BMP7 AS A MODULATOR OF BREAST CANCER GROWTH AND MIGRATION

Master's Thesis
Minna Ampuja
Institute of Biomedical Technology
University of Tampere
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Minna Ampuja

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Rintasyöpä on naisten yleisin syöpä länsimaissa ja maailmanlaajuisesti se koskettaa miljoonia ihmisiä. Rintasyövän syntyyn ja uusiin hoitokeinoihin liittyvä tutkimus onkin merkittävä osa syöpätutkimusta. Luun morfogeneettiset proteiinit (BMP:t) ovat joukko kasvutekijöitä, joiden on havaittu liittyvän useisiin erilaisiin syöpiin, kuten rintasyöpään. Alun perin BMP:t löydettiin perustuen niiden kykyyn indusoida luun muodostusta. Nykyään BMP:t tunnetaan monipuolisina kehityksen aikaisina solun jakautumisen, erilaistumisen, apoptoosin ja liikkumisen säätelijöinä. Tämän tutkimuksen tavoitteena oli tutkia BMP7:n vaikutusta rintasyövän kasvuun, migraatioon ja invaasioon käyttäen viittä rintasyöpäsolulinjaa.

Tutkimusmenetelmät: Rintasyöpäsoluja (MDA-MB-231, MDA-MB-361, HCC1954, SK-BR-3 ja BT-474) käsiteltiin ihmisen rekombinantti BMP7:llä (50 ng/ml) tai vehikkelikontrollilla ennen funktionaalisia testejä. Solujakautumista mitattiin laskemalla solut ja solusyklianalyyseissä käytettiin PI-värjäystä ja virtaussytometriaa. Migraatiota ja invaasiota tutkittiin transwell-formaatin avulla. Lopuksi BMP7:n aiheuttamaa signaalireitin aktivoitumista mitattiin käyttämällä Western blot -menetelmää.

Tutkimustulokset: BMP7-käsittelyn vaikutukset olivat solulinjaspesifisiä. BMP7 esti MDA-MB-361- ja HCC1954-solujen kasvua (MDA-MB-361 keskimäärin 9 % ja HCC1954 19 %), lisäsi MDA-MB-231-solujen kasvua (128 %), mutta ei vaikuttanut BT-474- eikä SK-BR-3-solujen kasvuun. Kasvueroista huolimatta solusyklissä ei huomattu eroa MDA-MB-361- tai HCC1954-solulinjoissa BMP7- ja vehikkelikäsiteltyjen solujen välillä. BMP7 lisäsi 1,1–1,6 kertaisesti MDA-MB-361-, SK-BR-3- ja BT-474-solujen sekä 7,4-kertaisesti MDA-MB-231-solujen migraatiota. Invaasiota tutkittiin BT-474-soluilla, mutta BMP7- ja vehikkelikäsiteltyjen solujen välillä ei havaittu eroa. Western blot osoitti, että SMAD-proteiinien kautta kulkeva BMP signalointireitti oli aktivoitunut MDA-MB-231-, BT-474- ja MDA-MB-361-solulinjoilla, kun taas MAPK-reitti oli aktivoitunut HCC1954- ja SK-BR-3-soluissa.

Johtopäätökset: BMP7:llä on vaihtelevia vaikutuksia rintasyöpäsolujen fenotyyppiin. BMP7 joko lisää kasvua, vähentää kasvua tai ei vaikuta kasvuun. Lisäksi BMP7 aiheuttaa kohtalaista migraation lisääntymistä. Yhteenvetona voidaan todeta, että BMP7:llä on monitahoinen ja tärkeä rooli rintasyövässä ja sen vaikutukset ovat solulinjaspesifisiä.

MASTER'S THESIS

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Abstract

Background and aims: Breast cancer is the most common cancer in women in Western countries and a global health concern affecting millions of people. Thus the search for causative agents and possible curatives continues to be important. Bone morphogenetic proteins (BMPs) are a group of growth factors that have been implicated in various cancers, including breast cancer. They were originally discovered due to their ability to induce bone formation, but are now recognized as multifunctional developmental regulators of cell proliferation, differentiation, apoptosis and motility. Dysregulation of BMP signaling pathways has been shown to contribute to cancer progression. The aim of this study was to examine the effect of BMP7 on breast cancer cells through proliferation, migration and invasion studies in five breast cancer cell lines.

Methods: Breast cancer cells (MDA-MB-361, MDA-MB-231, HCC1954, SK-BR-3 and BT-474) were treated with recombinant human BMP7 (50 ng/ml) or vehicle control followed by functional assays. Effects on proliferation were measured by counting the cells and cell cycle analyses were performed using PI staining and flow cytometry. Migration and invasion were studied using the transwell assay. Finally, signaling cascade activation upon BMP7 stimulation was analyzed with Western blotting.

Results: BMP7 treatment resulted in cell line-specific phenotypes. BMP7 decreased the growth of MDA-MB-361 and HCC1954 cells (on average 9% and 19%, respectively), increased the growth of MDA-MB-231 cells (128%) and had no effect on the two remaining cell lines. However, no differences in cell cycle were detected between BMP7- and vehicle-treated MDA-MB-361 and HCC1954 cells. BMP7 moderately increased migration in MDA-MB-361, SK-BR-3 and BT-474 (from 1.1-fold to 1.6-fold). A more dramatic increase (7.4-fold) was seen in MDA-MB-231 cells. Furthermore, invasion of BT-474 cells was examined but no changes were detected between BMP7- and vehicle-treated cells. Western blot showed that the canonical BMP signaling pathway through SMAD proteins was activated in MDA-MB-231, BT-474 and MDA-MB-361 cells, whereas in HCC1954 and SK-BR-3 cells the MAPK pathway was induced.

Conclusions: BMP7 has diverse effects on the growth of breast cancer cells. BMP7 induces both proliferative and anti-proliferative effects, as well as having no influence on cell growth. Moderate increases are seen in migration upon BMP7 stimulation. In conclusion, BMP7 has an important and complex role in breast cancer, and its effects are dependent on cellular background.

CONTENTS

Abbreviations

BMP	Bone morphogenetic protein
BRE	BMP response element
BSA	Bovine serum albumin
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ERBB2	Epidermal growth factor receptor
FBS	Fetal bovine serum
GDF	Growth and differentiation factor
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
MAPK	mitogen-activated protein kinase
PI	Propidium iodide
PR	Progesterone receptor
SMAD	Sma- and Mad-related protein
SMURF	SMAD ubiquitin regulatory factor
SBE	SMAD binding element
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGF-β	Transforming growth factor-β

1 Introduction

Worldwide, cancer is one of the leading causes of death together with cardiovascular and infectious diseases (Mathers et al., 2008). In women the most common cancer is breast cancer, which also causes more deaths than any other cancer type in women (Mathers et al., 2008). Cancer is a genetic disease, caused by mutations that release the cells from normal control and allow them to grow in an unrestricted manner. Cancerous cells are able to invade tissues and spread to other parts of the body, where they grow as metastases. In order to metastasize cancer cells must be able to invade through the basement membrane, pass into lymphatic or vascular system, attach to the lymph/blood vessel wall, extravasate to the target organ and subsequently proliferate in the target organ (Lu and Kang, 2007). Metastases are the major cause of death in cancer patients. Different cancer types prefer different tissues as the destination of metastases. Breast cancer metastasizes most frequently to bone, lung, liver and brain (Lu and Kang, 2007). Although treatment of breast cancer and cancer altogether has improved considerably, no definitive cure has been found and the treatments in use today, such as surgery, radiation and chemotherapy, are exhausting to the patients (Witt and Murray-Edwards, 2002; Schreier and Williams, 2004). Therefore, there are still many goals to be achieved in breast cancer research.

Both genetic and environmental factors contribute to cancer progression. Breast cancer can be divided in hereditary and sporadic cancers (Kenemans et al., 2004). In hereditary breast cancers a germline mutation in a tumor-suppressor (antitumorigenic) gene combined with inactivation of the other allele is the trigger for cancer progression. In sporadic cancers, on the other hand, activation of an oncogene can also be the early event that initiates the development of cancer. Nevertheless, many mutations in key cellular signaling pathways are needed for cancer progression (Karakosta et al., 2005). These mutations accumulate to the cells gradually; because of that cancer is a disease affecting mostly older people.

Bone morphogenetic proteins (BMPs) are a group of growth factors capable of regulating many cellular processes, such as proliferation and migration (Rider and Mulloy, 2010). They are excreted from cells and bind to their specific cell surface receptors inducing the SMAD (Sma- and Mad-related protein) or other signaling pathways. Due to their regulatory role they have been linked to the progression of many cancer types (Singh and Morris, 2010). However, their role in cancer is complex, with

both tumorigenic and antitumorigenic effects cited (Singh and Morris, 2010). In this study the role of BMP7 in breast cancer was studied. To this end five breast cancer cell lines were treated with recombinant human BMP7 in order to analyze its effects on proliferation, migration, invasion and signaling cascade activation.

2 Review of the literature

2.1 Breast cancer

Breast cancer affects mostly women (less that 1% of breast carcinoma patients are men) and typically develops at a later age (Fentiman et al., 2006). The malignant cells are most often the epithelial cells that form the milk ducts or lobules of the breast (Polyak, 2001). The two types of epithelial cells are the luminal cells that line the lumen and the myoepithelial cells that are in contact with the basement membrane (Figure 1). The milk ducts originate in the milk-producing lobules that form the twenty lobes of the breast. The ducts are surrounded by stromal connective tissue and the mammary fat. Experimental studies indicate the existence of mammary stem cells that could give rise to the epithelial cells of the ducts and thus enable the changes in the epithelia associated with puberty, pregnancy and lactation (Polyak, 2001).

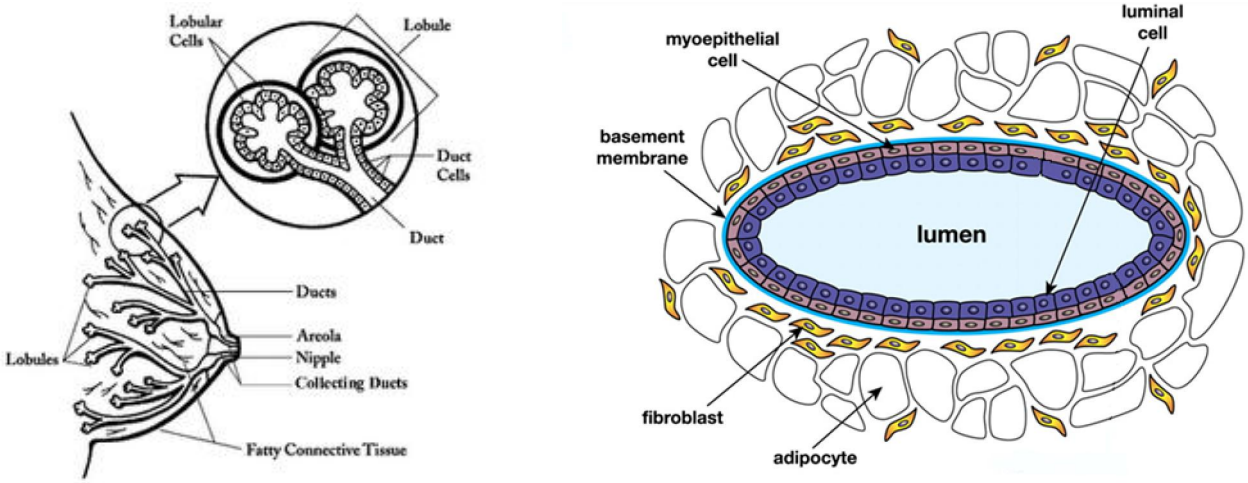

Figure 1. A diagram of breast structure. The milk ducts originate in the lobes and lobules of the breast. The ducts and lobes are enclosed by fat and stromal tissue. The lumen of ducts is surrounded by luminal and myoepithelial cells. A basement membrane surrounds the myopethelial cells separating them from stroma. Figure adapted from Visvader (2009) and the medical articles directory (http://www.medinik.com/cancer/breast-cancer-symptoms).

2.1.1 Breast cancer epidemiology and genetics

Breast cancer is the most common cancer among women. It has been considered an illness affecting mostly women in Western countries. However, breast cancer incidence in developing countries is on the rise. The increased incidence is often attributed to a high fat diet, obesity and lack of exercise, although experimental evidence is still lacking (Porter, 2009). Worldwide, 1 384 155 women were estimated to be diagnosed

with breast cancer in 2008 (Ferlay et al. 2008). In Finland 32% of cancers in women (4461 cases) were diagnosed as breast cancer in 2009 (Finnish Cancer Registry). The number of diagnosed cases has risen steadily since 1953 when documentation first started. In addition, 15.6% of cancer-related deaths in women were caused by breast cancer in 2009, making it the leading cause of death among women's cancers in Finland. The amount of deaths has been relatively steady since the beginning of 90's. The rise of incidence is predicted to have occurred due to unhealthy or altered life style and earlier diagnosis (Parkin and Fernandez, 2006). On the other hand, early diagnosis together with better treatment has ensured that cancer mortality has not risen in the same fashion (Porter, 2009).

Together with genetic changes, environmental agents are responsible for initiating cancer. Risk factors for developing breast cancer have been studied in numerous epidemiological surveys. A large twin study in Denmark, Sweden and Finland revealed that approximately one fourth of breast cancers can be explained by genetic factors (Lichtenstein et al., 2000). This leaves a substantial role for environmental contribution. Strong correlation has been found with breast cancer incidence and early menarche, having no children or having children late in life, late menopause and hormonal therapy (Parsa and Parsa, 2009). All these factors are connected to the hormonal balance of the body, and accordingly it has been found that overexposure to proliferative hormones and growth factors is associated with cancer (Reid et al., 1996). The combinatorial effect of early menarche and late menopause exposes the body to hormonal factors for a longer time. The benefit of bearing children has been associated with hormone levels, differentiation status of mammary gland cells and increased physical activity related to having many children, whereas hormonal therapy for the treatment of menopause increases hormone exposure making the cells susceptible to increased proliferation (Parsa and Parsa, 2009).

