating the membrane overnight at 4ºC and the tube was placed on a roller plate at 50 rpm.. Afterwards the membrane was washed 3 x 10 min with TBST to remove unbound primary antibodies and to reduce background. Secondary antibody was added after washing was completed, for 1 h at room temperature, placed on a roller plate and protected from light. The membrane was washed 1 x 10 mins. with TBST and 2 x 10 min with TBS, before it was left to dry on a piece of paper. To visualize the proteins on the membrane Odyssey Infrared Imaging System (LI-COR Biosciences) was used.

Table 1. Primary antibodies used for Western blot staining

Antibody	Species	Dilution	Molecular weight kDa	Manufacturer
GREM1	Goat IgG	1:500	21 kDa	R&D system (AF956)
BMP4	Mouse IgG	1:1000	47 kDa	Abcam (ab93939)
NQO1	Rabbit IgG	1:1000	31 kDa	Abcam (ab34173)
HO-1	Mouse IgG	1:1000	32 kDa	Enzo asa-110
NFE2L2	Rabbit IgG	1:1000	75 kDa	Cell Signaling Technology (#12721)
pSMAD1/5/9	Rabbit IgG	1:1000	60 kDa	Cell Signaling Technology (#13820S)
Actb	Mouse IgG	1:10 000	42 kDa	Abcam (ab6276)
ERK 1/2	Mouse IgG	1:2000	42,44 kDa	Cell Signaling Technology (#9107S)

Table 2. Secondary antibodies used for Western blot staining

Antibody	Dilution	Manufacturer
Donkey anti-goat IgG – IR Dye 800CW	1:10 000	LI-COR Bioscience #926-32214
Donkey anti-mouse IgG – IR Dye 800CW	1:10 000	LI-COR Bioscience #925-32212
Goat anti-rabbit IgG – IR Dye 800CW	1:10 000	LI-COR Bioscience #926-32211
Donkey anti-goat IgG – IR Dye 680RD	1:10 000	LI-COR Bioscience #926-68074
Goat anti-rabbit IgG – IR Dye 680RD	1:10 000	LI-COR Bioscience #926-68071
Goat anti-mouse IgG – IR Dye 680RD	1:10 000	LI-COR Bioscience #926-68070

3.3 SOFT-AGAR

Malignant transformed cells have the ability to become anchored-independent which means they can grow independently of a solid surface and is one of the hallmarks of carcinogenesis. The cells are capable of proliferating independently of external and internal signals that would normally inhibit cell growth. Soft-agar assay is a method used to characterize and monitor proliferation of transformed cells *in vitro*.

3.3.1 Agarose layer

To make the agar, Agarose-LE (Seakem, #50004) was mixed with autoclaved MilliQ water to obtain an end concentration of 0.75% and 1.5% agarose solution. 2x DMEM comprised of 10g D2902 powder, 3.7g NaHCO3, 3.5g glucose and 380ml MilliQ water. The pH was adjusted to 7.4 and a bottle top filtration unit (VWR International, #97066-202) was used to filter and get sterile medium before adding 20% FCS, 2% L-glutamine and 2% Penicillin/Streptomycin to the solution. The final pH of the solution was adjusted to 6.4.

To make the bottom layer 2x DMEM was mixed with 1.5% agarose in 1:1 ratio in a labeled 50 ml tube. The 1.5% agarose was kept at a temperature of 42°C to prevent it from solidifying. From this solution 1.5ml was transferred to each of the wells on a 6 wells plate. The plates were then left to harden at room temperature and stored up-side-down at 4°C up to seven days. The plates were pre heated to 37ºC before making the top layer. The top layer was made by 10 000 cells/well added to a 50ml tube containing 2x DMEM and 0.75% agarose in a 1:1 ratio. The

0.75% agarose was kept at a temperature of 42°C and 1.5ml of the solution was added to each of the wells on the 6 wells plate. The plates were then incubated for 15 days at 37ºC before staining of the cells.

The 6 wells plates were stained with a solution comprising of 0.04% Crystal violet (Sigma Aldrich, # 548-62-9), 2% ethanol and 2% PBS. 0.5ml of the solution was added to each well for 45 min, placed on a shaker and protected from light. The plates were washed 5 x 45 min with PBS. Photographs of the stained colonies were taken with EVOS Fl Auto 2 (Invitrogen, Thermo Fisher Scientific)

3.4 DNA SEQUENCING

Sanger DNA sequencing is a method used to determine the sequence of DNA molecules. The DNA sequence serves as a template for DNA synthesis and specific primer are designed for the initiation stage of the synthesis. The DNA fragments that is obtained at the end are measured and separated by each base.

3.4.1 Harvest gDNA

700 000 cells/10cm^2 were seeded and incubated at 37ºC for 2-4 days until reaching 80-90% confluence before DNA was harvested. Cells were washed with sterile PBS twice and added buffer that contained 0.8mM EDTA in 50ml PBS to each dish and incubated for 5 min at 37ºC. The cells were transferred to a 15ml tube and washed with 5ml PBS to collect the remaining cells attached on the dish and transferred to the same 15ml tube. The tubes were centrifuged at 1500 rpm. for 5 min and the supernatant was discarded. The pellet containing the cells were then resuspended in PBS to a total volume of 1.4ml and transferred to marked Eppendorf tubes. The Eppendorf tubes were centrifugated at 1500 rpm. for 5 min and the remaining supernatant was removed. Collected DNA samples were stored at -80ºC.

3.4.2 Isolation of gDNA

The genomic lysis buffer comprised of Tris HCl (50mM), NaCl (100mM), EDTA pH 8.0 (100mM), SDS (1%), Proteinase K (100 µg/ml) and RNaseA (100 µg/ml) were prepared. To each of the samples, 1ml genomic lysis buffer were added and each pellet was dissolved by inverting and vortexing the samples. The samples were then incubated at 55ºC overnight. The samples were taken out of the incubator the next day and 100µl of digested DNA samples were added to new Eppendorf tube containing 340µl genomic lysis buffer that did not comprise of

the enzymes Proteinase K and RNaseA. The remaining digested DNA samples were stored at -80 ºC. 310µl of 5M NaCl were added to the samples and shaken vigorously for a few min to mix the solution thoroughly. The samples were stored on ice for 30 min and then centrifuged for 10 min at 14 000 rpm. at 4ºC. A total volume of 700µl of the supernatant was transferred to a new Eppendorf tube and 700µl ice cold 100% ethanol was added to the samples. The samples were centrifuged for 40 min at 14 000 rpm. at 4ºC and the supernatant was discarded. Pellet was washed twice with 500µl 70% ethanol and centrifuged for 10 min at 14 000 rpm. at 4ºC between each wash. The DNA pellet was then left to dry for 3-5 min and resuspended in 50µl T1/10E buffer comprised of Tris HCl (50mM) and EDTA pH 8.0 (100mM) and incubated at 55ºC overnight. The next day samples were centrifuged for a few seconds and resuspended. DNA concentration were measured using NanoDrop ND-1000 Spectophotometer (ThermoFisher Scientific).

3.4.3 gDNA PCR. CUL3 mutation

Samples were diluted with MilliQ water to obtain a concentration of 20 ng/µl DNA. The samples were mixed with a reaction mixture that contained 2x Xtreme buffer, 2mM of each dNTP, 10µM forward primer CUL3,10µM reverse primer CUL3 and KOD Xtreme Hot Start DNA polymerase (Novagen. #71975, 200U). The samples were then sequenced/amplified using GeneAmp PCR System 9700 machine and the program were set according to table 3.

Table 3. PCR program: GeneAmp PCR system 9700 machine.

	Temperature [°C]	Time [s]	Cycles
Polymerase activation	94	120	
Denature	98	10	16
Annealing – Gradient	62-55	15	
Extension	68	20	
Denature	98	10	24
Annealing – Gradient	55	15	
Extension	68	20	
Final Extension	68	120	
Storage	4	∞	

PCR products were checked using 2% agarose gel that contained Agarose-LE (Seakem, #50004), 1x TAE buffer and GelRed nucleic acid gel stain (10 000x) (Biotium, #41003.) DNA samples were mixed with Loading Dye Blue 6x and H_2O before being loaded on the gel. Run the gel at 80V for 1-1.5h. To each PCR reaction 2µl ExoSAP-IT (Affymetrix USB USA, #78200) were added and the reaction were incubated at 37ºC for 15 min and 80ºC for 15 min.

The samples were then transferred to a new 1.5ml Eppendorf tube and added reverse primer and MilliQ water to get a total volume of 15µl. The samples were then sent to GATC Biotech for sequencing.

3.5 QUANTITATIEV POLYMERASE CHAIN REACTION

A real-time polymerase chain reaction (Real-Time PCR), also known as quantitative polymerase chain reaction (qPCR*)* is a highly sensitive method that can be used to achieve quantification of RNA, DNA or cDNA with detection of the PCR products in real time. At each PCR cycle the fluorescence emitted by the probes will be measured by the qPCR machine and with each PCR cycle the products will double. SYBR Green a fluorescence dye used in this method emit fluorescence signal when bound to double stranded DNA molecules. RT-PCR can be used for a variety of applications such as analyses of gene expression, SNP genotyping and discrimination between different alleles.

3.5.1 Harvesting and isolation of RNA

For each clone 100 000-200 000 cells/well were seeded on a 6 wells plate and incubated at 37ºC under normal growth conditions until it reached 80-90% confluence. RLT buffer were mixed with 10% β-mercaptoethanol (Sigma Aldrich, #M3148) of the total volume. Upon harvesting RNA, the cells were washed twice with cold PBS and 350µl RLT buffer with 10% β-mercaptoethanol were added to each well. The cell lysate was mixed by pipetting up and down a couple of times, transferred to marked Eppendorf tubes and put on ice. The samples were stored at -80ºC.