Research has been active on the effect of other environmental factors in breast cancer development. Obesity, high fat intake, alcohol and cigarette consumption and an inactive lifestyle have all been linked to cancer progression in general as well as breast cancer individually. Obesity in postmenopausal women seems to increase the occurrence of breast cancer. In particular, obese women had a higher incidence of aggressive tumors as well as having a worse overall outcome (Dawood et al., 2008; Litton et al., 2008). Obesity in younger women, on the other hand, seemed to have no effect. Dietary factors can also contribute to breast cancer progression. A diet containing

high saturated fat was associated with increased risk of breast cancer in some studies (Patterson et al., 2010). Excessive alcohol intake has been linked to increased risk of breast cancer (Terry et al., 2006). However, conflicting evidence has been found, with some studies showing no correlation between alcohol consumption and risk of developing breast cancer (Thygesen et al., 2006). Exercise seems to reduce the risk of breast cancer, as shown in numerous epidemiological studies, although results have not been completely consistent (Patterson et al., 2010). The effects of environmental and work-related mutagens and carcinogens, such as UV-light and tobacco toxins, can be manifested in the progression of many cancer types. Hormone-regulated cancers such as breast cancer can be affected by endocrine disrupting compounds, such as bisphenol A and phthalates, which mimic or block hormones causing hormone imbalance (Brody, 2010). In addition, people are subjected to a large amount of naturally occurring or synthetic chemicals for which no information regarding carcinogenicity is available (Weyandt et al., 2008). Finally, ionizing radiation is a significant risk factor for developing breast cancer (Khan et al., 2010).

Cancer results from hereditary or sporadic mutations that lead to uncontrolled growth of cells, formation of a primary tumor and dissemination of cells through the blood or lymphatic system to form metastases. The mutations caused by environmental agents are called sporadic mutations. Some mutations, however, can be inherited through the germ line and are referred to as hereditary mutations. Both types of mutations can contribute to cancer progression and thus cancer is often an interplay between environmental and genetic factors. Aberrant behaviour of cancer cells is due to disruption of many cellular pathways related to growth, apoptosis and other processes important to cancer. Five distinct pathways have been proposed based on the fact that disruption in these pathways transforms human cells into oncogenic cells (Hahn and Weinberg, 2002). These pathways are the p53 pathway (responsible for cell cycle and apoptosis control), Ras pathway (leads to mitogenic stimulation of cells), retinoblastoma pathway (involved in cell cycle control), telomere maintenance and PP2A (a protein phosphatase) pathway. Inactivation/activation of these pathways can be caused by mutations in different proteins of the pathway. Inactivation of genes is often a result of deletions, inversions, insertions or point mutations. Translocation under a strong promoter, increased copy number or point mutations, on the other hand, can lead to activation of genes (Vogelstein and Kinzler, 2004).

Among the most studied anti-tumorigenic agents in hereditary breast cancer are *BRCA1* and *BRCA2*. The protein products of these genes repair double-strand breaks in DNA. Consequently mutations in these genes confer a high risk for breast cancer (Mavaddat et al., 2010). In addition, mutation of *TP53*, a regulator of apoptosis and cell cycle, is associated with an increased risk (Ahmed et al., 2009). Sporadic mutational inactivation of these genes is rare because inactivation of both alleles is usually needed (Kenemans et al., 2004). ERBB2/HER2 receptor, the expression of which defines the breast cancer subtype ERBB2+ in molecular profiling of breast cancer (see section 2.1.2), is one of the most well-known proteins associated with tumor progression in sporadic cancers. Antibodies against this receptor significantly inhibit the growth of ERBB2 positive breast cancer cells and a therapeutic antibody trastuzumab is in clinical use (Menard et al., 2004). Other commonly mutated genes in sporadic breast cancer include the transcription factor Myc, the cell cycle-related cyclin D1/*CCND1* and the actin-binding protein cortactin/*EMS1* (Kenemans et al., 2004).

In addition to genomic changes, an increasing amount of attention is focused on epigenomic alterations. Epigenomic changes do not alter the sequence of DNA, but rather its structure. Epigenetic mechanisms, including methylation of promoter sequences, chromatin remodeling and modification of histones, influence regulation of gene expression. The contribution of epigenetic changes to cancer progression appears to be substantial and is now recognized widely (Herceg, 2007). Hypermethylation of anti-tumorigenic gene promoters prevents transcription, and this event occurs early in cancer progression (Baylin and Ohm, 2006). Likewise, hypomethylation leading to activation of pro-tumorigenic genes and chromosomal instability and breakage is connected to cancer development (Feinberg and Tycko, 2004). Finally, non-coding RNAs, such as micro RNAs, regulate gene expression. They are also involved in cancer, where they can have either tumor-suppressive or tumor-promotive roles (Le Quesne and Caldas, 2010).

The aforementioned concept that cancer is a genetic disease resulting from mutations in cancer-promoting and cancer-inhibiting genes (oncogenes and tumor suppressors, respectively) has been accepted for a long time. However, nowadays it has been recognized that this view may be too simple to describe the whole process of carcinogenesis. For example, epigenetic modulators are hard to fit into these categories. Furthermore, aberrant growth factor or cytokine signaling can influence cancer progression (Halper, 2010). The transforming growth factor-β (TGF-β) has been shown

to first inhibit growth of cells and later induce tumor progression (Moses et al., 1994). In addition, even though mutations in components of the TGF-β signaling pathway have been detected, mutations in the TGF-β ligand itself have not been found in cancer (Harradine and Akhurst, 2006). This makes placing TGF-β in either the class of oncogenes or tumor suppressors even harder, since traditionally oncogenes and tumor suppressors are cancer-causing genes only when mutated (Vogelstein and Kinzler, 2004).

2.1.2 Classification of breast cancer

Historically, breast cancer has been classified based on histology. The non-invasive breast tumors, such as ductal carcinoma *in situ*, are believed to precede the invasive stage (Hergueta-Redondo et al., 2008). The most common breast cancer type is infiltrating/invasive ductal carcinoma of no special type (IDC-NST) or not otherwise specified (IDC-NOS), which accounts for 50-80% of breast tumors (Weigelt et al., 2010). The other breast cancer types are referred to as special types and are rare compared to IDCs. The second most common type, infiltrating/invasive lobular carcinoma (ILC), accounts for only 5-15 % of tumors. It is important to note that the names of the cancer types do not reflect the origin of the tumor but rather the histological appearance (Weigelt et al., 2010). In addition to ILCs, there are other special types, such as medullary, apocrine and mucinous carcinomas, that are clinically important and often associated with specific outcomes (Weigelt and Reis-Filho, 2009).

In addition to histological type, breast tumors are clinically classified based on tumor grade and stage. In the Nottingham method for classification of tumor biology and prognosis, the histological grade of the tumor is evaluated using three different characteristics (Elston, 2005). Glandular differentiation, nuclear pleomorphism (abnormal nucleus) and mitotic counts are assigned a value from 1 to 3. Based on the sum of the values the tumors are assigned a grade from I to III. Histological grading can be combined with tumor size and lymph node status in Nottingham Prognostic Index (NPI) to predict the outcome of cancer. Additionally, the receptor status of the tumor is associated with prognosis and influences treatment decisions (Orlando et al., 2010). The estrogen (ER) and progesterone (PR) receptor status reflects the responsiveness of the tumor to endocrine therapy. Inhibiting the action of estrogen and progesterone in ER and PR positive tumors leads to decreased cancer progression. This inhibition can be achieved by blocking the receptor, downregulating its expression or suppressing

estrogen production. Tamoxifen is the most common drug used in endocrine therapy and it acts by competing with estrogen for receptor binding (Orlando et al., 2010). Taken together, the most relevant factors in clinical practice are tumor stage, grade and receptor status.

Besides histology, certain genetic aberrations have been suggested to serve as a basis for classifying primary breast cancers. Using gene expression profiles a new classification of breast cancer has been modulated. In this new classification system there are five categories that are luminal type A, luminal type B, ERBB2+/HER2+, basal type and normal-like breast cancer (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). These subtypes correspond to different clinical outcomes, thus providing a basis for more individualized treatment.

Luminal type A is characterized by the best prognosis of all five subtypes and a long period of disease-free time before the occurrence of metastasis (Sorlie et al., 2003). Luminal type B is characterized by moderate expression of luminal-specific genes and an intermediate prognosis compared to the other groups (Sorlie et al., 2001). Prognosis for patients with ERBB2+ subtype is poor. The subtype is defined by expression of several genes of the ERBB2 amplicon (Sorlie et al., 2001; Nakshatri et al., 2009). Basal type breast cancers are also called triple negative, because they are characterized by lack of expression of ER, PR and ERBB2. This subtype of cancer is associated with the worst prognosis from all the five classes (Sorlie et al., 2003). Normal like breast cancers express many genes that are also expressed by the non-epithelial cells, such as adipose cells (Sorlie et al., 2001). In addition, they express strongly basal epithelial genes and weakly luminal epithelial genes. It is possible that this subgroup comprises tumors in which normal cells are over-represented and therefore is not a true subgroup (Hergueta-Redondo et al., 2008).

The differences between the molecularly defined breast cancer subtypes could be explained by the theory that they originate from different cell types (Sorlie et al., 2003). In fact, there is some evidence that the gene expression profiles of the different subtypes correlate with the gene expression profiles of different types of cells in breast tissue; luminal epithelial cells with luminal subtype and basal epithelial with basal subtype (Sorlie et al., 2003). The differences can additionally be connected to the differentiation status of breast cancer cells (Hergueta-Redondo et al., 2008). The basal type of cancer might originate from progenitor cells whereas differentiated or progenitor luminal cells could give rise to luminal breast cancer types.

2.1.3 The origin of breast cancer

Even as the nature of cancer genes is being discussed, the cell of origin in cancer is also under debate. Traditionally cancer progression has been explained by the theory of clonal evolution and only recently the possibility of cancer stem cells has presented another view (Shackleton et al., 2009). The theory of clonal evolution states that in the event of beneficial mutations accumulating in a single cell or a small population of cells, that cell/cell population continues to expand at the expense of other cells in the population, creating heterogeneity (Campbell and Polyak, 2007). The instability of cancer cell genomes, the heterogeneity of cancer cells, their unlimited proliferative ability and the appearance of drug-resistant cancer cells after treatment can all be explained by clonal evolution and have been used as evidence for its validity in explaining cancer evolution (Campbell and Polyak, 2007).

The theory of cancer stem cells proposes that a small amount of the tumor mass contains cancer stem cells, which give rise to the other cells of the tumor mass (Shackleton et al., 2009). Despite many experimental tests supporting the existence of cancer stem cells, the issue remains controversial with scientists both opposing and advocating this concept. The pioneering experiment performed by Bonnet and Dick (1997) showed that acute myeloid leukemia (AML) was initiated in mice after progenitor-like AML cell transplantation and not after transplantation of more differentiated AML cells. In 2003 Al-Hajj et al. discovered a subset of breast cancer cells, characterized by high CD44 and low CD24 expression ($CD44^+/CD24^-$), capable of inducing cancer in immunodeficient mice. The rest of the breast cancer cells, representing the majority of cancer cells, were unable to initiate cancer even in large amounts. Additionally, activation of stem cell-associated pathways (such as Hedghehog, Wnt and Notch) in cancer cells and the theory that recurrence of cancer may result from slowly proliferating cancer stem cells surviving treatment are in support of the cancer stem cell model (Campbell and Polyak, 2007).

Stem cells in the human breast have been identified using keratin profiling and flow cytometry with specific cell surface markers. Commonly used cell surface markers are CD44, CD24 and CD133 (Nakshatri et al., 2009). Normal breast stem-cell like cells have been characterized by increased expression of aldehyde dehydrogenase 1 (ALDH1) and basal keratins K5/K6 (Ginestier et al., 2007). Also $CD44^+/CD24^-$ phenotype has been connected to stem cell-like status, both in normal breast cells and breast cancer cells (Al-Hajj et al., 2003; Shipitsin et al., 2007). Usually two or three

markers are used to identify cancer stem cell-like cells, but such a limited amount of markers may result in ambiguity and is one of the reasons for doubt against the existence of cancer stem cells (Campbell and Polyak, 2007).

To date putative cancer stem cells have been found in many different types of cancers (Shackleton et al., 2009). However, some characteristics of cancer stem cell-like cells can be attributed to clonal evolution. For example resistance to treatment can be due to genetic and epigenetic differences resulting from clonal evolution rather than the existence of therapy-resistant cancer stem cells (Shackleton et al., 2009). It has been proposed, therefore, that cancer stem cells could explain the progression of some of the cancer types, while clonal evolution contributes and in some cancer types seems to be the only model of creating heterogeneity (Shackleton et al., 2009).

Prior to the theory of stem cells the general consensus was that breast cancer arose from transformation of the luminal epithelial cells. Recognition of stem cells capable of differentiating both to luminal epithelial and myoepithelial cells questions the cells of origin of breast cancer (Hergueta-Redondo et al., 2008). However, concerning cancer stem cells there is no consensus on whether the observed cancer stem cells originate from normal stem cells or cancer cells which have reverted to stem cell-like condition (Nakshatri et al., 2009). In conclusion, there is still a lot of research to be done to improve the treatment of breast cancer. Knowing the origin of breast cancer and the genetic and epigenetic aberrations associated with tumor progression is a crucial aspect of the process.

2.2 Bone morphogenetic proteins

2.2.1 The structure and function of bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are a family of secreted signaling molecules belonging to the transforming growth factor-β (TGF-β) superfamily (Kingsley, 1994). The BMP family consists of over 20 members that are named BMP or GDF (growth and differentiation factor). Precursor BMPs are 400-500 amino acids long and contain an N-terminal signal domain, a prodomain that helps in proper folding, and a C-terminal mature peptide (Figure 2) (Bragdon et al., 2010). All BMPs share the same structure, the cystine knot motif of the TGF-β superfamily. The cystine knot involves seven cysteine residues, six of which form three intramolecular disulfide bonds (Daopin et al., 1992; Schlunegger and Grutter, 1992). Mature BMPs exist as dimers. Dimerization occurs

when the remaining cysteine of a BMP monomer forms an intermolecular bond with another monomer. Subsequently the N-terminal prodomains are proteolytically cleaved and the dimer is secreted as a biologically active, 50-100 amino acids long BMP molecule (Bragdon et al., 2010). In addition to homodimers, heterodimers of some BMPs, notably BMP2/BMP7 have been observed (Little and Mullins, 2009).