For RNA isolation the RNeasy Mini kit (250) (Qiagen, #74106) was used. To the lysate 350µl of 70% ethanol were added and mixed by pipetting up and down a few times. The sample of 700µl was then transferred into a RNeasy spin column that was placed on top of a collection tube. This was then centrifuged for 15 s at 8000 g and the flow-through were discarded. 700µl of buffer RW1 were added to the column of each sample, centrifuged for 15 s at 8000 g and the flow-through were discarded. Afterwards the samples were washed 2 x 500µl with RPE buffer, centrifuged for 15 s for the first step and for 2 min for the last step at 8000g and the flow-through were discarded. The column was transferred to a new 2ml collection tube and centrifuged for 2 min at 8000 g. After this step the column was placed in a new marked 1.5ml collection tube and eluted 2 x 30µl with RNase-free water and centrifuged for 1 min at 8000 g.

The RNA concentration of the samples was then measured using NanoDrop ND-1000 Spectrophotometer. The purity of the isolated RNA can be measured by how much light the

samples absorbs at 260nm and 280nm (260/280 absorbance ratio). An absorbance ratio of approximately 2.0 indicates a purity of the isolated RNA, while higher or lower measurements than this can indicate a present of proteins, DNA or other chemicals in the sample. The concentration (ng/µl) of the samples was measured by using the program for nucleic acid on NanoDrop ND-1000. The instrument was initiated and blanked by using RNase-free water before the samples were measured. The samples were then stored at -80ºC.

3.5.2 qPCR analyses

For this technique the QuantiTech Reverse Transcription Kit (Qiagen, #205313) were used for the cDNA synthesis. For each reaction 500ng RNA were used. The samples were diluted with H_2O to obtain 500ng RNA in a total volume of 12µl and 2µl DNA wipeout was added to each of the samples. This was then placed in a C1000 Thermal Cycler machine (BioRad, Thermo Fisher Scientific) and incubated for 2 min at 42ºC and placed on ice immediately afterwards. For each sample a total volume of 6µl of reverse-transcription master mix containing Quantiscript Reverse Transcriptase (1x), Quantiscript RT buffer (5x) and RT Primer Mix (1x) were added. This was then incubated for 15 min at 42ºC and 3 min at 95ºC and placed on ice immediately for 2-3 min. The cDNA was diluted in a ratio of 1:5 with H_2O afterwards.

For the qPCR reaction the QuantiTect SYBR Green PCR kit (Qiagen, #204141) were used. The reaction mix containing QuantiTect SYBR Green PCR master mix (2x), different targeted QuantiTect Primer Assay (10x) and H_2O were mixed in an Eppendorf tube and transferred to a 96 wells plate (table 4). The samples were then placed in a RT-PCR machine (Thermo Fisher Scientific, Applied Biosystem) for quantification of the product using the ΔΔCT program. The setting for the cycle sequence program were adjusted according to table 5.

Table 4. Primers used for qPCR

Primer	**Manufacture**
Actb	QuantiTect Primer Assay #QT01136772
BMP4	QuantiTect Primer Assay #QT00111174
Gclc	QuantiTect Primer Assay #QT00130543
Grem1	QuantiTect Primer Assay #QT01039983
Hmox1	QuantiTect Primer Assay #QT00159915
Nqo1	QuantiTect Primer Assay #QT00094367
TBP	QuantiTect Primer Assay #QT00198443

Table 5. Cycle sequence program. Minutes (mins), seconds (s)

PCR initial activation step	15 mins.	95ºC	**This step activates HotStarTaq DNA Polymerase**
3-step cycling:			
Denaturation	15s	94ºC	
Annealing	30s	55 ºC	
Extension	30s	72 ºC	Perform fluorescence data collection
Number of cycles	40 cycles		

3.6 PROLIFERATION ANALYSES

Counting cells are usually carried out prior to cell culture or before carrying out an experimental process that require an accurate and consistent number of input cells. To see if there is an increase in the cell population a proliferation experiment can be conducted. Here we use a direct cell counting method. The rate of the cell growth was calculated and presented in a linear graph.

A 24 wells plate were used to seed 10 000 cells/ well and 4 wells for each clone. The plates were incubated at 37 ºC until different time points 31.5 h, 44.5 h, 56.5 h, 68.5 h and 80.5 h and counted. Prior counting the cells were washed twice with PBS, trypsinated, resuspended in DMEM medium and transferred to marked Eppendorf tube. Each sample was counted three to four times on the Z1 Beckman counter.

3.7 FLOW CYTOMERTY

Flow cytometry is a technique that can be used to detect different biomarkers. The fluorescence molecules are usually in the form of antibodies or dyes and cells that are bound to these fluorescence molecules flow in a liquid stream through a laser or light beam. The measurement of the fluorescence is carried out when cells pass a sensing area right after flowing through the laser or light beam. Focusing on 66cl4 clones WT, A6 and C6 we tested them for the expression of CD24 antibody (Biolegends, #101808). All the centrifugation steps in this method was carried out at 1500 rpm. for 5 min at 4ºC.

Cells were seeded and harvested when 80-90% confluent was reached. The cells were washed twice with PBS and trypsinated before resuspended with medium and transferred to a

15ml tube. The tubes were then centrifuged and afterwards washed and resuspended with MACS buffer comprised of PBS (without Ca^{2+} and Mg^{2+}), 2mM EDTA and 0.5% BSA before being centrifuged again. The supernatant was then discarded, and the pellet was resuspended with 1ml MACS buffer. The cells were counted by adding 20µl cell suspension in 10ml isotone II dilution on Z1 Beckman counter to get a sufficient cell number for the next steps in the experiment.

In each FACS tube 350 000 cells were added together in 1ml MACS buffer, mixed and centrifuged. The supernatant was discarded leaving 100µl which was resuspended with the pellet. Three tubes were prepared for each clone where one tube containing CD24 antibody and cells, another tube contained cells together with PE isotype control and the last tube contained cells that were unstained. 1µl FC block were added to the tubes that contained either CD24 antibody or PE isotype control and placed on ice for 10 min protected from light. After the tubes had been incubated with FC block, 1µl of CD24 antibody or PE isotype control was added to the respective tubes and put on ice for 30 min protected from light. Compensation beads (BD™ CompBeads Biosciences, #552845) were prepared by adding 1 drop of positive control (BD™ CompBeads, #51-90-9001291) and 1 drop of negative control (BD™ CompBeads, #51-90-90000949) in 100µl MACS buffer. To this tube 1µl of CD24 antibody was added and placed on ice for 30 min together with the other tubes. The samples were then washed with 1ml MACS buffer and centrifuged. The supernatant was discarded before the pellet was resuspended in 300µl MACS buffer. The samples were analyzed on BD LSR II flow cytometer machine (BD Bioscience)

3.8 STATISTIC ANALYSES

Statistical analyses have been performed on the experiments. An unpaired t-test was performed to determine the significance of the findings. Standard derivation is marked as error bars to show the differences around the mean value that was conducted form three independent experiments.

4 RESULTS

The BALB/cfC3H-Mouse 4T1 breast cancer model was utilized in this thesis to investigate the heterogeneity between 66cl4 sub-clones. Based on previous results in the group, RNA sequence data from the metastatic 66cl4 and non-metastatic 67NR cells were obtained to identify potential candidates for gene products that might contribute to metastasis. From these studies some candidate genes were selected, and their expression changed by both shRNA and CRISPR/Cas9. These initial experiments indicated that sub-clones of 66cl4 showed sign of heterogeneity and varied in phenotype even in the absence of any genetic manipulation (unpublished data, Ulrike Neckmann and Camilla Wolowczyk). In order to assess the possible heterogeneity between the 66cl4 sub-clones in a firmer manner, sub-clones arising from single cells were established and analyzed by different approaches.

4.1 Expression of biomarkers in 66cl4 sub-clones

The 66cl4 cells were sorted into single cells and cultured to sub-cell lines. Twenty single cell clones were obtained and split in duplicates or triplicates to get identical copies and to store multiple vials at low passages. Ten sub-clones were randomly chosen, expanded to new cell lines and analyzed together with the mother 66cl4 cell line. The heterogeneity of different 66cl4 sub-clones were investigated by looking into differentiation and oxidative stress pathway that might be important in growth and metastases of the tumor. Based on earlier findings in the group it was observed that among the members of the TGF-β superfamily, BMP4 display the most distinguished differences. BMP4 and its antagonist GREM1 is important in many aspects of embryonic development and a loss of function could lead to lethality [44, 47, 61, 62] In addition, BMP4 signaling at protein level can be measured by phosphorylated level of phospho-Smad1(Ser463/465)/Smad5(Ser463/465)/Smad9(Ser465/467). The other pathway analyzed in this thesis is the NFE2L2 pathway. NFE2L2 is an important transcription factor that regulates the expression of several cytoprotective genes including NQO1 and HMOX1 [84, 87, 88].

4.1.1 BMP4 and pSMAD1/5/9 protein level did not differ but GREM1 level might display variation

BMP4 stimulate epithelial cell differentiation and loss of such contribute to oncogenic transformation. We asked if the sub-clones displayed differences in protein expression levels of BMP4, phosphorylated SMAD1/5/9 and GREM1. To test this the BMP4 and GREM1 protein level were measured in total protein extracts from the 66cl4 cell line and sub-clones. Since both proteins are secreted, the cell cultures were treated with PTI a mixture of Brefeldin A and

Monensin that interferes with protein to allow the proteins to accumulate. We cultured the cells and added 1x PTI for 6h before cells were collected. Immunoblots from the sub-clones were performed on untreated and treated cells. The findings of untreated sub-clones showed a weak, nearly undetectable BMP4 band. However, an accumulation of the BMP4 protein level was observed in all the sub-clones that were treated with PTI. The results suggested similar level of BMP4 in all the sub-clones but lower BMP4 level compared to the 66cl4 cell line (figure 7). Furthermore, immunoblotting analyses of GREM1 protein expression indicated variation between the sub-clone A6, D9, D12, E1, E5 and F4 that showed lower GREM1 level contra the 66cl4 cell line. On the other hand, sub-clone B2, C6, D2 and D7 might have higher expression of the protein compared to the 66cl4 cell line. Surprisingly, the results indicated that the protein level of GREM1 did not accumulate when sub-clones were treated with PTI. Even though the variation was insignificant sub-clone A6 and D9 showed a tendency of lower GREM1 protein expression compared to 66cl4 cell line (figure 7).

It was observed that the protein expression of BMP4 and GREM1 varied quite much between the three independent experiments performed. Therefore, the results might indicate that there was no variation in BMP4 protein level between the sub-clones. Even though GREM1 level was not significantly different it might indicate sign of variation between the sub-clones, but it is difficult to conclude precisely how different the sub-clones are.

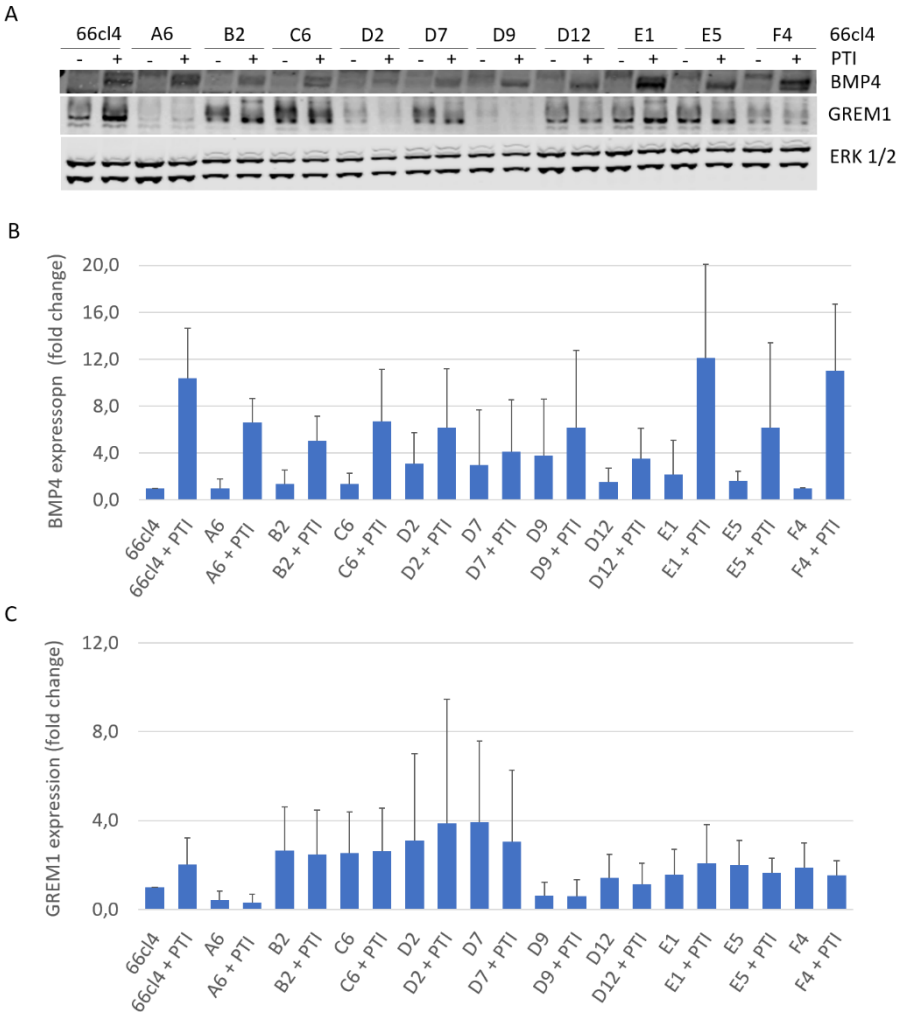


Figure 7: Similar BMP4 but varied GREM1 protein level between the 66cl4 sub-clones.

The figures are an illustration of one out of three independent experiments carried out. A) Immunoblot analyses of BMP4 and GREM1 in the 66cl4 sub-clones, both untreated and after 6h stimulation with 1x PTI treated cells. ERK 1/2 was used as loading control. The blots represented one out of three independent experiments executed. B) Quantification of the average expression of BMP4 of the three independent experiments. The standard derivation is marked by the black line. C) Quantification of GREM1 protein expression (n=3). An unpaired t-test were performed and revealed that there were no significant differences between the sub-clones.

Having observed a tendency of variation in GREM1 level we wondered whether there were any differences in phosphorylated SMAD1/5/9 protein level as well. To test this, the phosphorylated level of SMAD1/5/9 were analyzed. Except for sub-clone A6, D9 and F4 that might illustrate a higher levels the other sub-clones showed comparable phosphorylated SMAD1/5/9 level. However, similar to the previous results above the phosphorylated level varied quite much indicating that the level between the sub-clones might not differ (figure 8).

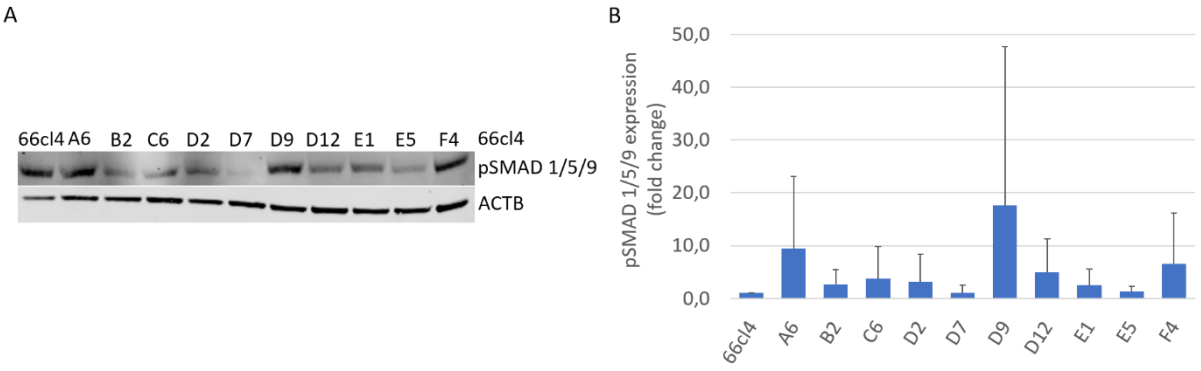


Figure 8: pSMAD1/5/9 level did not vary between the 66cl4 cell line and the sub-clones. A) The immunoblot represent one out of three experiments performed of phosphorylated SMAD1/5/9 protein expression. ACTB were utilized as loading control. B) Quantification of phosphorylated SMAD1/5/9 protein level (n=3). Statistical analyses revealed no significant differences between the sub-clones.

4.1.2 Similar mRNA expression of BMP4 and GREM1 between 66cl4 sub-clones

The results from the immunoblots illustrates that the sub-clones might have displayed sign of variation in protein level. We speculated if the mRNA level would move in the same direction and show differences in BMP4 and GREM1 expression. To test this quantification of mRNA were carried out utilizing qPCR. We chose to focus on two clones in addition to the 66cl4 cell line for further investigation. Based on the results obtained from immunoblotting we selected one clone that showed high (C6) and one clone that showed low (A6) expression level of GREM1in comparison to the 66cl4 cell line. The finding revealed that the sub-clones had similar mRNA level of BMP4 as the 66cl4 cell line. In addition, comparing 66cl4 cell line and the two sub-clones A6 revealed to have a lower GREM1 mRNA level. mRNA level of GREM1 in sub-clone C6 might be higher than 66cl4 cell line but the variation between the experiments was high making it difficult to conclude (figure 9).

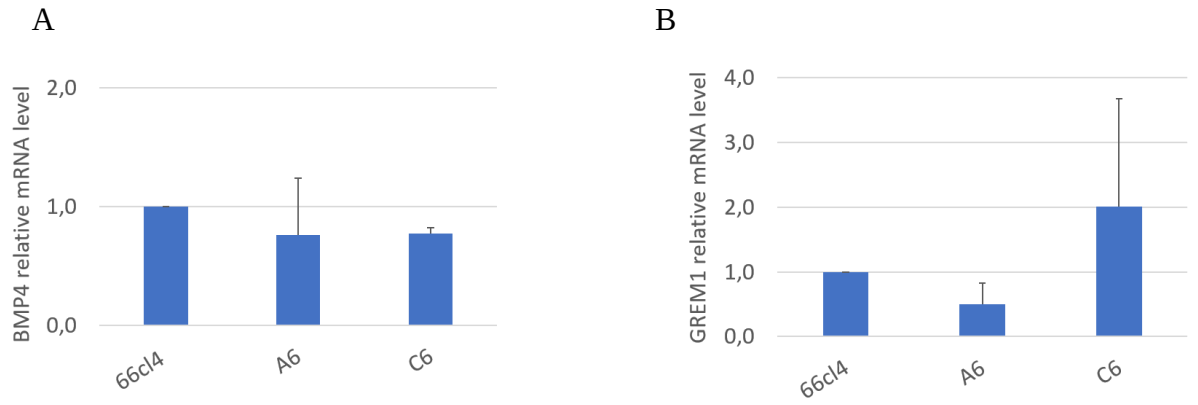


Figure 9: Quantification of BMP and GREM1 was similar in 66cl4 cell line and sub-clones A6 and C6. Three independent experiments were performed, and B-ACTIN and TUBULIN were utilized as controls. A) mRNA level of BMP4. B) mRNA level of GREM1. The statistical significance of mRNA expression of BMP4 and GREM1 displayed no differences.