BMPs were originally found based on their ability to induce extraskeletal bone and cartilage formation (Gazzerro and Canalis, 2006). Later they were found to be important regulators of developmental processes. BMPs act as diverse morphogens in different tissues based on their ability to regulate cell proliferation, differentiation, apoptosis and motility. Numerous *in vivo* studies have demonstrated the importance of BMPs in organogenesis. Knock-out of *bmp2*, *bmp4* or *bmp10* in mouse is embryonically lethal (Winnier et al., 1995; Zhang and Bradley, 1996; Chen et al., 2004). Lack of other BMPs cause aberrant but viable phenotypes, often displaying skeletal abnormalities. For example, knock-out of *bmp3* led to increased trabecular bone formation, knock-out of *bmp6* caused delayed ossification in the developing sternum and knock-out of *bmp8a* resulted in spermatogenesis malfunction (Zhao et al., 1996; Solloway et al., 1998; Bahamonde and Lyons, 2001). The evident diversity of BMP function has led to the suggestion of renaming BMPs as body morphogenetic proteins (Wagner et al., 2010).

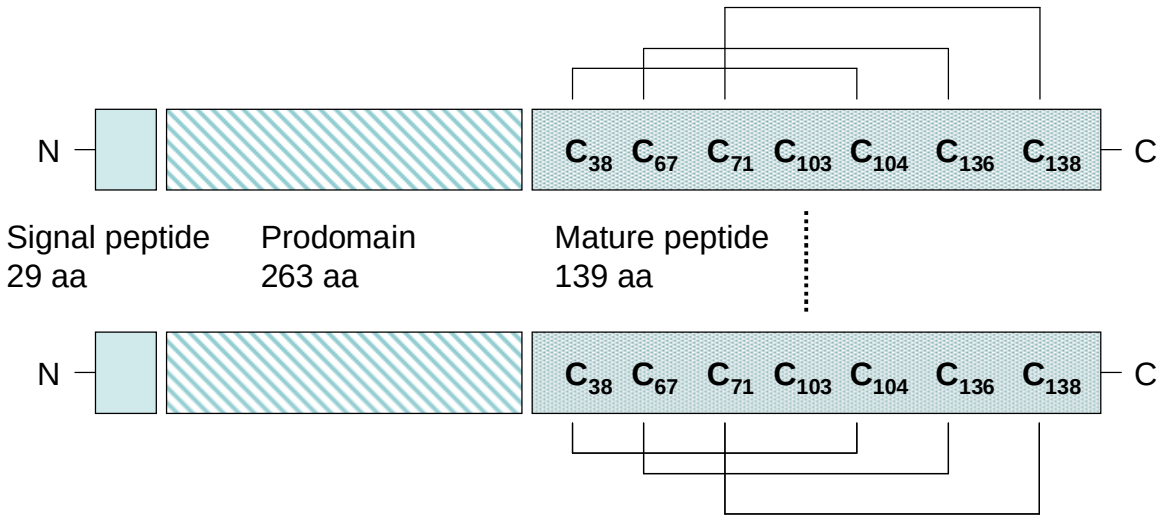

Figure 2. BMP7 precursor domain structure. The BMP7 precursor molecule contains a signal sequence, a prodomain and the mature domain. The number of amino acids in each domain of BMP7 is indicated (Celeste et al., 1990; Ozkaynak et al., 1990). Six cysteine residues of the monomer form three intramolecular cysteine bonds. The remaining cysteine forms a bond with another monomer in the mature BMP dimer. The intra- and intermolecular bonds are indicated with dashes (Griffith et al., 1996).

BMP7, also known as osteogenic protein -1 (OP-1), was discovered by Özkaynak et al. in 1990. Its three-dimensional structure has been solved to a 2.8-Å resolution (Griffith et al., 1996). The prodomains of BMP7 proteins are proteolytically cleaved, but they remain associated with the secreted dimers, a phenomenon also confirmed for BMP4, -5 and -10 and GDF5 and -8 (Sengle et al., 2008a; Sengle et al., 2011). However, the prodomains of BMP4, -5 and -7 do not prevent signaling, in contrast to the prodomains of TGF-β, GDF-8 and BMP10, because BMP receptors are able to compete with the prodomain (Sengle et al., 2008b; Sengle et al., 2011). BMP7 is expressed in a wide variety of tissues, including breast, thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas and prostate (Alarmo et al., 2006; Bragdon et al., 2010). Homozygous deletion of *Bmp7* in mice results in perinatal lethality, with renal dysplasia, anophthalmia and minor defects in the skeleton (Dudley et al., 1995).

2.2.2 BMP signaling

Receptor assembly

BMPs bind as dimers to a receptor complex that consists of two type I receptors and two type II receptors. The three type I (BMPRIA or ALK3, BMPRIB or ALK6 and ACVR1A or ALK2) and three type II (BMPR2, ACVR2A and ACVR2B) receptors used by BMPs are all serine/threonine kinases (Sieber et al., 2009). They contain an extracellular ligand binding domain, an intramembrane segment and an intracellular kinase domain. Ligand binding leads to phosphorylation of type I receptor by the constitutively active type II receptor. The signal is transmitted to the nucleus mainly using the SMAD (Sma- and Mad-related protein) pathway (shared by the TGF-β family of growth factors) or mitogen activated protein kinase (MAPK) pathway. BMP receptors have been found to be expressed at least in thymus, bone marrow, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas and prostate (Bragdon et al., 2010).

Typically BMPs bind to type I receptors with different specifities and affinities. BMP7 binds strongly to ACVR1A and weakly to BMPRIA and BMPRIB (Liu et al., 1995; Macias-Silva et al., 1998). Binding to the type I receptor is affected by binding to the type II receptor, as observed by deletion of BMPR2 receptor leading BMPs to use different type I receptors (Yu et al., 2005). Interestingly, disruption of BMPR2

enhanced BMP7 signaling, which proceeded through ACVR1A and ACVR2A (Yu et al., 2005).

BMPs signal to the nucleus through intracellular SMAD proteins or through other pathways, including the MAPK pathway. There is evidence that the signaling pathway activated depends on whether the BMP binds to a preformed complex of type I and type II receptors or first to a type I receptor followed by recruitment of a type II receptor (Nohe et al., 2002). The SMAD pathway is triggered upon ligand binding to a preformed complex whereas binding of BMPs first to a type I receptor (the formation of BMP-induced signaling complex) leads to activation of the MAPK pathway. In addition, considerable crosstalk exists between these and other signaling pathways (Miyazono et al., 2005). The restriction of BMP and BMP receptor expression to a specific tissue or specific time, in addition to the paracrine manner of BMP expression and the action of BMP antagonists together with signaling crosstalk create the possibility of diverse signaling despite the relatively small amount of receptors and the promiscuity of receptor binding (Rider and Mulloy, 2010).

The SMAD pathway

The major signaling route of BMPs is the canonical SMAD pathway (Figure 3). The active ligand-receptor complex phosphorylates receptor-SMADs (R-SMADs), three of which (SMAD1, SMAD5 and SMAD8) are employed by BMPs (Miyazono et al., 2005). R-SMADs in turn pair with the common SMAD, SMAD4 or co-SMAD, resulting in translocation of the complex to the nucleus. SMADs contain two conserved domains, the mad homology 1 (MH1) and MH2 domain, and a variable linker region in between (Gazzerro and Canalis, 2006). In order to regulate gene expression the SMADs bind directly to DNA in the promoter regions of BMP responsive genes. MH1 domain is responsible for interaction with DNA and MH2 domain binds to various intracellular regulators and mediates R-SMAD oligomerization (Gazzerro and Canalis, 2006). The C-terminal SXSS-sequence, the site of phosphorylation by the receptor, is also located in the MH2 domain (Sieber et al., 2009). The linker region is subject to modifications regulating the activity of SMADs (Eivers et al., 2008).

The MAPK pathway and signaling cross-talk

It has been shown that BMPs can signal through MAP kinases in a SMAD-independent manner (Figure 3). The ERK1/2, p38 and JNK pathways have all been implicated as

alternative routes to the canonical SMAD signaling (Bragdon et al., 2010). The mechanisms and the extent of MAPK signaling under different circumstances remain poorly characterized. XIAP and BRAM1, however, have been shown to mediate SMAD-independent signals from the BMP receptors to other signaling components of the MAPK pathway (Kurozumi et al., 1998; Yamaguchi et al., 1999; Wu et al., 2006). For example, BMP4 and BMP2 stimulation activates through XIAP the MAP kinase kinase kinase TAK1, which can in turn be bound by TAB1 (Shibuya et al., 1998; Kimura et al., 2000). In addition, BMP2 stimulation leads to activation of ERK and RAS in osteoblasts (Lou et al., 2000; Lai and Cheng, 2002). However, more information is available for signaling crosstalk between SMAD and other pathways than MAPK pathway alone.

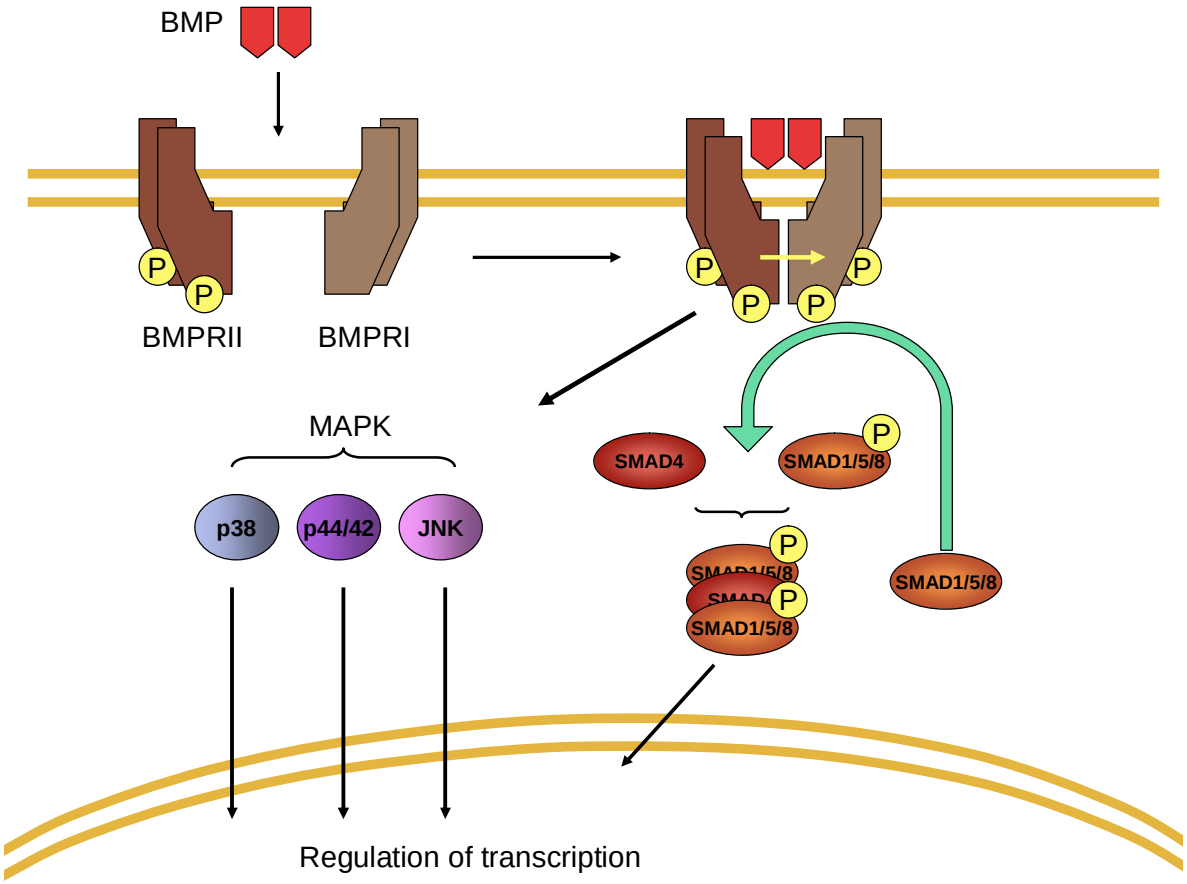

Figure 3. BMP signaling pathways. A BMP dimer binds to a receptor complex, resulting in phosphorylation of the type I receptors by the type II receptors. SMAD1, -5 or -8 is phosphorylated and pairs with SMAD4 and another SMAD1/5/8, eventually translocating to the nucleus and regulating gene expression. Alternatively the MAPK pathway may be activated.

MAP kinases can cross-talk with the SMAD pathway by phosphorylating R-SMADs or SMAD4 and thus regulating BMP signaling. ERK1/2 kinase activated by various growth factors can phosphorylate the linker region in SMAD1, which leads to repression of BMP signaling through nuclear exclusion (Kretzschmar et al., 1997; Eivers et al., 2008). This repression results from inhibition of nuclear translocation of the MAP kinase phosphorylated SMAD or interaction with the SMURF ubiquitinases leading to degradation. Oncogenic Ras signaling through MEK/ERK decreases the stability of SMAD4 (Saha et al., 2001). JNK and p38 seem to preferentially phosphorylate tumor-derived mutant SMAD4 and promote its proteasomal degradation (Liang et al., 2004). ERK, JNK, and p38 have all been implicated in the transcriptional regulation of SMAD7, an inhibitor of BMP signaling, therefore indirectly regulating TGF-β signaling (Uchida et al., 2001; Dowdy et al., 2003).

In addition, signaling cross-talk has been found between the SMAD pathway and the Wnt/β-catenin, Notch, Ca+/calmodulin and JAK/STAT pathways (Nohe et al., 2004; Miyazono et al., 2005). These signaling pathways can either affect BMP signaling directly or through other regulators of BMP signaling (Miyazono et al., 2005). BMP signaling is therefore a complex process involving a network of different pathways and signaling molecules.

2.2.3 Regulation of BMP signaling

Extracellular signaling regulation

Based on current knowledge extracellular regulation of BMP signaling is mostly inhibitory in nature, with numerous extracellular BMP antagonists deterring BMP signaling by binding to BMPs and preventing their association with their receptors (Table 1) (Rider and Mulloy, 2010). Antagonism of BMP signaling is especially important during development, when antagonists regulate the BMP gradient that determines the organization of tissues. Many BMP antagonists share the similar structure of cystine knot with the BMP ligands. Based on the size of the cystine ring the extracellular antagonists have been divided into three subgroups: 1) the chordin and noggin family, 2) twisted gastrulation and 3) the DAN family (Avsian-Kretchmer and Hsueh, 2004).

BMP antagonists are important in development, for example null mutations of *noggin*, *gremlin* and *chordin* genes in mice are embryonically or perinatally lethal (Gazzerro and Canalis, 2006). The antagonists have also been studied in cancer, where

they can either promote or inhibit cancer progression (Walsh et al., 2010). Many antagonists are able to bind several BMPs (Gazzerro and Canalis, 2006). Furthermore, some antagonists can interact with each other or regulate the activity of another antagonist. For example, sclerostin is able to bind to noggin, which results in abolishment of both their antagonistic effects and twisted gastrulation promotes chordin degradation (Walsh et al., 2010). Clearly in addition to their individual actions, the different antagonists form an intricate web of connections in order to regulate the biological availability of BMPs.

Table 1. BMP signaling regulation. BMP signaling is regulated at multiple levels by different factors. The antagonists recited in the table do not form a comprehensive list but are instead used as examples. Involvement in BMP7 signaling regulation is indicated.

Location	Signaling regulator	Function	BMP7	References
Extracellular				
Antagonists	Follistatin	Inhibition of BMP-receptor interaction	+[a]	Yamashita et al. (1995), Rider and Mulloy (2010)
	Gremlin	—‖—	+	Merino et al. (1999), Wordinger et al. (2008)
	Noggin	—‖—	+	Groppe et al. (2002), Krause et al. (2011)
	Tsg	—‖—	+	Zakin et al. (2005), Gazzerro and Canalis (2006)
	Chordin	—‖—	+	Piccolo et al. (1996), Gazzerro and Canalis (2006)
	Sclerostin	—‖—	+	Kusu et al. (2003), Yanagita (2005)
Glycosaminoglycans	Heparan sulfate	Retention of BMPs to ECM/cell surface	+	Irie et al. (2003), Rider (2006)
	Chondroitin sulfate	—‖—	n/a[b]	Manton et al. (2007), Miyazaki et al. (2008)
Membrane				
Pseudoreceptors	BAMBI	Inhibition of BMP-receptor interaction	n/a	Onichtchouk et al. (1999)
Co-receptors	Endoglin	Enhancement of BMP-receptor interaction	+	Barbara et al. (1999), Scherner et al. (2007)
	RGMa	—‖—	-[c]	Babitt et al. (2005)
	DRAGON	—‖—	+/ -[d]	Samad et al. (2005), Andriopoulos et al. (2009)
	Hemojuvelin	—‖—	n/a	Malyszko (2009)
Intracellular				
I-SMADs	SMAD6	Inhibition of SMAD signaling		Park (2005)
	SMAD7	—‖—		Park (2005)
SMURFs	SMURF1	Ubiquitination of SMADs		Inoue and Imamura (2008)
	SMURF2	—‖—		Inoue and Imamura (2008)
Nucleus	TFs[e]	Transcriptional repression or activation		Zwijsen et al. (2003), Miyazono et al. (2005)

[a] +, BMP7 is regulated by the signaling factor in question, [b] n/a, not available, [c] - , BMP7 is not regulated by the signaling factor in question
[d] +/- , contradictory results, [e] TF = transcription factor

Another class of extracellular BMP signaling regulators is proteoglycans, which consist of core proteins linked to carbohydrate glycosaminoglycan moieties capable of binding BMPs and regulators of BMP signaling, such as noggin (Ruppert et al., 1996). A basic constituent of extracellular matrix (ECM), proteoglycans are able to bind to many proteins including cytokines, growth factors and transmembrane proteins (Muramatsu et al., 2006). Their role in regulation of BMP signaling is controversial, however, since both inhibitory and activatory mechanisms have been observed (Umulis et al., 2009). By binding BMPs and preventing their association with receptors, proteoglycans can inhibit BMP signaling. On the other hand, it has been postulated that when BMP concentration is low, proteoglycans could prevent BMPs from diffusing away and essentially concentrate them near the cell surface (Umulis et al., 2009).

Signaling regulation at the membrane

BMP signaling regulation at the membrane level is achieved by BMP co-receptors and pseudo-receptors (Table 1). Members of repulsive guidance molecules (RGM), which include RGMa and DRAGON, are glycosylphosphatidylinositol (GPI)-anchored co-receptors for BMPs. They can bind BMP type I or II receptors together with BMP2 and -4 and enhance signal transduction (Miyazono et al., 2010). BMP7 and various other ligands are bound by the transmembrane protein endoglin, which enhances BMP7 signaling (Scherner et al., 2007). In contrast, BMP receptor associated molecule 1 (BRAM1) binds to BMPR-IA and negatively regulates BMP signaling (Zeng et al., 2010).

The pseudo-receptor BMP and activin membrane-bound protein (BAMBI) inhibits BMP signaling (Zeng et al., 2010). The extracellular domain of BAMBI resembles BMP type I receptors, but the pseudo-receptor has no intracellular domain for signal transmission. BAMBI is able to bind to type I receptors but downstream signaling is inhibited due to the lack of kinase domain. Expression of BAMBI is induced by BMPs, generating a feedback loop for BMP signaling (Miyazono et al., 2010).

Intracellular signaling regulation

There are multiple ways of regulating BMP signaling inside the cell, including SMAD ubiquitin regulatory factors (SMURFs), inhibitory SMADs (I-SMADs), protein phosphatases and other regulatory factors (Table 1). Inhibitory SMADs, SMAD6 and

SMAD7, have different ways of regulating BMP availability (Zeng et al., 2010). SMAD6 competes with SMAD4 preventing its association with R-SMADs and thus inhibiting SMAD signaling. SMAD7 in turn binds to type I receptors and blocks the receptor-R-SMAD interaction and activation of the signaling cascade. In the nucleus SMAD7 can bind to the SMAD-responsive DNA element and inhibit the formation of R-SMAD-DNA complexes, while SMAD6 antagonizes signaling through its interaction with transcriptional co-repressors (Sieber et al., 2009; Miyazono et al., 2010). In addition, both inhibitory SMADs recruit the ubiquitinases SMURF1 and -2 promoting SMAD4 and R-SMAD ubiquitination and degradation (Zeng et al., 2010). The expression of I-SMADs is induced by BMPs, creating a feedback loop that prevents prolonged signaling.

SMURF1 and -2 are HECT type E3 ubiquitin ligases that target R-SMADs (Miyazono et al., 2010). The WW domains of SMURFs are responsible for interaction with the PPXY sequences in the linker region of SMADs. Ubiquitination by SMURFs leads to proteasomal degradation of R-SMADs. A RING type E3 ligase Arkadia interacts with the inhibitory SMAD7 to promote its degradation (Miyazono et al., 2005).

SMAD7 can mediate dephosphorylation of TGF-β type I receptors through recruitement of protein phosphatases, which results in deactivation of the receptors (Miyazono et al., 2005). Whether such action is possible for BMP receptors remains unknown. In addition, several phosphatases are capable of dephosphorylating R-SMADs (Miyazono et al., 2010).

Other regulatory factors include Tob, SANE, AMSH and transcriptional co-repressors. Tob suppresses BMP signaling through interaction with R-SMADs, I-SMADs and BMP type I receptors (Miyazono et al., 2005). SMAD1 antagonistic effector (SANE) binds to SMAD1/5 and type I receptors blocking BMP signaling. Associated molecule with the SH3 domain of STAM (AMSH) enhances BMP signaling by binding to SMAD6 and inhibiting its function. In conclusion, an intricate web of connections is formed by the multiple regulators of BMP signaling working at different levels.

2.2.4 BMP transcriptional regulation and target genes

In the nucleus, interaction of SMADs with other DNA-binding molecules provides differential regulation of BMP signaling in a time- and cell-specific manner. In addition, the weak affinity of SMADs to DNA is complimented by SMAD-interacting molecules

capable of binding DNA (von Bubnoff and Cho, 2001). Runx, Schnurri, Menin, OAZ, MyoD and Vent2, among many other proteins, interact with SMADs (Miyazono et al., 2010). Ski (identified as oncogene of avian Sloan-Kettering retrovirus) and SnoN (Ski like novel protein) are transcriptional repressors that inhibit TGF-β signaling by binding to SMAD2 and -3. In addition, they inhibit both TGF-β and BMP signaling through association with SMAD4 (Miyazono et al., 2005). Some SMAD-interacting proteins, such as c-Ski and SnoN, can recruit epigenetic modulators like histone deacetylases in order to regulate transcription. Furthermore, there are R-SMAD-interacting proteins that are themselves epigenetic modulators, such as the histone acetyltransferases p300 and CPB (Miyazono et al., 2010). In addition to transcriptional regulation, R-SMADs have been shown to regulate miRNA processing (Davis et al., 2008).

In order to regulate transcription, SMADs bind to BMP responsive regions containing SMAD binding elements (SBE) and GC-rich boxes (also referred to as BMP responsive elements, BRE) in their target gene promoters (Miyazono et al., 2005; Nakahiro et al., 2010). Common target genes for many BMPs include *Id1-3* (*inhibitor of differentiation 1-3*), *SMAD7* and *Runx2*, which are induced by BMPs in a cell type-specific manner. Recently, microarray methods have been used in order to identify additional BMP target genes in cell lines of different tissues of both human and mouse origin (Locklin et al., 2001; Vaes et al., 2002; Miyoshi et al., 2008; Fessing et al., 2010). For example, new targets of BMP2 in human marrow stromal cells include *STAT1*, *JunB* and *HES-1* and *tropomyosin 1*-α is a target gene of BMP4 in human keratinocytes and fibroblasts. The amount of target genes varied from 50 to over 300 depending on the cell type and the criteria used for defining target genes. Except for BMP7 function as an inducer of cytokine and chemokine signaling in kidney (Gould et al., 2002), very little research has been done about possible BMP7-specific target genes or alterations in cancer. More research is needed for creating a clear picture of the target genes of different BMPs and their regulation in normal and cancer tissues.

2.3 BMPs and cancer

BMPs have been studied in numerous cancer types, including breast, prostate, pancreatic, renal cell, colon and lung cancer (Thawani et al., 2010). BMPs are able to regulate cellular processes such as osteogenic, chondrogenic and neural crest stem cell, as well as embryonic stem cell self-renewal (Varga and Wrana, 2005). Therefore it is

not surprising that they play a role in cancer progression. However, BMPs act in concert with other signaling pathways, which makes the elucidation of their specific role in cancer difficult. Both tumor-promoting and inhibiting roles have been associated with BMPs (Singh and Morris, 2010). BMPs are also implicated in epithelial-to-mesenchymal transition (EMT), a process that results in epithelial cells acquiring more mesenchymal-like characters (Bailey et al., 2007). The process of EMT thus gives epithelial cells migratory characteristics and it is considered to be a prerequisite for metastasis formation.

BMP expression, function and role in cancer have been studied both *in vivo* and *in vitro* in many cancer types (Singh and Morris, 2010). Most studies have focused on BMP2, -4, -6 and -7 (Thawani et al., 2010). Very few studies have been published on mutations of BMP genes in cancer. BMP2 and BMP4 mutations have been associated with colon and colorectal cancer (Lubbe et al., 2011; Slattery et al., 2011). However, overexpression of BMPs has been detected in many cancer types, for which examples are given here. Both BMP2 mRNA and protein are overexpressed in lung cancer and adenomas of salivary glands (Kusafuka et al., 1998; Langenfeld et al., 2003; Langenfeld et al., 2005). BMP4 is expressed in prostate cancer tissue (Yang et al., 2005). BMP4 and -7 are widely expressed in both breast cancer and melanoma tissue samples and cell lines (Rothhammer et al., 2005; Alarmo et al., 2007). Immunohistochemistry of tumor samples showed that BMP6 is overexpressed in esophageal squamous cell and prostate carcinoma (Raida et al., 1999; Yuen et al., 2008).

The function of BMPs has been studied using cell lines and animal models. BMPs inhibit cancer cell growth in some cell lines and promote growth in others, while no effect is seen in some cell lines. BMPs often increase migration and/or invasion of cancer cell lines both in *in vitro* studies and animal models (Singh and Morris, 2010). BMP2 enhances migration and invasion in lung, prostate and breast cancer and decreases proliferation in breast and gastric cancer cells (Arnold et al., 1999; Langenfeld et al., 2003; Wen et al., 2004; Clement et al., 2005; Feeley et al., 2005). BMP4 inhibits growth in breast, prostate and lung cancer (Brubaker et al., 2004; Buckley et al., 2004; Ketolainen et al., 2010). In addition, it promotes migration and invasion in breast, melanoma, colorectal and hepatocellular carcinoma cancer cells (Rothhammer et al., 2005; Deng et al., 2007; Maegdefrau et al., 2009; Ketolainen et al., 2010). BMP5, -6 and -7 inhibit growth in myeloma cells (Ro et al., 2004). BMP6 appears to be an anti-metastasis agent in breast cancer and BMP7 inhibits growth and

migration in melanoma cells (Takahashi et al., 2008; Na et al., 2009; Yang et al., 2009). BMP9 and -10 inhibit the growth and invasion of prostate cancer cells (Brubaker et al., 2004; Ye et al., 2008; Ye et al., 2009). Ketolainen et al. (2010) showed that BMP4 is able to simultaneously inhibit growth and promote migration of breast cancer cells. Although other BMPs in some cancer types seem to be able to act in this way, the results are derived from separate studies with different cell lines and are thus not conclusive. It is important to note that the examples stated above present a simplified view of the BMP data and results from all studies are not always consistent. Taken together, the effects of BMPs depend on the cancer type, cell line and model used.