4.1.3 Correlation between GREM1 and CD24

BMPs and GREM1 is known to have key role in regulating the developmental processes for instance by inducing differentiation of certain cell lineages and an uneven balance between their activity can lead to lethal diseases [34, 44, 47, 61, 62]. In CSC high expression of GREM1 is shown to prevent the BMP-induced differentiation of CSC [75]. In this way GREM1 can prevent the loss of CSC within the tumor and might contribute to CSC proliferation [75]. Stem cells can be characterized by presence of different protein markers. One of the markers is CD24 [78]. Analyses of the transcriptome data demonstrated that the CD24 mRNA level is significantly higher in metastatic 66cl4 contra 67NR cells both in culture and in the primary tumor (table 6). Thus, possible heterogeneity in GREM1 mRNA and protein expression levels between the sub-clones could result in altered differentiation.

Table 6: Transcriptome data over CD24 stem cell marker expressed in 66cl4 and 67NR. Log_2 = indicate the differential expression between 66cl4 and 67NR cell line or primary tumor. A positive value indicates higher expression in 66cl4 and a negative value indicates higher expression in 67NR. p-value = p-value form t-test of comparison of expression between cell lines and primary tumors of 66cl4 and 67NR.

	Comparison of cell lines				**Comparison of primary tumor**				**66cl4 metastasis**	
Gene	**66cl4**	**67NR**	**Log_2**	**p-value**	**66cl4**	**67NR**	**Log_2**	**p-value**	**66cl4**	**p-value**
CD24	173.6	0.04	12,02	0,00031	74,92	5,54	3,76	4,74E-05	102,1	0,00017

Kaplan Meier (KM) plotter is an online database and was used to estimate the clinical relevance of CD24 mRNA expression in breast cancer patients [130]. KM-plotter showed analyzed data form 3951 patients and the database divided the patient samples into two cohorts of low or high expression of mRNA levels of CD24. The KM-plotter revealed that high CD24 mRNA level was found to correlate with reduced survival (figure 10).

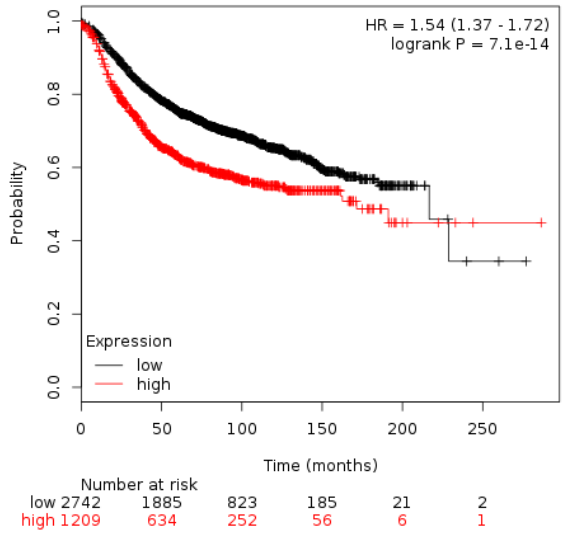


Figure 10: CD24 is correlated with poor prognosis in breast cancer patients. Survival analysis for breast cancer patients where patients are divided into two cohorts of high or low CD24 expression. HR is the ratio between the two groups. P-value is the significance of differences between the two cohorts with a 95% confidence interval. X-axis show the time (months) after started study. Y-axis show estimated survival probabilities. The KM-plotter illustrated high expression of CD24 leads to reduced survival in relapse-free survival patients

We therefore wanted to determine the correlation between GREM1 and stem cell marker CD24 and hypothesis that GREM1 might affect the expression of CD24. Flow cytometry were performed to analyze whether expression of CD24 was present or absent in the sub-clones A6 and C6 in addition to the 66cl4 cell line and 67NR cell line. The gating strategies for the experiments are included in appendix 1. In line with the transcriptome data, 67NR did not express CD24 protein while the 66cl4 cells were clearly positive. However, analyses of the two clones that seems to express different GREM1 levels did not differ from each other and both had an CD24 level indistinguishable from the mother 66cl4 cell line (figure 11).

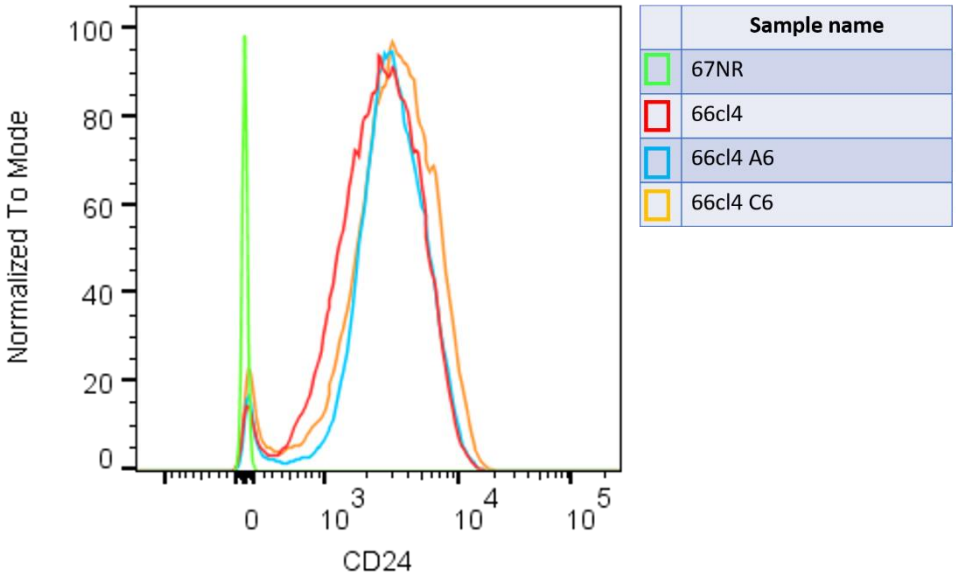


Figure 11: CD24 is expressed in sub-clone A6 and C6 and the 66cl4 cell line cell line but not in 67NR. The expression of CD24 does not vary when comparing the sub-clone A6 and C6 to the 66cl4 cell line cells. CD24 was not expressed in 67NR. Color of clones: green = 67NR cell line, red = 66cl4 66cl4 cell line cell line, blue = 66cl4 sub-clone A6 and orange = 66cl4 sub-clone C6

Even if there were no differences in CD24 signal of the A6 and C6 clones, we still asked if a possible difference in GREM1 expression could have different ability to neutralize exogenously added, recombinant BMP4, and different ability to block BMP4 induced differentiation and leading to loss in CD24. Thus, the different cells were treated with 100 ng/ml rBMP4 for 24h and 48h and CD24 levels determined. The results revealed no reduction in CD24 staining in any of the 66cl4 or 66cl4 sub-clones in response to recombinant BMP4 (figure 12). Surprisingly, both 66cl4 mother cells and the two sub-clones responded by a slight increase in CD24 level in response to 24 and 48 hours stimulation of recombinant BMP4.

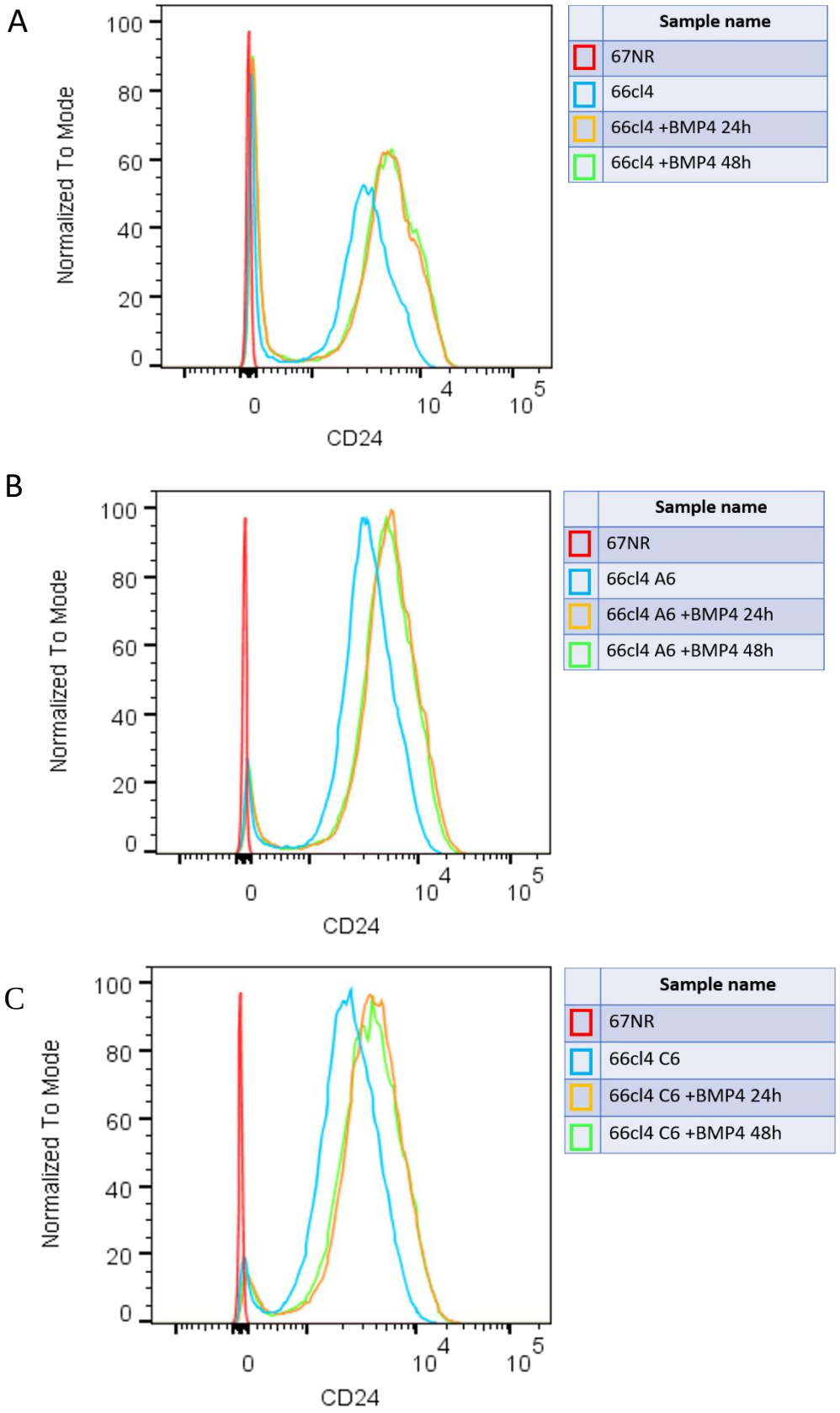
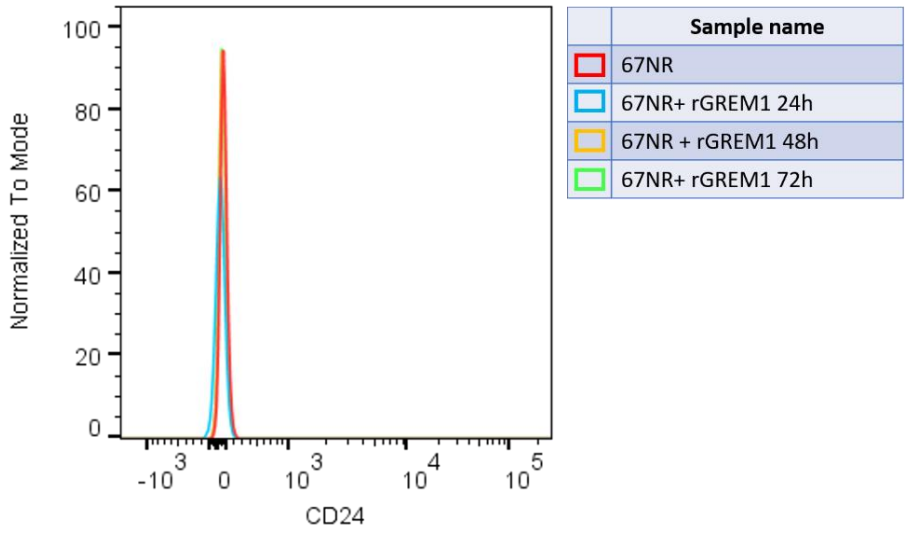