In addition to BMP ligands, BMP receptors, BMP antagonists and intracellular mediators, including SMAD4, can have a role in cancer progression. Overexpression of BMP receptors may lead to increased binding of BMP ligands, which results in aberrant cell behavior associated with cancer (Singh and Morris, 2010). The expression levels of BMP receptors have been studied in many cancer types. 22 breast cancer cell lines and four of five prostate cancer cell lines expressed the BMP receptors (Yang et al., 2005; Alarmo et al., 2007). In lung cancer lower levels were found in cancer than in normal tissue and in pancreatic cancer receptors were overexpressed (Kleeff et al., 1999; Langenfeld et al., 2003). BMP antagonists could be potential anti-tumorigenic agents against cancer-promoting BMPs, although both tumorigenic and antitumorigenic properties have been ascribed to antagonists (Haudenschild et al., 2004; Walsh et al., 2010). A direct indication of the role of BMP signaling in cancer is the fact that germline loss-of-function mutations in *SMAD4* and *BMPR1A* cause Juvenile Polyposis Syndrome (Zhou et al., 2001; Yang and Yang, 2010). In addition, loss of *SMAD4* expression often occurs in the cancers of pancreas, gastrointestinal tract and skin (Yang and Yang, 2010).

BMPs appear to have a role in bone metastasis. Patients with BMP-positive osteosarcomas had a higher incidence of lung and bone metastases compared to BMP-negative osteosarcomas (Yoshikawa et al., 1988). BMP6 is associated with bone metastasis in prostate cancer (Autzen et al., 1998; Singh and Morris, 2010). Furthermore, BMP4 enhances attachment of prostate cancer cells to the endothelium of bone marrow (Cooper et al., 2003). In contrast, Buijs et al. (Buijs et al., 2007b) found BMP7 to be an inhibitor of bone metastasis in prostate cancer. In addition to prostate cancer, BMP7 is linked to bone metastasis in breast cancer (Buijs et al., 2007a; Alarmo et al., 2008).

The role of growth factors in cancer is not always simple, as illustrated by the example of TGF-β. A bifunctional role has been suggested for TGF-β based on its ability to first inhibit growth of cells and later induce tumor progression (Moses et al., 1994; Reiss and Barcellos-Hoff, 1997). At first TGF-β is able to both inhibit growth and induce apoptosis in cancer cells, but later cancer cells become insensitive to these signals (Ikushima and Miyazono, 2010). At this stage TGF-β is able to induce epithelial-to-mesenchymal transition and promote cancer. A dual role of action, similar to TGF-β, has been proposed for BMPs as well (Alarmo and Kallioniemi, 2010). However, more studies are needed in order to determine whether a dual role for BMPs in cancer could explain some of the paradoxical findings of BMPs in cancer.

2.4 BMP7 and breast cancer

The expression patterns of BMP7 have been studied at mRNA and protein levels using both breast cancer cell lines and primary tumors. The first study on BMP7 in breast cancer found no *BMP7* mRNA expression in MCF-7 and MDA-MB-231, the two cell lines used (Arnold et al., 1999). Schwalbe et al. (2003) detected *BMP7* mRNA in two out of three breast cancer cell lines, whereas immunohistochemistry and Western blot analysis revealed BMP7 protein in all three cell lines. In a study of 22 cell lines, 14 cell lines had a higher expression of *BMP7* mRNA compared to normal human mammary epithelial cells, whereas no *BMP7* was detected in 4 cell lines (Alarmo et al., 2006). In addition, Alarmo et al. (2006) found BMP7 protein expression in all of the 11 breast cancer cell lines examined using immunohistochemistry.

BMP7 expression is also found in primary tumors. Schwalbe et al. (2003) found BMP7 protein in all 170 tumor samples examined. In addition, they found BMP7 expression to be associated with EGF receptor and PR status. Buijs et al. (2007a) found that *BMP7* mRNA levels in primary tumors developing bone metastases were lower than in primary tumors developing lung and/or liver metastases. However, there was no difference between tumors with metastases compared to tumors without metastases. In a study including 409 primary tumors, BMP7 protein was expressed in 47% of the tumors, more often in lobular than ductal carcinomas (Alarmo et al., 2008). Furthermore, primary tumors expressed BMP7 significantly more often than the corresponding local recurrences (Alarmo et al., 2008). Finally, BMP7 expression in

primary tumor proved to be an independent prognostic factor for bone metastasis development (Alarmo et al., 2008).

Studies about BMP7 function are currently limited. Alarmo et al. (2009) studied BMP7 function in eight breast cancer cell lines. Silencing BMP7 expression in one of the three BMP7 expressing cell lines resulted in growth inhibition. Adding BMP7 to the medium of five BMP7-negative breast cancer cells led to growth enhancement in one cell line and inhibited growth in four cell lines. The mechanisms of growth change were reflected either in the distribution of cell cycle phases or apoptosis patterns. Thus, BMP7 can promote or inhibit the growth of breast cancer cells depending on the cell line (Alarmo et al., 2009). This discrepancy can be at least partly explained by the heterogeneous nature of breast cancer as well as the complexity of BMP signaling.

In addition to growth properties, another aspect of tumor progression is migration and invasion capability. Overexpression of BMP7 in MDA-MB-231 cell line inhibited osteolytic lesions in mice (Buijs et al., 2007a). Furthermore, BMP7 treatment of mice inoculated intraosseously or in fat pads with MDA-MB-231 cancer cells inhibited breast cancer growth at both sites. In contrast, BMP7 treatment enhanced both migration and invasion of MDA-MB-231 cells *in vitro* (Alarmo et al., 2009). Based on the results of functional experiments, it is clear that multiple cell lines and models are required in order to reliably study BMP7 function.

The differences and contradictions in the role of BMP7 in cancer may in part be explained by other factors that influence the action of BMPs. For example, BMP7 has been shown to be a target gene of Lim-only protein 4 and p53 (Wang et al., 2007; Yan and Chen, 2007). In addition, BMP7 knockdown in p53-deficient but not in p53-proficient breast cancer cells led to growth inhibition (Yan and Chen, 2007). Numerous regulators of BMP signaling, such as BMP antagonists, are able to modulate the effect of BMP7 on its target cells (Table 1). Furthermore, epigenetic regulation can influence BMP signaling. For example, BMP7 promoter is hypermethylated in brain cancer (Ordway et al., 2006). In addition, miR-155 inhibits BMP2-, BMP6-, and BMP7-induced *Id3* expression (Yin et al., 2010). The complex results of BMP signaling have prevented making any decisive conclusions about BMP function in cancer.

3 Aims of the research

The aim of this project was to study the effects of BMP7 on proliferation, migration and invasion of breast cancer cells using five commercial breast cancer cell lines. In addition, cell cycle analyses were performed in order to find out whether potential changes in cell proliferation were due to alterations in the distribution of cells in the cell cycle phases. Western blot analysis was used as a means to discover which signaling pathway is activated upon BMP7 treatment in these breast cancer cells.

4 Materials and Methods

4.1 Cell lines

All five breast cancer cell lines (BT-474, SK-BR-3, MDA-MB-361, MDA-MB-231 and HCC1954) used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell line MDA-MB-231 was used as a positive control. The cell lines were cultured under recommended conditions. The basal media used were McCoy's (SK-BR-3), L-15 (MDA-MB-361 and MDA-MB-231) and DMEM (HCC1954 and BT-474), all purchased from Sigma-Aldrich (St. Louis, MO, USA). Basal media were supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). In addition, HCC1954 medium contained 10 mM HEPES (Lonza, Basel, Switzerland), 1 mM sodium pyruvate (Sigma-Aldrich), 4.5 mg/ml glucose, 0.27 IU/ml insulin (Novo Nordisk, Bagsværd, Denmark) and 1.5 mg/ml sodium bicarbonate. The cells were cultured at 37°C and 5% CO_2. In functional assays using 24-well plates, L-15 was replaced with DMEM since L-15 medium is suitable only for use in CO_2-free atmosphere. Additionally, all functional tests with MDA-MB-231 cells were performed in medium containing 1% FBS.

4.2 BMP7 treatment

For the functional assays cells were incubated with recombinant human BMP7 (R&D Systems, Minneapolis, MN, USA) for the indicated time periods (Figure 4). BMP7, diluted in 4 mM HCl containing 0.1% BSA, was used in a concentration of 50 ng/ml. An equivalent volume of vehicle (BMP7 dilution buffer) was used as a control. For each assay and cell line the appropriate number of cells to be used was tested in a way that allowed sustained growth for the entire analysis period and provided a sufficient amount of material for the measurements (Table 2).

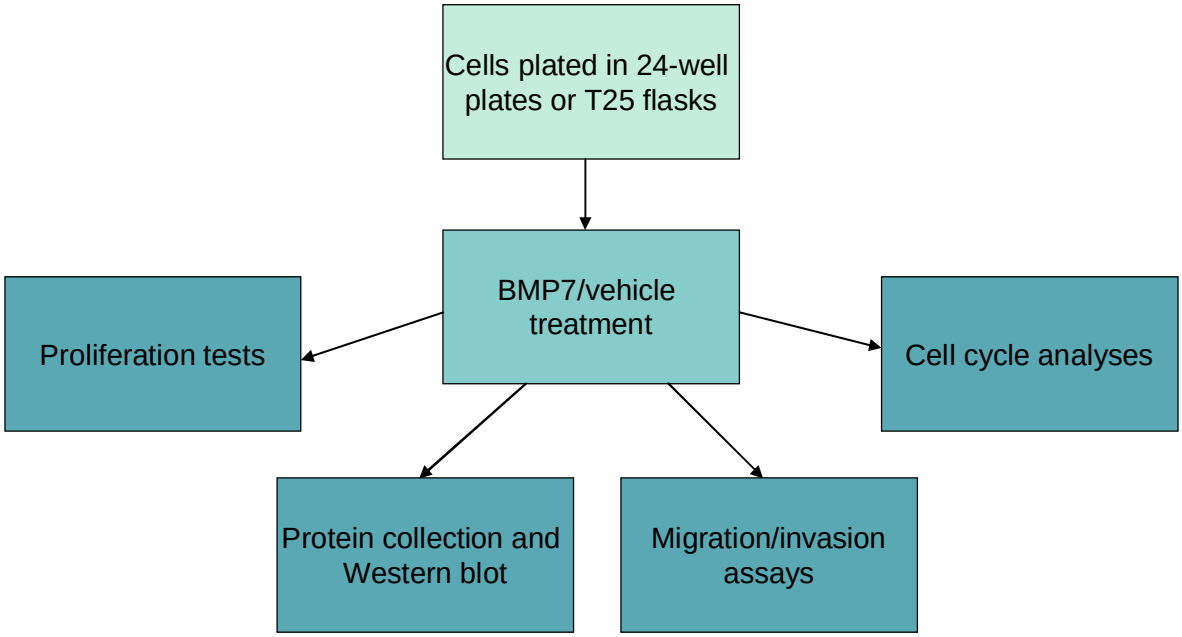

Figure 4. Outline of the study. Cells were first plated either on 24-well plates or T25 flasks and BMP7 or vehicle was added 24 h later. Following a treatment period characteristic to each assay, the cells were subjected to the different experiments.

4.3 Proliferation assays

Proliferation assays were conducted using 24-well plates. The appropriate numbers of cells (Table 2) were seeded on day 0. After 24 h (day 1), the cells were given medium containing BMP7 or vehicle. In the 7-day experiments, fresh medium with BMP7 or vehicle was added to the cells at day 4.

The rate of cell proliferation was assessed by counting cells after three (day 4) and six days (day 7) of BMP7 or vehicle treatment. The cells were detached using 400 µl of trypsin and following detachment trypsin was neutralized with 400 µl of medium. The cell suspension was mixed with Coulter ISOTON III Diluent electrolyte solution in a 1:10 or 1:40 ratio (Beckman Coulter, Fullerton, CA) and cells were counted using the Z1 Coulter Counter (Beckman Coulter). All experiments were performed in three to six replicates and repeated at least twice, except for MDA-MB-231 proliferation assay, which was done once.

Table 2. Cell numbers in functional assays. The appropriate number of cells plated in 24-well plates (cell number/well), T25 flasks (subculture ratio) or migration/invasion inserts.

	Proliferation	Cell cycle	Migration/Invasion		Migration	Invasion
Cell line	24-well plate	24-well plate	24-well	T25 flask	insert	insert
HCC1954	25000	60000	50000	ND	25000	ND
MDA-MB-361	100000	100000/125000	100000	1:3	75000	100000
MDA-MB-231	25000	ND	25000	ND	25000	ND
SK-BR-3	30000	ND	50000	1:3	100000	100000
BT-474	50000	ND	100000	1:2	100000	150000

ND = not done

4.4 Cell cycle analyses

In order to determine whether changes in cell proliferation were due to changes in cell cycle, the cells were stained with propidium iodide (PI). PI is a dye that binds to DNA stoichiometrically. Therefore, the cells in G2 phase of the cell cycle have twice the amount of bound PI than cells in the G1 phase, due to duplication of DNA during S-phase. Consequently, the phases of the cell cycle can be separated based on the intensity of PI fluorescence signal.

Prior to ligand treatment cells were plated on 24-well plates (Table 2). The cells were treated with BMP7 or vehicle for 2, 4 or 7 days, before they were collected and stained. The cells were detached with trypsin and collected by sentrifugation at 1000 x g for 5 min. 500 µl of hypotonic staining buffer (0.1 mg/ml sodium citrate tribasic dehydrate, Triton X-100, 2 µg/ml ribonuclease A and 50 µg/ml PI) was added and the cells pipetted to achieve single cell suspension. Stained cells were kept on ice and in the dark for 30 min until analysis by flow cytometer (Figure 5).

The stained samples were analyzed using Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA). A blue laser of 488 nm was used for excitation of PI and emission was detected at 585 nm in the FL-2 channel. A total of 20 000 events were collected from each sample. The default threshold setting of 80 000 for forward scatter was used to exclude small particles that represent debris. The cell cycle phase distribution was analyzed using ModFit LT 3.0 (Verity software house, USA, Figure 6). All experiments consisted of three to six replicates and were performed at least three times.

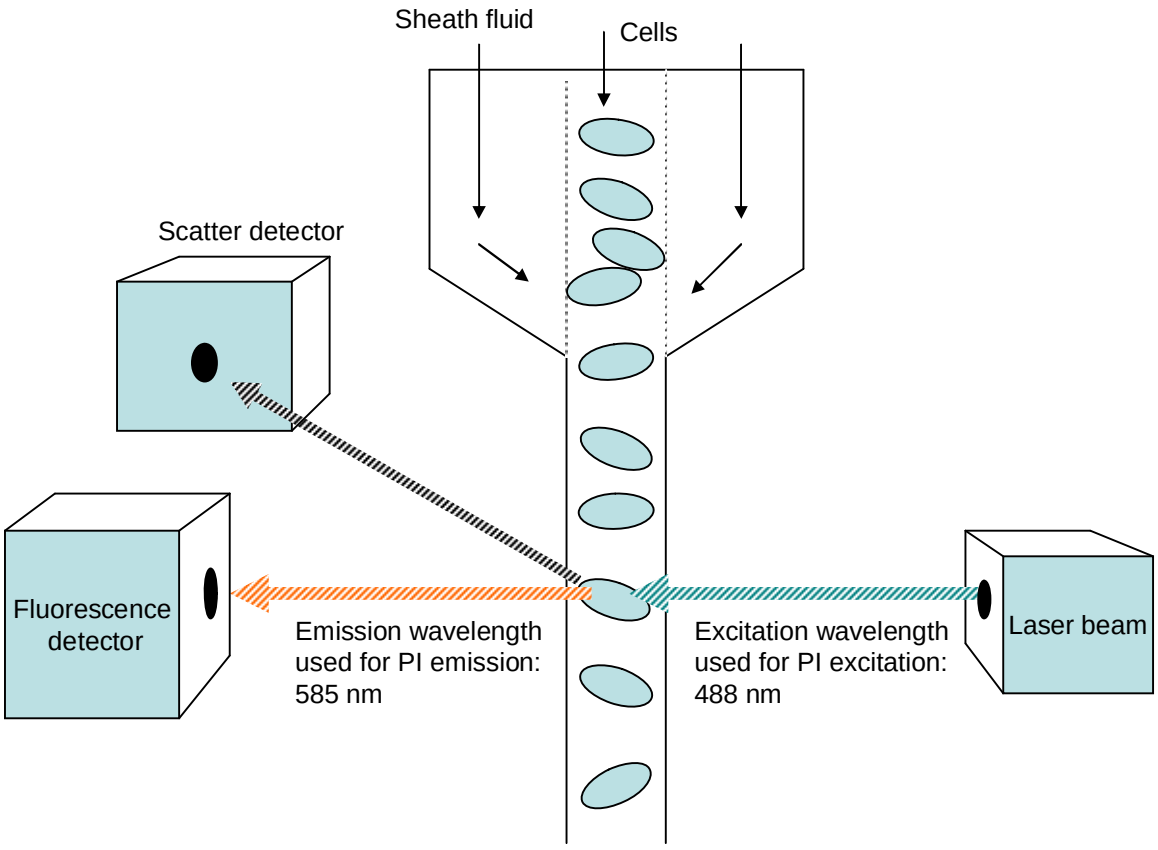

Figure 5. The principle of flow cytometry. Cells pass through the intersecting laser beam as a single file. The laser excites the fluorochromes in the cells (in this case propidium iodide), and the fluorochromes emit fluoresescent light that is detected by a fluorescence detector. Scattered light caused by laser photons colliding with other structures of the cells is gathered by the scatter detector. Forward scatter is an indication of the size of the particle, whereas side scatter represents the morphology of the cell.

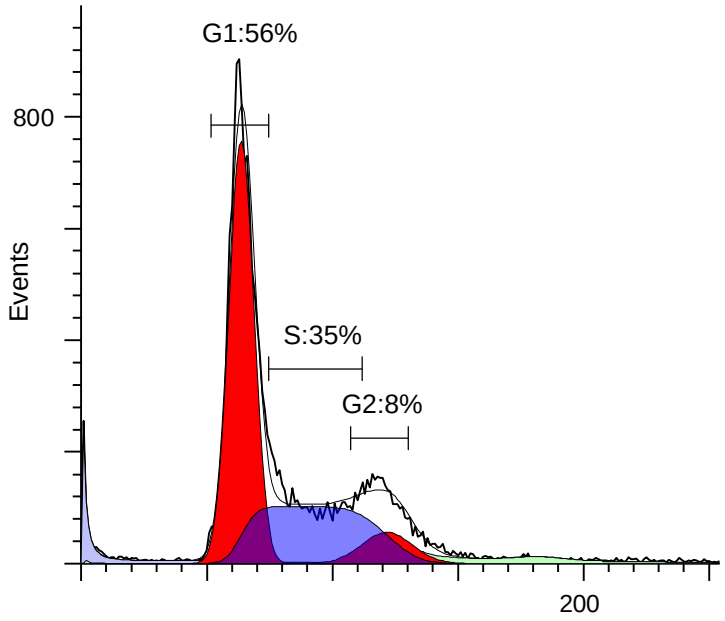

Figure 6. The distribution of cell cycle phases. The phases of cell cycle were analyzed by ModFit. The percentage of cells in G1, S and G2 phases is calculated by the program.

4.5 Migration and invasion

The effect of BMP7 on the migration and invasion of breast cancer cells was tested in an assay where the cells were allowed to migrate or invade through membranes in a transwell/Boyden chamber format (Figure 7).

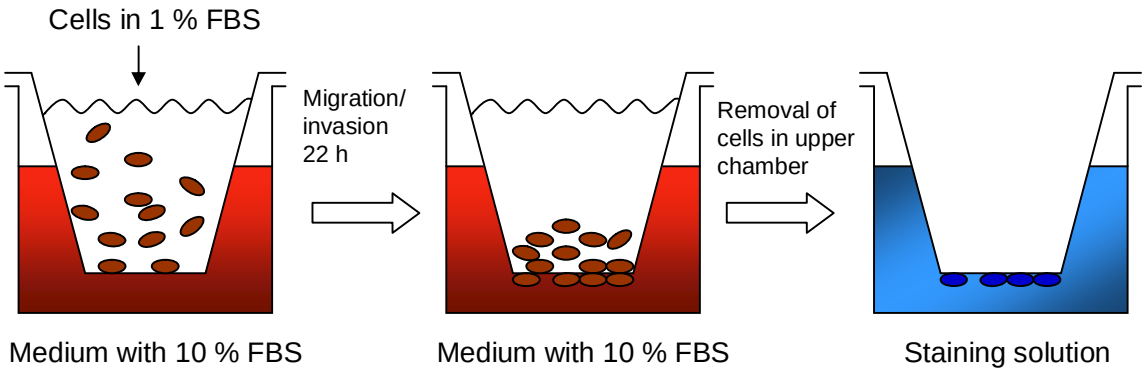

Figure 7. Migration/invasion assay. Cells were suspended in medium containing 1% FBS and placed in the migration or invasion inserts. The bottom of the inserts is a porous membrane through which cells can migrate/invade towards the greater serum concentration. Additionally, invasion inserts contain a layer of matrigel on top of the membrane. After 22 h of migration/invasion the cells that have moved through the membrane are stained and quantitated.

First, cells were plated either on 24-well plates or T25 flasks (Table 2) and subjected to 72 h of BMP7 or vehicle treatment. BMP7- and vehicle-treated cells were collected and counted as described before (chapter 4.3) and subsequently suspended in medium containing 1% FBS. A 24-well cell culture insert companion plate (BD Biosciences, Franklin Lakes, NJ, USA) was used for migration and invasion inserts. For each cell line, 750 µl of cell line-specific medium containing 10% FBS was added to the wells prior to placing the BD Falcon cell culture inserts for migration (8.0 µm pore side, BD Biosciences) and BD BioCoat Matrigel invasion chambers (8.0 µm pore side, BD Biosciences) to the wells. Invasion inserts were allowed to rehydrate before use in DMEM for two hours at 37°C, according to the manufacturer's instructions. The appropriate number of cells (Table 2) was added to the migration inserts in a volume of 350 µl and to the invasion inserts in a volume of 500 µl in medium containing 1% FBS. The cells were then allowed to migrate or invade for 22 hours at 37°C towards media with a greater serum concentration.

The cells were stained after 22 h of migration/invasion. The cells that failed to migrate/invade through the membrane were removed by scrubbing the inside of the inserts first with a pipette tip and then with a dry cotton stick and a cotton stick soaked in medium. After that the inserts were fixed in methanol for two minutes and stained with 1% toluidine blue in 1% borax for two minutes. The inserts were allowed to dry before the membranes were cut with a surgeon knife. Finally, the membranes were mounted on microscope slides using immersion oil.

The membranes were scanned with ScanScope XT (software version 9) (Aperio, Vista, CA, USA). Four images were captured from each membrane using JVSview (Tuominen and Isola 2009). The numbers of cells in the four images were counted with ImageJ (Rasband WS (1997-2009) ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA) using the cell counter plugin and were then added together to represent one membrane/insert. All experiments consisted of three to six replicates and were performed at least twice.

4.6 Protein collection

Proteins from BMP7- and vehicle-treated cells were collected for Western blot analyses. Cells were dispensed in T25 flasks and treated with BMP7 or vehicle for three hours.

After treatment the cells were collected by trypsinization and centrifuged for 8 min at 800 x g. The supernatant was discarded and the cell pellets stored at -80°C.

The cell pellets were placed on ice to melt and 150 µl of RIPA-buffer (1% PBS, 1% non-idet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing PhosSTOP phosphatase inhibitor (Roche Diagnostics GmbH, Steinheim, Germany) and Complete mini protease inhibitor (Roche) was used to lyse the cells. The samples were suspended with repeated passage through a syringe and a needle (20 G). After lysis, the samples were incubated on ice for 20 min and periodically vortexed. Following incubation the samples were centrifuged for 10 min at 10 000 x g and at 4°C. The supernatant containing proteins was collected and stored at -20°C.

The protein content of the samples was measured with the Bradford method, which is based on binding of the dye Coomassie Brilliant Blue G-250 to protein leading to a shift in the absorbance maximum of the dye (Bradford 1976). Five different concentrations of BSA (0, 0.25, 0.50, 1.0 and 1.4 mg/ml, Sigma-Aldrich) were used to create a standard curve. 1.5 ml of Bradford reagent (Sigma-Aldrich) at room temperature was added to 50 µl of standards and samples diluted 1:10 in water. The samples and standards were then incubated at room temperature protected from light for 5 minutes. Absorbances were measured with Ultrospec 3100 *pro* spectrophotometer (GE Healthcare, Waukesha, WI, USA).

4.7 Western blot

Western blot is a technique for recognizing and visualizing a protein of interest. The protein is transferred from a polyacrylamide gel to a membrane after electrophoretic size fractionation. Subsequently the protein is marked with a specific antibody for recognition. In this work, this primary antibody is recognized by a secondary antibody conjugated with the enzyme horseradish peroxidase. Upon addition of a substrate, the horseradish peroxidase catalyzes a light-producing reaction that exposes photographic films allowing detection of the protein of interest.

An SDS-PAGE gel consisting of a 12% resolving gel and 5% stacking gel was prepared for protein separation, using Tris-HCl-buffer-based acrylamide-bisacrylamide (Bio-Rad Laboratories, Hercules, CA) gels with TEMED and ammonium persulfate (Sigma-Aldrich) for initiation of polymerization. Fifty µg of each protein sample was mixed with an equal volume of sample buffer (10% SDS, 20% glycerol, 0.2 M Tris-HCl

pH 6.8., 0.05% bromophenolblue) and β-mercaptoethanol (Sigma-Aldrich) was added to a final concentration of 0.7 M (53 mg/ml). The mixture was boiled for 5 min to denature proteins. The samples and PageRuler Prestained Protein Ladder Plus (Fermentas, Burlington, Canada) were loaded into the gel. Gels were run for 10-20 min under 100 V and for 50-60 min under 120 V using Bio-Rad Mini-PROTEAN 3 Cell (Bio-Rad).

The proteins separated by their molecular weight were transferred from the gel to a PVDF membrane (Roche, Basel, Switzerland) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The membrane was first wetted with methanol and then it and the gel were incubated in blotting buffer (48 mM Tris, 39 mM glycine, 20% methanol) for 15 min. The blotting papers were also wetted with blotting buffer. The gel was blotted for 30 minutes under 15 V and 400 mA.

After blotting, the membrane was placed in a blocking solution containing 10% blocking reagent in 1 x TBS (BM Chemiluminescence Western Blotting Kit, Roche) for 1 h under agitation at room temperature or overnight at 4°C. Primary antibody treatment was performed as indicated in Table 3. The membrane was washed in 0.5% Tween-TBS, 0.1% Tween-TBS and 0.05% Tween-TBS (10 min each) under agitation. The secondary anti-mouse/anti-rabbit antibody of the Chemiluminescence Kit (Roche) was diluted 1:5000 in 1 x TBS, 0.05% Tween-20 and the membrane was incubated in the antibody solution for 1 hour under agitation at room temperature. The membrane was washed as described above and placed in 1 x TBS.

Proteins were visualized using the Chemiluminescence kit (Roche). The secondary antibody of the kit is labeled with horseradish peroxidase which catalyzes the oxidation of its substrate luminol in a light-producing reaction. The detection solution was prepared by mixing substrate solution A with starting solution B in 100:1. The membrane was placed in the detection solution for 60 seconds and then inserted in the film cassette between two transparency sheets. Various exposure times (3-30 s) were used for the photographic film (Kodak BioMax MR Film, Sigma) to obtain optimal visualization of the proteins.