Figure 12: rBMP4 do not affect the surface level of the stem cell marker CD24 in 66cl4 or any of the 66cl4 sub-clones. The cells were treated for up to 48h with rBMP4 prior harvesting. Cells were analyzed for CD24 expression. A) 67NR and 66cl4 66cl4 cell line. B) 67NR and 66cl4 sub-clone A6. C) 67NR and 66cl4 sub-clone C6

BMP4 is able to induce differentiation in cells and thus create a loss of CD24 in tumor cells. 67NR does not express GREM1 and thus, are not able to block the differentiation of cells which should also have low CD24 level. This was tested by adding recombinant GREM1 to 67NR cells for 24, 48 and 72 hours and the CD24 levels was determined. The results showed that the 67NR cells did not express CD24 regardless of the treatment (figure 13 A). We therefore asked if GREM1 expression in the 66cl4 cells could be important to the CD24 level. To check this, two 66cl4 GREM1 knockout clones utilizing CRISPR/Cas9 method that were previously obtained in the group were analyzed for the CD24 level. The result illustrated that the CD24 level was quite similar in both GREM1 KO clones and was not different from the 66cl4 cell line (figure 13 B).

A



B

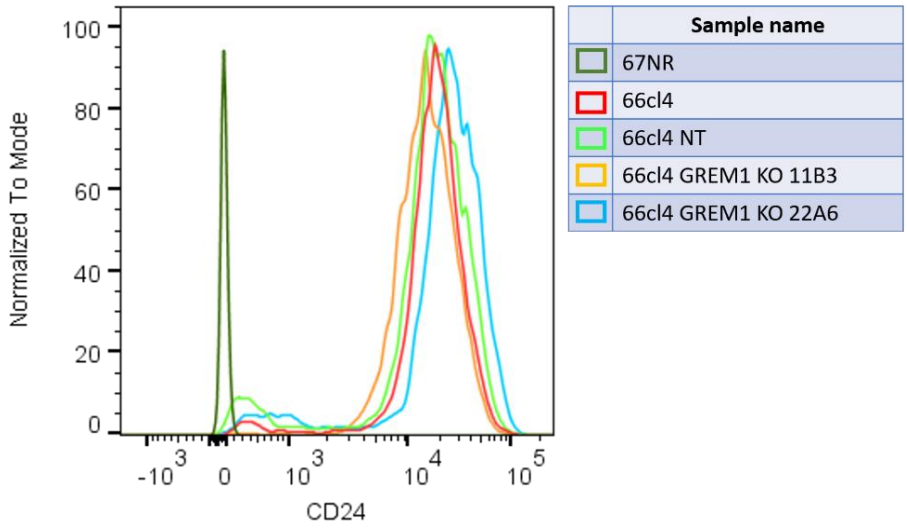


Figure 13: 67NR cells do not express CD24 though treated with rGREM1 and 66cl4 KO cells expressed CD24. A) Two 66cl4 with GREM1 knockout clone and one non-target clone show CD24 expression. B) 67NR cells were stimulated with rGREM1 for up to 72h prior harvesting yet the cells do not express CD24.

4.1.4 CUL3 mutation is present in all the 66cl4 sub-clones

Earlier findings in the group revealed that 66cl4 tumor cells had CUL3 mutation that was not found in any of the other four cell lines. CUL3 mutation is present on exon 14 where a Cytosin (C) base is replaced with a Guanie (G) base. This lead to further investigation of the sub-clones and we speculated if the genotype would be the same in all the sub-clones. Thus, DNA was isolated from 66cl4 clones and the 66cl4 cell line and subjected to CUL3. The results from the DNA sequence revealed that all the sub-clones had the same CUL3 mutation in their genes demonstrating homogeneity (figure 14).

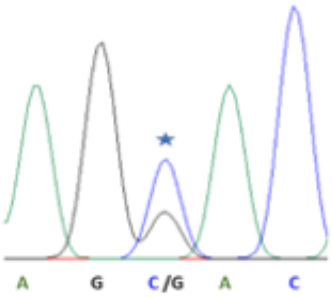


Figure 14: CUL3 mutation is present in the 66cl4 sub-clones. The figure is a representation of the sub-clones CUL3 mutation on exon 14 where a C base is exchanged with a G base. All the 66cl4 sub-clones had the mutation

4.1.5 Similar NFE2L2 and NQO1 but different HMOX1level between the 66cl4 sub-clones

CUL3 forms a complex with KEAP1 and ubiquitin ligase E3 and together control the oxidative stress response by regulating NFE2L2 transcription factor. By this CUL3 contribute to defense against several pathologies [84, 85]. Having found the CUL3 mutation present in all the sub-clones we asked if this would lead to equal protein expression of NFE2L2. To test this immunoblotting were performed. It was observed a tendency of similar level with no significant variation in the protein expression of NFE2L2 were observed between the sub-clones (figure 15).

NFE2L2 controls the expression of antioxidant genes NQO1 and HMOX1 and together these genes are important in the cellular defense mechanism. Even though the sub-clones did not show sing of heterogeneity in CUL3 mutation and NFE2L2 protein level we still asked if NQO1 and HMOX1 protein level would indicate diversity between the sub-clones. Thus,

immunoblotting for NQO1 and HMOX1 were performed. NQO1 protein expression might have indicated similarity between the sub-clones. Interestingly it was observed that HMOX1 protein level was different. Except for sub-clones C6 and D2, the other sub-clones had lower HMOX1 protein expression than the 66cl4 cell line. Also, the sub-clone C6, D2, D9 and F4 showed no significant differences, while sub-clone A6, B2, D7, D12, E1 and E5 revealed to be very significant when compared to the 66cl4 cell line.

Further, when comparing HMOX1 and NQO1, except for sub-clone E1, there might be a tendency that the sub-clones with low HMOX1 protein expression showed a slightly higher NQO1 protein level. Together the results indicate that the sub-clones showed sign of heterogeneity in their protein expression of HMOX1 but not of NFE2L2 and NQO1 (figure 15).