Table 3. Antibody treatment. All antibodies are from Cell Signaling Technology (Danvers, MA, USA), except for β-tubulin, which was obtained from Sigma-Aldrich and the secondary antibody, which was from the Roche Chemiluminescence kit.

			Incubation time	
Antibody	Dilution	Dilution buffer	Blocking	Antibody
Primary antibody				
Phospho-SMAD1/5/8	1:1000	5% w/v BSA, 1xTBS, 0.1% Tween-20	1 h	o/n
SMAD5	1:1000	—‖—	1 h	o/n
Phospho-p44/42	1:1000	—‖—	1 h	o/n
p-44/42 (ERK1/2)	1:1000	—‖—	1 h	o/n
Phospho-p38 MAPK	1:1000	—‖—	1 h	o/n
p38 MAPK	1:1000	—‖—	1 h	o/n
β-tubulin	1:2000	1xTBS, 0.05% Tween-20	o/n	1 h
Secondary antibody				
Chem.lum.kit	1:5000	1xTBS, 0.05% Tween-20	none	1 h

In order to reprobe the membranes for loading control or total protein levels they were stripped with a 2% SDS, 0.1 M (7.8 mg/ml) β-mercaptoethanol solution in 1 x TBS for 30 minutes at 50°C under agitation. After stripping the membranes were washed in 0.5% Tween-TBS and 0.05% Tween-TBS, 15 minutes in each solution. Blocking and antibody treatment were done as described above.

4.8 Statistical analyses

Mann-Whitney test was used to evaluate the difference between BMP7- and vehicle-treated cells. A P value of less than 0.05 was considered significant. Statistical analyses were conducted with GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA).

5 Results

5.1 BMP7 has diverse effects on the proliferation of breast cancer cells

The effect of BMP7 on the growth of breast cancer cells was examined in five breast cancer cell lines, one of which (MDA-MB-231) was used as a positive control. Cells were treated with 50 ng/ml BMP7 or vehicle and the cell numbers were determined using the Coulter counter. An average of 9% growth reduction (day 7, $p<0.05$) was seen in BMP7-treated MDA-MB-361 cells compared to vehicle-treated cells (Figure 8). Similarly, HCC1954 showed an average of 18% decline in growth ($p<0.05$) at day 7. In contrast, a dramatic growth induction was seen in MDA-MB-231 cells (20% at day 4, $p<0.05$ and 128% at day 7, $p<0.05$). BMP7 had no effect on the growth of the two remaining cell lines, SK-BR-3 and BT-474 (Figure 8).

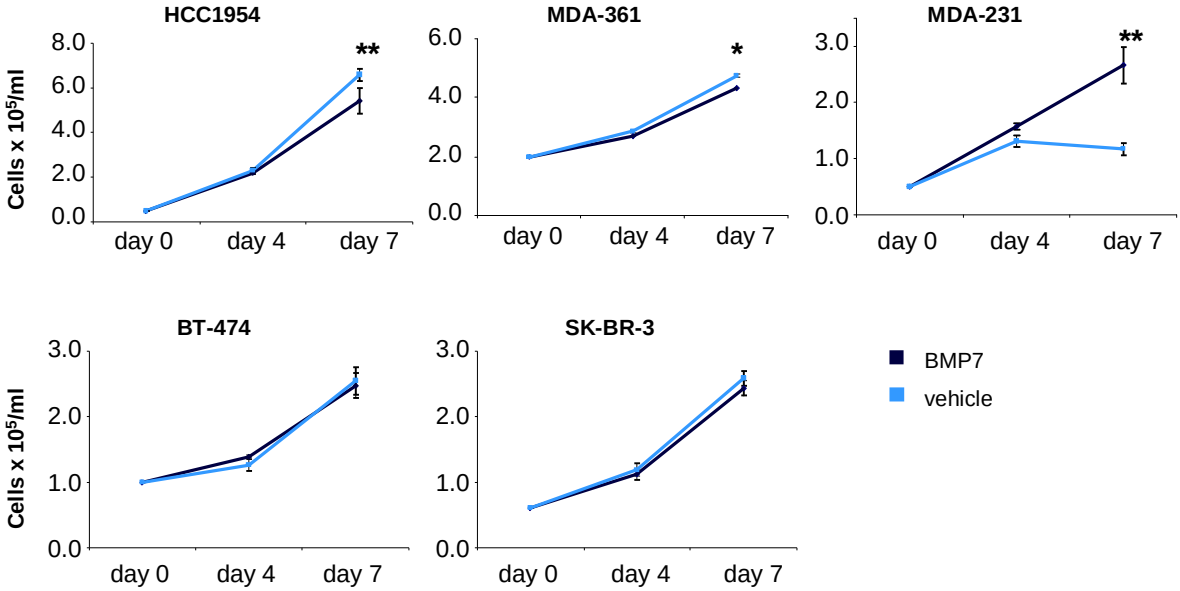

Figure 8. BMP7 has both growth inhibitory and stimulatory effects in breast cancer cells. Cells were seeded at day 0 and BMP7- (50 ng/ml) or vehicle-containing media was added at day 1 and replenished at day 4. Cell numbers were counted at day 4 and 7. Representative examples are shown with error bars indicating +/- SD of six replicates. Asterisks *$p < 0.05$, **$p < 0.005$

5.2 Cell cycle is not affected by BMP7 stimulation

Cell cycle analyses were performed in order to find out whether the growth inhibition seen in HCC1954 and MDA-MB-361 cells after BMP7 treatment was due to alterations in the distribution of cells in different cell cycle phases. Cells were treated for 48 hours with 50 ng/ml BMP7 or vehicle. For HCC1954, no consistent changes in cell cycle phases between BMP7- and vehicle-treated groups were observed (Table 4). In the case

of MDA-MB-361, BMP7 treatment was extended to last for 4 and 7 days in addition to the 48-hour treatment. However, no consistent differences were detected in the cell cycle phases between BMP7- and vehicle-treated cells after 4 or 7 days of treatment (Table 4).

Table 4. Cell cycle is not affected by BMP7 treatment. A table listing data from all cell cycle analyses performed with HCC1954 and MDA-MB-361. The cells were subjected to 2, 4, and 7 days of BMP7 or vehicle treatment before cell cycle analyses. The number of replicate measurements, their mean value, time point (day) and standard deviation are shown.

Cell line	No of repl.	Time point	Treatment	G1	S	G2
HCC1954	3	2 d	BMP7	53.8 ± 1.2	36.6 ± 4.9	9.6 ± 4.0
			Vehicle	53.2 ± 1.1	32.1 ± 3.3	14.7 ± 2.2
	6	2 d	BMP7	58.1 ± 1.1	30.0 ± 1.4	11.8 ± 0.9
			Vehicle	52.8 ± 1.0	32.2 ± 1.1	15.0 ± 0.9
	6	2 d	BMP7	58.3 ± 1.5	33.3 ± 2.2	8.4 ± 0.9
			Vehicle	57.9 ± 0.5	29.8 ± 1.0	12.3 ± 0.8
MDA-MB-361	3	2 d	BMP7	63.0 ± 0.9	35.1 ± 1.2	1.9 ± 0.5
			Vehicle	56.5 ± 0.7	39.2 ± 0.9	4.4 ± 1.0
	6	2 d	BMP7	70.1 ± 0.7	24.6 ± 2.2	5.3 ± 1.5
			Vehicle	69.3 ± 0.6	25.2 ± 0.8	5.5 ± 0.9
	4	2 d	BMP7	63.9 ± 1.7	32.1 ± 0.8	4.0 ± 1.2
			Vehicle	58.2 ± 0.7	35.8 ± 1.2	6.0 ±1.3
	3	4 d	BMP7	73.0 ± 1.1	20.6 ± 1.0	6.0 ± 0.0
			Vehicle	72.9 ± 0.7	20.4 ± 1.0	6.7 ± 0.4
	6	7 d	BMP7	65.1 ± 1.3	26.1 ± 1.9	8.8 ± 0.6
			Vehicle	64.2 ± 0.6	26.8 ± 1.2	9.0 ± 0.7

5.3 BMP7 affects migration of breast cancer cells

The possible effects of BMP7 on breast cancer cell migration were studied after 72-hour treatment with BMP7 or vehicle. In three of the cell lines (MDA-MB-361, BT-474 and SK-BR-3), BMP7 treatment induced a slight increase in cell migration (Figure 9). As

compared to vehicle-treated cells, a very small but statistically significant 1.1-fold (p=0.0079) increase in cell migration was detected in MDA-MB-361 cells, 1.2-fold (p=0.0043) in SK-BR-3 cells and 1.6-fold (p=0.0022) in BT-474 cells (Figure 9). BMP7 treatment had no effect on migration of HCC1954 cells, whereas a large increase (7.4-fold) was observed in migration of MDA-MB-231 cells.

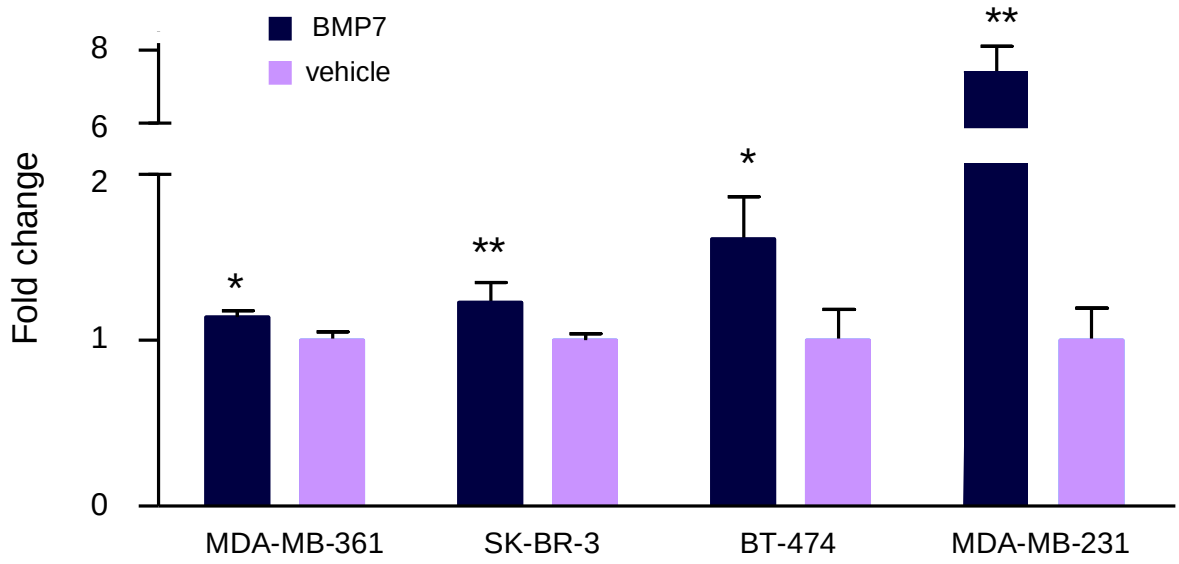

Figure 9. BMP7 induces cell migration. After 72-hour treatment with BMP7 or vehicle the cells were allowed to migrate for 22 hours. The changes in migration are represented as fold change bars with standard deviations. Asterisks * $p<0.05$, ** $p<0.005$

Based on these migration results, three cell lines (MDA-MB-361, BT-474 and SK-BR-3) were selected for invasion assays. No consistent changes were detected in MDA-MB-361 and SK-BR-3 cells that had an inherently low invasion capability. Despite the increase in migration, BMP7 did not have any effect on the invasion of BT-474 cells (data not shown). In the case of HCC1954 invasion tests were not conducted because no effect was seen in migration. A positive control (MDA-MB-231 cells) was used only for migration and not invasion testing.

5.4 Western blot reveals activation of signaling pathways in the cell lines

Possible activation of SMAD and MAPK signaling cascades in response to BMP7 treatment was determined by Western blot. An antibody against phosphorylated SMAD1, SMAD5 and SMAD8 (SMAD1/5/8) was utilized to find out whether the canonical SMAD pathway used by BMP proteins was activated. In addition, antibodies against two MAP kinases, p38 and p44/42 (ERK1/2), were employed to detect the activation of the mitogen-activated protein kinase (MAPK) pathway. Cells were treated

with BMP7 or vehicle for three hours (based on previous data from the research group) and then collected for protein extraction.

SMAD signaling pathway was activated in three of the five cell lines as seen by the difference in the intensity of phosphorylated SMAD1/5/8 bands between BMP7- and vehicle-treated cells (Figure 10). BMP7 induced prominent phosphorylation of SMAD1/5/8 in both MDA-MB-361 and MDA-MB-231 cell lines with very low basal phosphorylation levels. In contrast, the basal phosphorylation level in BT-474 cells was more prominent. Nevertheless, a clear induction of the SMAD pathway can be seen in this cell line as well. No differences were detected in the phosphorylation levels of SMAD1/5/8 in HCC1954 or SK-BR-3 cell lines between BMP7- and vehicle-treated cells. However, in these two cell lines MAPK activation was detected. Slight activation of p38 could be seen in SK-BR-3 cells in response to BMP7 treatment (Figure 10). Similarly, in HCC1954 cells BMP7 induced a small increase in p44/42 phosphorylation (Figure 10). P38 or p44/42 activation could not be seen in any other cell lines (data not shown). Antibodies against SMAD5, p38 and p44/42 proteins were used to confirm that there was no difference in the amount of unphosphorylated proteins between BMP7- and vehicle-treated cells (Figure 10). β-tubulin was used as a loading control for overall total protein.