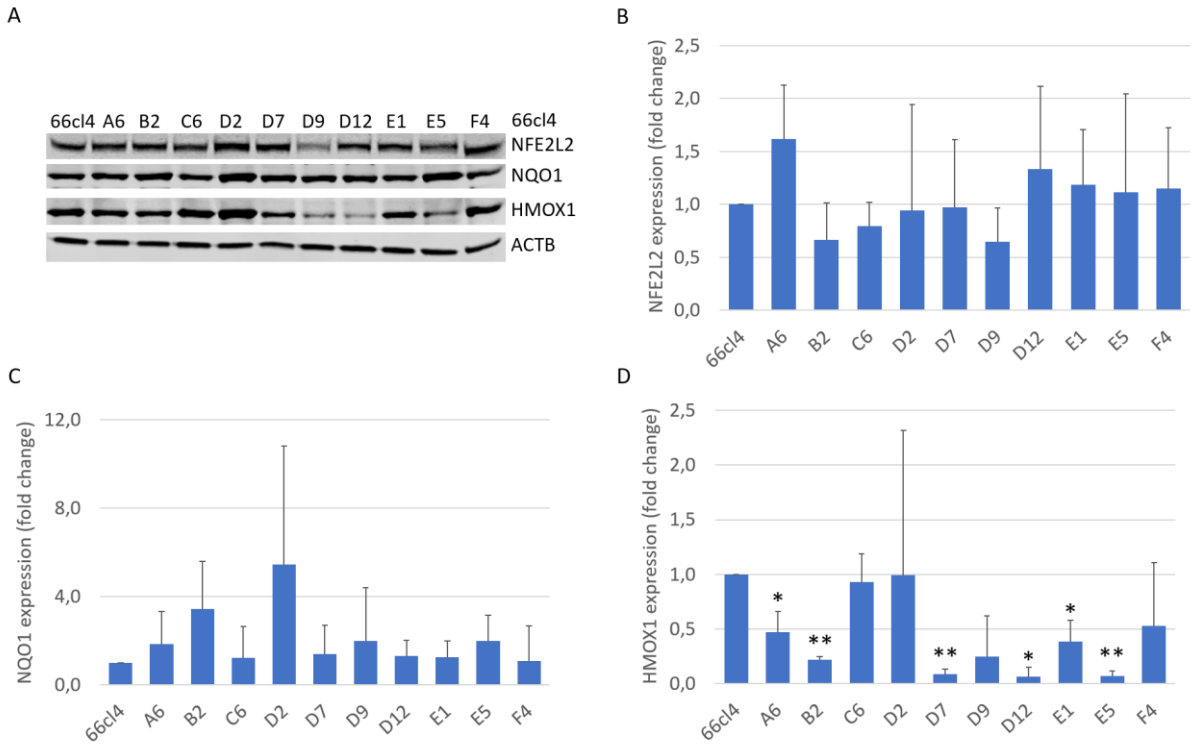


Figure 15: significant differences in HMOX1 but not in NFE2L2 and NQO1 protein level between the sub-clones and 66cl4 cell line. A) Immunoblot of NFE2L2, NQO1 and HMOX1 (n=3). ACTB was utilized as loading control. B) Quantification of NFE2L2 protein level of three independent experiments C) Quantification of NQO1 protein expression (n=3). D) Quantification of the average HMOX1 protein level of three independent experiments. Unpaired t-test were conducted on the different markers. NFE2L2 and NQO1 protein expression did not have any statistical variation. HMOX1 protein level showed statistical variation between the sub-clones. One star = very significant with p-value <0.05. Two stars = extremely significant with p-value <0.01

Having found sign of heterogeneity between the sub-clones with respect to HMOX1, we speculated that the mRNA level of HMOX1 would also be different. Thus, quantification of HMOX1 and NQO1 mRNA level were performed and results showed that NQO1 mRNA level did differ. The sub-clone A6 had a significantly higher while sub-clone C6 had similar mRNA level compared to the 66cl4 cell line. The findings for sub-clone A6 and C6 did not differ in mRNA expression of HMOX1 (figure 16). When comparing NQO1 and HMOX1 there were a tendency of sub-clone A6 having slightly higher expression of both markers contra sub-clone C6 and 66cl4 cell line. The sub-clone C6 seems to have comparable mRNA level for both NQO1, HMOX1 and similar tendency might be seen on the protein level of the markers as well.

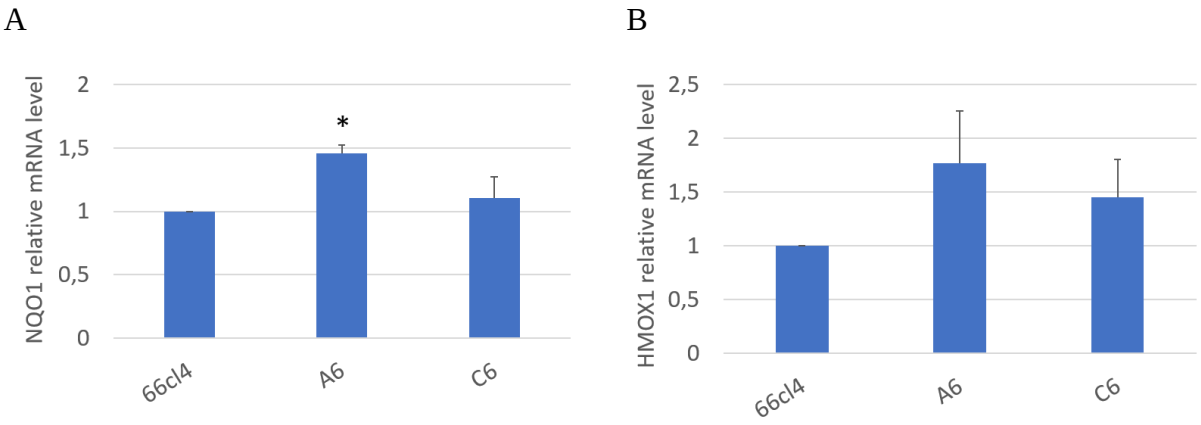


Figure 16. Sub-clones differed in mRNA level of NQO1 but showed no variation in HMOX1 mRNA level. A) mRNA level of NQO1. B) mRNA level of HMOX1. The statistical significance of mRNA expression of BMP4, GREM1 and HMOX1 displayed no differences. mRNA expression of NQO1 for sub-clone A6 was statistically significant compared to the 66cl4 cell line with a p-value <0.05.

4.2 Phenotype characteristics of the 66cl4 sub-clones

It was observed in the transcriptome data that GREM1 is highly expressed in metastatic cell line 66cl4 contra non-metastatic 67NR. GREM1 is thought to have an important role in tumor metastasis and enhance tumor cell survival. We therefore asked if differences in GREM1 level in the sub-clones could would resulted in different proliferation rates and ability to form colonies in soft agar. The proliferation results illustrated that the sub-clones had similar doubling time showing no significant differences from each other or the 66cl4 cell line (figure 17). Like the proliferation experiment there were no variation between the sub-clones. However, the sub-clones did display significant reduced ability of colony formation when compared to the 66cl4 cell line. Comparing the two methods a tendency of sub-clone C6 have a higher doubling time and form bigger colonies than sub-clone A6 was observed. This might indicate that sub-clone C6 have higher GREM1 level which points to the same direction as the mRNA and immunoblots results.

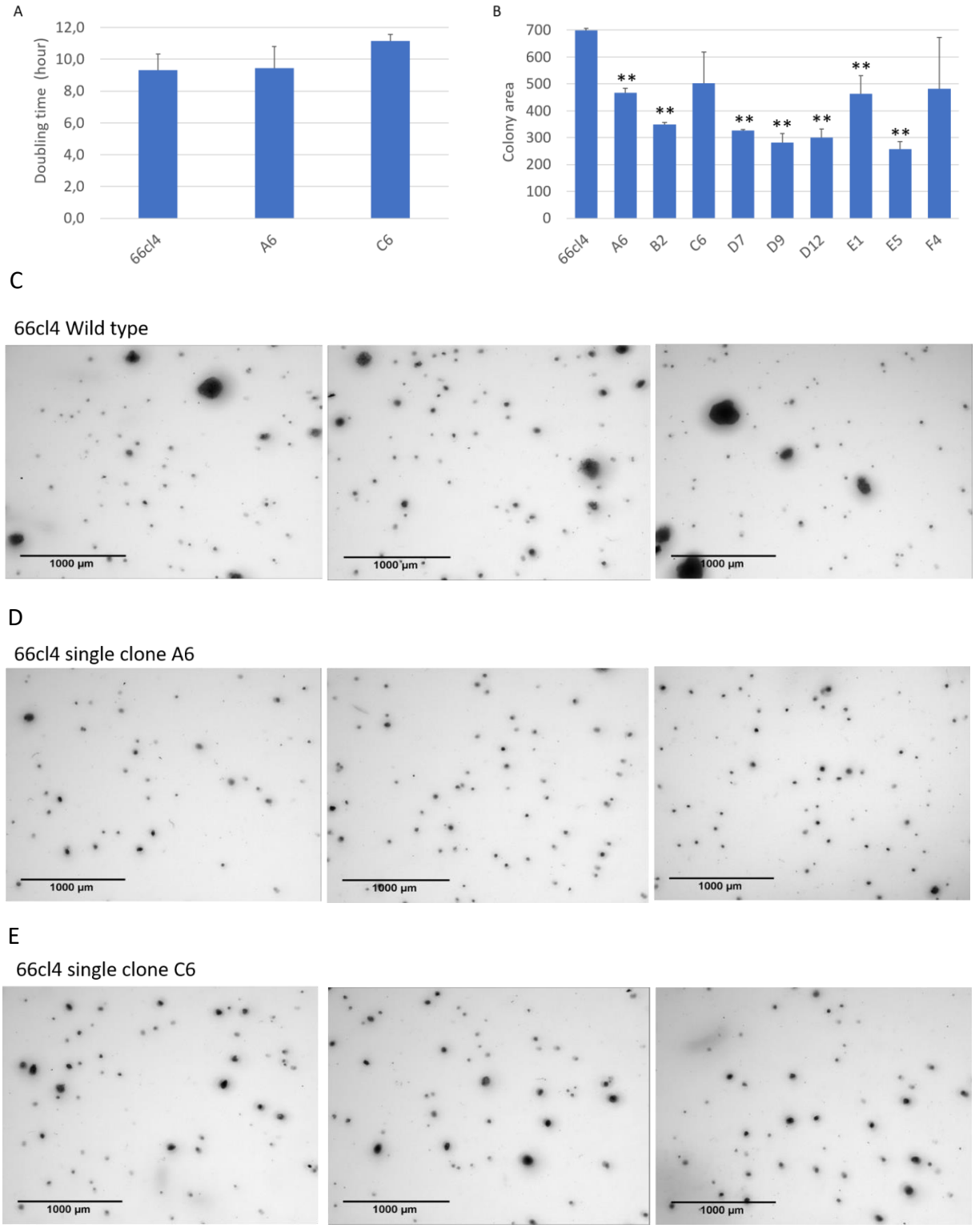


Figure 17: No differences in proliferation but reduced colony formation capacity in sub-clones compared to 66cl4 cell line. A) The 66cl4 sub-clone proliferation characteristics showing the doubling time (hour) of the population. The graph represents the average time it takes for the clones to double in population in three independent experiments (n=3). B) The average colony shape formed by 66cl4 sub-clones illustrating different size of the colonies (n=2). Two stars = extremely significant with p-value <0.01. C) The illustration represents one out of two experiments from soft agar colony formation of the 66cl4 66cl4 cell line. D) Colony formation of 66cl4 sub-clone A6 E) Colony formation of 66cl4 sub-clone C6

5 DISCUSSION

Cancer related deaths is primary caused by metastasis in breast cancer patients [24, 25]. This emphasizes the essential it is to understand the underlying mechanism behind metastasis. In this thesis the immunocompetent 4T1 breast cancer mammary tumor mouse model was used with the focus on the metastatic 66cl4 cell line. Previous studies from the group from knockout experiments indicated that 66cl4 derived sub-clones showed sign of heterogeneity. To investigate the possible heterogeneity, 66cl4 cells were sorted into single cells and cultured to sub-cell lines. Ten sub-clones were chosen for further analyses. In this study, it was determined is the 66cl4 sub-clones varied in mRNA level and protein expression of BMP4, phosphorylated SMAD1/5/9, GREM1, CUL3, CD24, NFE2L2, NQO1 and HMOX1. In addition, we tested whether the sub-clones of 66cl4 differ with respect to proliferation rate and ability to form colonies in soft-agar.

The results indicated that the sub-clones show sign of heterogeneity with respect to GREM1 protein and mRNA level. Comparable protein and mRNA level of BMP4 and phosphorylated SMAD1/5/9 were observed for all the 66cl4 clones. All the sub-clones had the same CUL3 mutation in their genes and NFE2L2 protein and mRNA level did not vary. However, the level of NFE2L2 controlled HMOX1 and NQO1 varied between the sub-clones at both protein and mRNA level. The phenotype of the 66cl4 sub-clones did not differ with respect to proliferation rate but there might be a tendency of diversity from soft-agar assay.

5.1 Technical variation

The data obtained in this thesis were quite different between the experiments. Ten sub-clones and 66cl4 cell line were cultivated at the same time and it was observed that the cells grew at different rates. Getting all the sub-clones equally confluent prior harvesting was therefore a challenge. Several reasons could cause the varying results and one possible explanation could be biological noise such as physical environment or different passages between the sub-clones and 66cl4 cell line. The differences in how confluent the sub-clones were could also affect the outcome. In addition, the serum in the medium comprise of components such as growth factors, hormones and amino acid. Serum gives nutrition to the cells and these factors are essential for normal growth. Therefore, differences in change of medium between the sub-clones were could lead to different cell growth.

To minimize the variances, sub-clones should not be kept in culture over a long period and the sub-clones should be split at the same time to avoid different passages. However, to test if

the confluency is the reason for inconsistent results we could examine the total protein level of cells at different densities and observe if there is a difference in the results. If there is a difference the reason is most likely caused by cells confluency. Similar experiments could be performed on medium supply and pH.

Technical variation can occur which might lead to low reproducibility between each replicate. It is therefore important to be precise and specific when performing the experiments. On the other hand, the characteristics in a cell line can change as tumor cells are genetically unstable [131]. The spontaneous mutation rate is much higher *in vitro*, and the phenotype of the cultivated cells can in some cases differentiate. The differences might be due to selective overgrowth or adaptive responses which causes variability from one passage to the next [131].

We sorted 66cl4 cells in 96 well plates and only 10% of the cell were able to grow, form colonies and become new sub-clone cell lines. Tumor cells proliferation, survival and invasion can be influenced by the microenvironment, tumor cells adapt and responses to different surroundings which might alter the cells behavior. The sorting process might be too rough on the single cells that in the end the cells might have lost some of its properties leading to reduced aggressiveness of the cell. The reduced abilities could be observed in soft agar assay where sub-sub clones had similar but decreased colony formation properties compared to the 66cl4 cell line.

Measurements of protein and mRNA level between the sub-clones varied quite much in the experiments carried out. Thus, it is difficult to say how much the sub-clones really displayed differences between them and the 66cl4 mother cell line. Further analyses should be performed before concluding.

5.2 Differences in GREM1 level indicating sign of heterogeneity between 66cl4 sub-clones

BMP4 are important in the development both during embryogenesis and in adult life by inducing differentiation of cells [44, 46]. GREM1 is a BMP4 antagonist and controls BMP4 signaling [48, 61, 62]. Together their active role contributes to keep homeostasis during the developmental process. Previously obtained data in our group showed highly upregulated levels of BMP4 and GREM1 in cell line 66cl4 contra 67NR (unpublished data, Ulrike Neckmann).

We asked whether the sub-clones differ in their expression of BMP4, pSMAD1/5/9 and GREM1. The immunoblotting results of BMP4 protein levels indicated an upregulated level of

BMP4 and no differences between the sub-clones and 66cl4 cell line were observed. Similar to BMP4, the phosphorylated level of SMAD1/5/9 suggested no variation between the sub-clones. Interestingly the sub-clones might differ in GREM1 protein level where sub-clone A6 stood out showing lower GREM1 level compared to 66cl4 cell line. Having seen a tendency of variation in GREM1 protein expression sub-clones were analyzed for BMP4 and GREM1 mRNA level. From the results it was observed similar BMP4 mRNA level while GREM1 showed a potential sign of variation between the sub-clones. Sub-clone A6 revealed to have lower GREM1 mRNA level than both 66cl4 cell line and sub-clone C6. This suggest that sub-clone A6 have a lower production of GREM1 as qPCR and immunoblots results seems to validate each other moving in the same direction.

BMP4s function in tumorigenesis might explain why BMP4 level is upregulated as shown in the results and in the transcriptome data. A study in breast cancer found highly metastatic cell line had an overexpression of BMP4 compared to cell lines with little invasive properties [52]. The elevated BMP4 level was found to increase migration and invasion cancer cells. BMP4 has also been associated with epithelial-to-mesenchymal transition (EMT) that lead to cancer cells mobility and metastatic abilities [52]. BMP4 was found to be one of the most frequent and highest expressed BMPs in breast cancer [42, 46].

5.3 CD24 was expressed independent of GREM1 in 66cl4 cells

Cancer development is tightly connected to differentiation and proliferation status of cancer cells. It is well established that low differentiated cancer cells of epithelial origin are more metastatic and lead to poor prognosis. GREM1 inhibit BMP driven differentiation and overexpression of GREM1 may directly contribute to a less differentiated phenotype. A study showed that glioma cancer stem cells highly secreted GREM1 that inhibit the BMP-induced differentiation by inhibiting BMP signaling and promote the CSC maintenance within the tumor hierarchy [75]. The transcriptome data was searched, and we found that CD24, a marker of low differentiation, was significantly higher in metastatic 66cl4 contra 67NR both in culture and in the primary tumor (unpublished data, Bjørkøy group). In line with such a notion we asked if GREM1 had any correlation with stem cell markers.

Flow cytometry were carried out and it was observed that CD24 level was clearly highly expressed in 66cl4 contra 67NR cells. Analyses of the two sub-clones that differ in GREM1 mRNA and protein expression did not vary from each other or from the 66cl4 cell line with respect to CD24 expression level. Manipulating GREM1 levels by treatment with rBMP4 did

not affect the CD24 levels of 66cl4 cell line or any of the sub-clones. If GREM1 and CD24 had any correlation, GREM1 would lead to loss in CD24 level in 66cl4 cell line and the sub-clones. Also, 67NR cells were treated rGREM1 and the 67NR cells did not respond to by changing CD24 levels.

The high level of CD24 that was clearly expressed in 66cl4 cells might be due to its role as a ligand for p-selectin. The interaction between CD24 and p-selectin enhances tumorigeneses by facilitating passage of tumor cells in the circulation during metastasis [76, 77]. Also, a study found that high level of CD24 in breast cancer was correlated to poor prognosis [80]. This could also be seen in survival curves in Kaplan Meier plotter where high CD24 mRNA level correlated to poor prognosis. This might suggest that CD24 have a role in metastasis.

Together the results indicated that GREM1 and CD24 was not correlated. By manipulating cells with BMP4 and GREM1 it was not possible to get 67NR cells to dedifferentiate and becoming more stem cell like thus expressing CD24, and 66cl4 which are low differentiated cells did not differentiate into epithelial cell and lose CD24 expression. One possible reason could be that the regulation of CD24 might happen at an early stage and it is irreversible. It is possible that GREM1 is only affecting the differentiation to a certain stage. However, there is no support for the CD24 level to depend on GREM1. Also, the process has not been tested in the group earlier. Therefore, it is unknown whether the concentration of rGREM1 was significant enough to activate the process. It is also unknown if stimulating cells with only rGREM1 is enough to initiate CD24 expression or additional factors are required. In addition, the time that the cells were stimulated with recombinant GREM1 might not be long enough to initiate the process. Due to time limit we did not perform additional experiments testing the different parameters, therefore optimization of the procedure should be performed.

5.4 GREM1 as therapeutic target

As GREM1 is highly expressed in metastatic 66cl4 cell line and correlates to poor prognosis it could be a potential therapeutic target. An upregulation of GREM1 has also proven to enhanced cancer cells survival and expansion in cancer associated stroma cells of different carcinomas [69]. The results obtained revealed that the sub-clones did not differ in in the level of the stem-cell marker CD24. Also, our results indicate that interfering with GREM1 alone is not sufficient to inhibit BMP signaling and change the differentiation status of the cancer cells.

GREM1 is not the only antagonist of BMPs. Of the BMP antagonists analyzed in the RNA sequencing data it was observed that the antagonists were differently expressed in 66cl4 and

67NR cell lines and primary tumors. A study from Gao et al. where the 4T1 breast cancer mouse model was used showed that COCO, a BMP antagonist, promoted reactivation of inactive tumor cells and metastatic relapse to the lungs [132]. In addition, COCO sustained the expression of several embryonic stem cell markers, Nanog, Oct4 and Sox2, that is often reactivated in metastatic breast cancer. The finding in this study is correlated to poor overall survival, however when comparing the antagonists in KM-plotter, it is clear that high COCO mRNA level is not as clinically relevant as GREM1. This suggests that there is some specificity in GREM1 that contributes to poor prognosis. Thus, the role of GREM1 and its correlation to CD24 should be examined *in vivo* as there might be limitation *in vitro*.

5.5 Variation of NQO1 and HMOX1 but not NFE2L2 level between 66cl4 sub-clones

CUL3 forms a complex with KEAP1 and ubiquitin ligase E3 to regulate NFE2L2 [93, 94]. Earlier obtained data in our group revealed that 66cl4 tumor cells had CUL3 mutation that was not found in any of the other four cell lines. The DNA sequencing revealed that all the sub-clones had the same CUL3 mutation. It is believed that the CUL3 mutation must have occurred early in 66cl4 and has not been subjected to selection or loss. Hence, it might be the reason why the mutation is carried in the genes of newly formed cancer cells when clones expand.

A mutation in CUL3 is a loss of function as leads to constant activation of NFE2L2 transcription factor [93, 94]. This results in transactivation of its downstream genes and was shown to provide a potential better environment for the cancer cells to proliferate [93, 94]. Since all the sub-clones had the same CUL3 mutation we also expect to have an increase of NFE2L2 level. It was observed that all the sub-clones had upregulated NFE2L2 protein level. Activation of NFE2L2 is important in the defense of oxidative damage in the cells [87, 88]. When checking for NQO1 and HMOX1 protein and mRNA level the results showed no correlation between NQO1 and HMOX protein and mRNA levels. All the sub-clones had a tendency of similar elevated protein level of NQO1 but differed in mRNA level compared to the 66cl4 cell line. On the other hand, the sub-clones showed varied protein level of HMOX1 but demonstrated similar level of mRNA when compared to each other and to the 66cl4 cell line. Further, comparing NQO1 and HMOX1 there were a tendency of sub-clone A6 having slightly higher expression of both markers contra sub-clone C6 and 66cl4 cell line. Since NQO1 and HMOX1 both are dependent of NFE2L2 this suggest that HMOX1 might need

other cofactors or components to get activated. Also, there are no correlation between mRNA and protein level. Not all NFE2L2 regulated genes are elevated and in line with this the transcriptome data showed that some NFE2L2 genes are upregulated in the 66cl4 cells but a wide range of known NFE2L2 genes are not upregulated. Together, these results indicate that HMOX1 protein level and NQO1 mRNA level between the sub-clones did differ demonstrating sign of heterogeneity. The findings for NFE2L2 and NQO1 protein expression might suggest similar levels for both markers between all the sub-clones.

The heterogeneity between the sub-clones in this thesis might be supported by another study that utilized the same 4T1 mouse model. The study demonstrated that when co-implanting the same amount of two 4T1 sub-clones they displayed differences in their metastatic potential [12]. Another study have demonstrated that different populations have different ability to form metastasis displaying heterogeneity among the cancer cells [133].

NQO1 is a ubiquitous cytosolic flavoenzyme that catalyzes the reduction of quinone substrates in one single step by using NADH or NADPH as substrates [98]. The direct two-electron reduction of quinone substrates prevent the production of cell damaging radical species [99-101]. The upregulation of NQO1 shown in the results could be due to NQO1s enzymatic function and might contribute to tumors aggressive phenotype. In KM-plotter high NQO1 mRNA level is correlated to poor prognosis. A study of breast cancer where overexpression of NQO1 shows poor differentiation and enhance tumor cell survival and the ability to metastasize to the lymph node. This was associated to a lower disease-free survival and five-year overall survival rate [107]. The highly expressed level of NQO1 in 66cl4 and its contribution in tumorigenesis makes it a target for therapeutic treatment. In line with this NQO1 inhibitors has been developed to prevent tumor growth and induce tumor cell apoptosis [134, 135]. Further experiments with NQO1 knockout can be carried out using CRISPR/Cas9 to study its role in metastasis.

5.6 Phenotype of 66cl4 sub-clones did not display heterogeneity

BMPs stimulate differentiation of epithelial cells. Thus, GREM1 as an BMP antagonist should be expected to block differentiation and to keep the cells in a low-differentiated state. In normal epithelial cells, there is an inverse correlation between proliferation and differentiation. The proliferation rate normally decreases as the cells differentiate. Since the sub-clones seemed to vary in GREM1 expression level we asked if this could affect the differentiation status and change the proliferation rate. However, there was no significant

difference in proliferation rate suggesting that the cells are also differentiated similarly. Such an observation is consistent with the observation that the sub-clones did not differ in in the level of the stem-cell marker CD24. Together, the results collectively suggest that interfering with GREM1 is not alone sufficient to change the differentiation status of the cancer cells and this limits the possibility of using GRME1 as a drug target.

Tumor heterogeneity can be one among many challenges that arise when finding the most suitable treatment for patients. For instance, when performing a biopsy only a small part of the tumor is taken as sample and analyzed. This might cause a loss of relevant lesions that could be of therapeutic importance [13]. In addition, it might be one of the reasons that creates low level of reproducibility in clinical research. However, by understanding the cause and characterization of tumor heterogeneity it may lead to better progression and knowledge for therapeutic therapy [9]

Functional analyses involve manipulating the protein levels by genetic approaches, isolate single clones harboring the manipulation and check for functional consequence. Such experiments rely on that sub-clones are similar to the mother cell so that eventual functional changes are due to the genetic manipulation and not from random variations in sub-clones. The study of a phenotype that cause tumors to become more aggressive is often studied by manipulating the gene of interest using CRISPR/Cas9 method. Given the results obtained in this thesis it is important for future experiments that the difference is due to the manipulation and not random variation. Therefore, it is important to reintroduce the gene in the mouse and see the phenotype change back. The findings in this thesis demonstrates that sub-clones of 66cl4 are similar in some indications (CUL3 mutation and BMP4 expression) but different in others (GREM1 and HMOX1 protein levels). The differences did not result in any variations with respect to proliferation rate or ability to form colonies in soft agar. Still, such heterogeneity is very important to be aware of when analyzing results from single cell clones after genetic manipulation. The conclusions drawn from such experiments also have to take into account that there might be an underlying variation between sub-clones that is independent of the specific manipulation performed. Thus, based on the results in this thesis, it is strongly recommended that genetic manipulations should also be reversed by adding back the gene or by inducible systems that allow a transient and reversible change in genotype and phenotype.

6 CONCLUSION AND FUTURE PERSPECTIVES

The study of tumor heterogeneity is important as it might how it might disturb therapeutic resistance as what might work for some cells in the tumor might have no effect on other cells. In addition, it might also be one of the reasons that creates low level of reproducibility in clinical research. Cells form a cell line is considered to be homogenous, but cancer cells are mutated all the time which might lead to sub-clone heterogeneity. The main aim of this study was to investigate the heterogeneity within 66cl4 cell line. 66cl4 cells were sorted to single cells and analyzed for differences based on markers from two different pathways. Our results indicated that the sub-clones might show sign of heterogeneity with respect to GREM1 and HMOX1 level. Comparable protein and mRNA level was observed in BMP4, NFE2L2 and NQO1 level for all the 66cl4 sub-clones. In addition, CD24 was expressed independent of GREM1. CUL3 mutation was also homogenously present in the sub-clones. The phenotype of the 66cl4 sub-clones did not differ with respect to proliferation rate but a reduced ability of colony formation in soft-agar was observer. However, measurements of protein and mRNA level between the sub-clones varied quite much between the experiments. It is difficult to say how much the sub-clones really displayed differences between them and the 66cl4 mother cell line. Therefore, further analyses should be performed.

To elucidate this further, based on the transcriptome data the heterogeneity between 66cl4 sub-clones could be examined for other markers that might have a role in metastasis. Amongst other methods, ELISA could be performed on proteins that are secreted to see if there are any differences between the sub-clones. *In vivo* experiments could be performed testing out the correlation between GREM1 and CD24. Also, we obtained 20 new sub-clones and only half of them were analyzed in this thesis. It could therefore be interesting to see if the other half show sign of heterogeneity based on the same markers that were analyzed in this study. It could also be interesting to see if the phenotype of the sub-clones shows sign of heterogeneity by performing experiments such as invasion assays as well as migration assays.

7 REFERENCES

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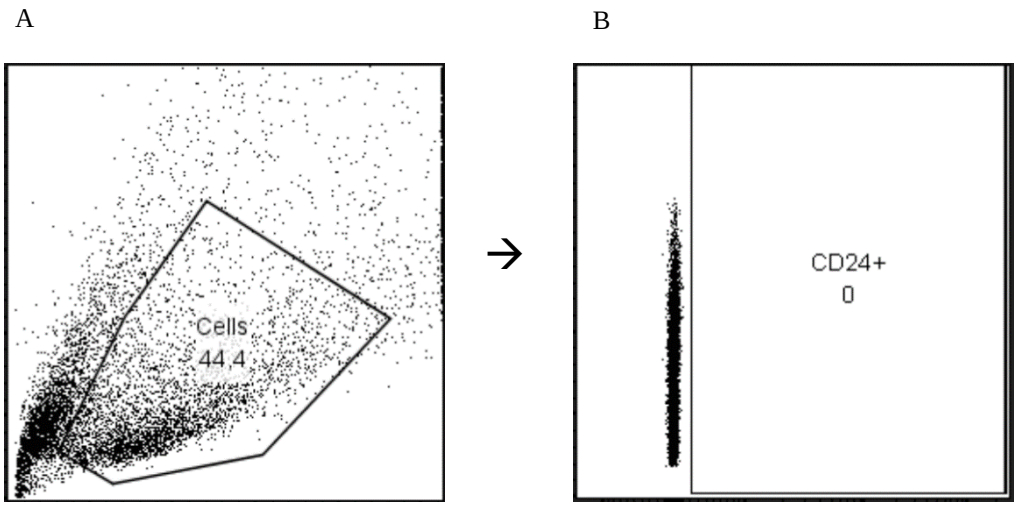
8 APPENDIX



Representative images of the gating strategies utilized in flow cytometry. A) Gating of live cells. B) Image represent the gating of negative control (left) and positive CD24 control (right square marked CD24+)