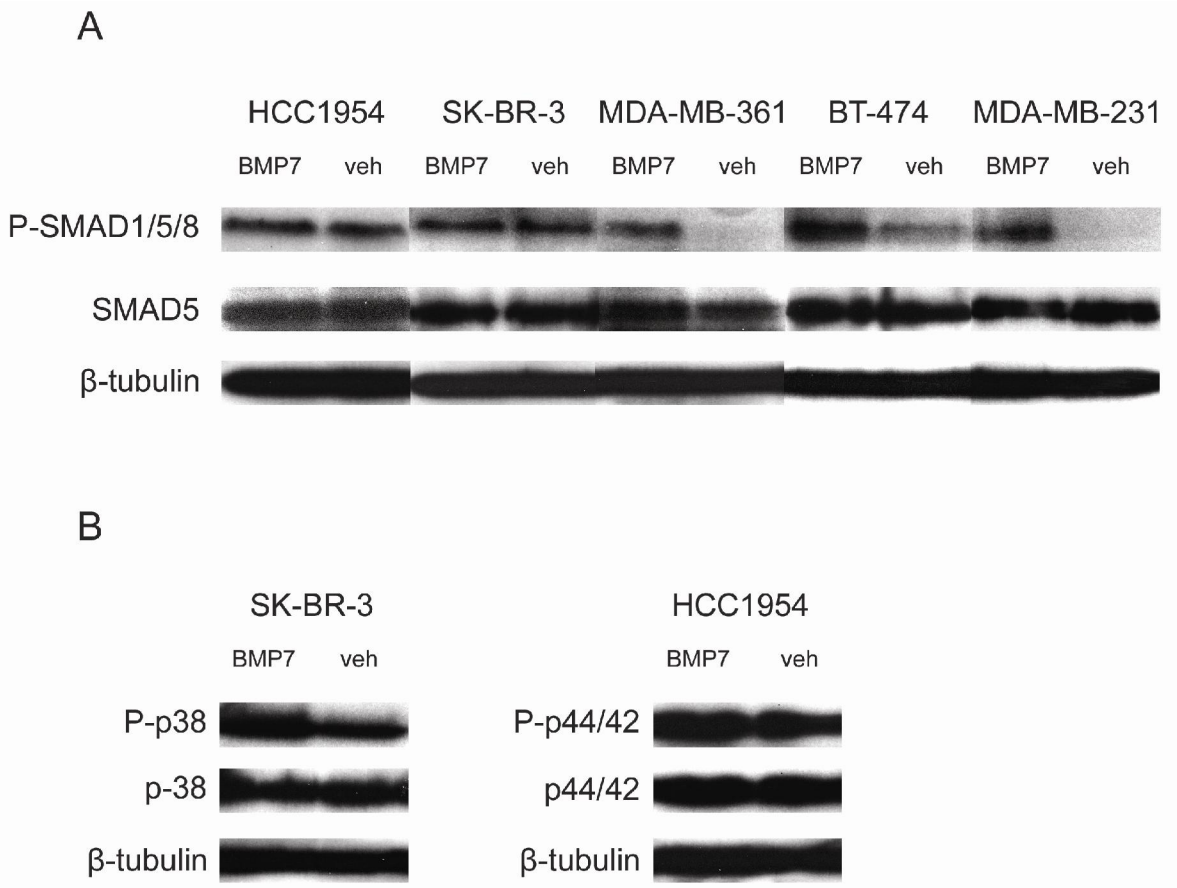

Figure 10. Either SMAD or MAPK pathway is activated upon BMP7 treatment in breast cancer cells. A, Phosphorylated and total levels of SMAD1/5/8 in the five cell lines. B, Phospho-p38 and p38 levels in SK-BR-3 cells and phospho-p44/42 and p44/42 levels in HCC1954 cells. The cells were treated with BMP7 or vehicle for three hours followed by protein collection and Western blot. β-tubulin was used as a loading control for all experiments. (P-SMAD1/5/8 stands for phospho-SMAD1/5/8, P-p38 stands for phospho-p38, P-p44/42 stands for phospho-p44/42.)

6 Discussion

6.1 BMP7 is able to inhibit or increase proliferation

BMP7 treatment of breast cancer cells with low endogenous BMP7 expression levels was previously shown to either inhibit or promote growth and induce migration (Alarmo et al., 2009). In this study the function of BMP7 was examined more extensively and cell lines with high endogenous expression levels were also included in BMP7 treatment. Five breast cancer cell lines were employed in assessing the function of BMP7 in breast cancer.

BMP7 function has been previously studied with the cell line MDA-MB-231 (Alarmo et al., 2009). Here the cell line was used as a positive control. Accordingly, the proliferation and migration results with MDA-MB-231 were comparable to the previous findings (Alarmo et al., 2009). BMP7 increased the proliferation of MDA-MB-231 cells 128% when the cells were grown in medium containing 1% serum. The reduced serum level was used in order to reveal the influence of BMP7 in MDA-MB-231 cells, since the effects of BMP7 were more modest in 10% serum. Most likely other growth factors present in the serum hide the action of BMP7 by allowing the cells to grow fast enough to override the potential effects of BMP7.

A previous study showed that BMP7 inhibits the growth of MDA-MB-361 and HCC1954 but no experiment was conducted on the mechanisms responsible for proliferation changes (Alarmo et al., 2009). In order to study cell cycle phase distribution, proliferation assays with MDA-MB-361 and HCC1954 were first repeated. As expected, BMP7 reduced the growth of MDA-MB-361 (9% at day 7) and HCC1954 (18% at day 7). However, cell cycle tests showed no consistent changes between BMP7- and vehicle-treated cells. For MDA-MB-361 cell line the cell cycle analyses were performed at several different time points after BMP7 treatment (2, 4 and 7 days) but no changes in cell cycle were detected. The cell cycle experiment was subsequently performed with BMP4 treatment, which has been shown to induce a prominent G1 arrest in this cell line (Ketolainen et al., 2010). The same results were obtained in this study (data not shown), thus confirming that the method was applied correctly. There are at least two possible explanations for the lack of discernible effects in cell cycle. First, it is possible that the growth effect was not large enough to be seen as a clear difference in the cell cycle phases between BMP7- and vehicle-treated cells. The cells could be synchronized prior to the start of the experiment in order to bring them to the

same cell cycle phase. This might reduce variation between BMP7- and vehicle-treated cells and thus reveal a change in cell cycle distribution. Secondly, the growth difference in MDA-MB-361 and HCC1954 might be due to increased apoptosis, which was not evaluated here.

BMP7 did not induce changes in growth in either SK-BR-3 or BT-474 cells, both of which had high endogenous BMP7 levels. For both cell lines a medium with 1% serum was tested to find out whether the removal of serum-derived growth factors would bring out an effect. However, no changes were observed in either 1% or 10% serum. In a previous study BMP7 expression was silenced in SK-BR-3 and BT-474 cells in order to observe potential proliferation changes (Alarmo et al., 2009). No effects were seen in SK-BR-3, which is consistent with the fact that addition of BMP7 had no effect. However, abolishing BMP7 expression from BT-474 cells led to reduced growth. Thus one would expect the addition of BMP7 to enhance the growth of BT-474 cells. The fact that no such change was seen suggests that a threshold is present so that addition of more BMP7 does not result in increased proliferation. However, BMP signaling must be functional, since Western blot data shows that the SMAD signaling pathway is activated. Instead, other signaling pathways or regulatory factors taking part in regulating growth could prevent the growth promoting or growth enabling effects of BMP7 in BT-474 cells.

The effect of BMP7 on the growth of other cancer types has been studied only minimally. BMP7 has been implicated in growth inhibition in melanoma and depending on the cell line it can either inhibit the growth of prostate cancer cells or have no effect on their growth (Feeley et al., 2005; Yang et al., 2005; Feeley et al., 2006; Buijs et al., 2007b; Hsu et al., 2008; Na et al., 2009; Morrissey et al., 2010). In addition, BMP7 seems to reduce growth in colon cancer but not affect the growth of lung cancer cells (Beck et al., 2006; Chen et al., 2010). Thus the effects of BMP7 on proliferation are dependent on the cancer type and and at least in breast and prostate cancer depend on the cell lines used.

6.2 BMP7 increases migration of breast cancer cells

The effect of BMP7 on migration was tested in all five cell lines. In accordance with previous results (Alarmo et al., 2009), migration of MDA-MB-231, the cell line used as a positive control, increased significantly. In addition, BMP7 moderately enhanced

migration of MDA-MB-361, SK-BR-3 and BT-474 cells (1.1, 1.2 and 1.6-fold, respectively). Interestingly, upon BMP7 treatment migration increased in almost all cell lines, regardless of the effects BMP7 had on growth. For example, proliferation of SK-BR-3 and BT-474, the cell lines with high BMP7 expression, was not affected by BMP7 although BMP7 increased migration in these cell lines. It has been shown that BMP4 simultaneously inhibits the growth of breast cancer cells and promotes their migration (Ketolainen et al., 2010). Even though the changes were small, a similar dual function can be seen in MDA-MB-361 cells.

MDA-MB-361, SK-BR-3 and BT-474, the cell lines in which BMP7 treatment increased migration, where chosen for invasion testing. However, the invasive potential of MDA-MB-361 and SK-BR-3 was not sufficient to achieve reliable results. BMP7 induced no effects on the invasion of BT-474 cells. Invasion requires activation of matrix metalloproteinases that are able to degrade ECM allowing cells to move through tissues (Gialeli et al., 2011). Invasion is therefore a more intricate process requiring that the cells be able to both migrate and degrade ECM.

In prostate cancer BMP7 appears to inhibit migration and/or invasion, although opposite findings have been reported (Corey and Vessella, 2007; Fournier and Guise, 2007). In lung cancer and melanoma BMP7 seems to be able to inhibit invasion, whereas in kidney and colon cancer cells BMP7 increased invasion (Grijelmo et al., 2007; Na et al., 2009; Chen et al., 2010). Thus the effects of BMP7 in migration and invasion depend on the cancer type.

A previous study with mouse models showed that over-expression of BMP7 in MDA-MB-231 cells inhibits metastasis, suggesting an antimigratory role for BMP7 (Buijs et al., 2007a). It was shown by Alarmo et al. (2009) and affirmed here that addition of BMP7 to the medium of MDA-MB-231 cells increases their migration *in vitro*. In addition, BMP7 increased migration in three other cell lines of this study, albeit only moderately. The contradictions likely result from the differences between using cell lines and a mouse model, and measuring invasion instead of migration. It is also possible that using a *BMP7* transgene, as done by Buijs et al. (2007a) creates a different situation from adding exogenous BMP7 protein.

6.3 BMP7 activates SMAD or MAPK signaling

Western blot data showed that BMP7 induces strong SMAD1/5/8 activation in MDA-MB-231, MDA-MB-361 and BT-474 cells. Activation of the signaling pathway in MDA-MB-231 clearly led to the dramatic changes in growth and migration. However, only minor changes in growth and migration were seen MDA-MB-361 cells and no effect on growth was evident in BT-474 cells. Nevertheless, BMP7 signaling was clearly strong in both cell lines. It is possible that the effects of BMP7 on these cell lines are reflected in other phenotypic changes that were not measured here. Those changes could affect, for example, the cytoskeleton, the metabolism of the cell, excretion of different factors or the condition of the cell making it more/less susceptible to hypoxia, temperature changes etc. In addition, changes downstream of SMAD1/5/8 signaling can alter the response of the cell to BMP7 (Table 1), but those changes were not measured in this study.

SMAD signaling was not induced in SK-BR-3 and HCC1954, where MAPK pathways were activated. MAPK signaling induction due to BMP7 has been detected in colon cancer but has not been studied in other cancer types (Grijelmo et al., 2007). BMP7 induced MAPK signaling in some normal tissues, such as kidney, brown adipose tissue and rat brain (Cox et al., 2004; Motazed et al., 2008; Tseng et al., 2008). Because BMP7 inhibited growth in HCC1954 and increased migration in SK-BR-3, it seems that both growth and migration effects can be signaled through SMAD and MAPK pathways, instead of either pathway being responsible for a particular phenotypic effect. Finally, activation of the signaling pathway did not correlate with endogenous BMP7 levels. For example, HCC1954 and MDA-MB-361 both had low levels of BMP7 expression but SMAD pathway was only activated in MDA-MB-361.

6.4 Possible factors involved in BMP7-induced phenotypic effects

There are several intrinsic and extrinsic factors that can be considered as possible explanations for the differences seen in proliferation and migration between the cell lines upon BMP7 treatment. First, it should be noted that the BMP7 concentration used here was not tested specifically with the cell lines of this study. Previously it was shown that 50 ng/ml was a sufficient amount of BMP7 to induce a phenotypic response in several breast cancer cell lines (Alarmo et al., 2009). In other cancer types the BMP7 concentration used ranges from 10 ng/ml to 500 ng/ml, with a few studies also using 50

ng/ml (Yang et al., 2005; Beck et al., 2006). Nevertheless, this amount might have been too low or high in some of the cell lines in this study, making tests with different BMP7 concentration levels warranted. However, activation of SMAD or MAPK signaling pathway could be seen in all cell lines, which verifies that the dose of 50 ng/ml was at least sufficient for induction of signaling.

The endogenous level of BMP7 varied between the cell lines, which raises the question of whether the effects of exogenous BMP7 are dependent on endogenous levels. Indeed, the effects of BMP7 on growth seem to correlate with endogenous BMP7 levels to a degree. BMP7 was able to increase the growth of the cell line with no BMP7 expression (MDA-MB-231) and inhibit the growth of the cell lines with low levels of BMP7. The cell lines which had high levels of BMP7 expression were not affected by BMP7 treatment. Moreover, in a previous study it was shown that BMP7 reduced the growth of ZR-75-30, another cell line with no BMP7 expression (Alarmo et al., 2009). Thus it appears that BMP7 is able to regulate growth if it is not endogenously expressed at high levels. However, no such correlation is seen migration. BMP7 enhanced migration significantly both in MDA-MB-231, which had no BMP7 expression, and in BT-474 and SK-BR-3, which had high levels of BMP7 expression. Migration was also increased to a lesser degree in MDA-MB-361, the cell line with a low expression level. Therefore, proliferation but not migration seems to correlate with endogenous BMP7 levels, although more cell lines would be needed to confirm the results and to find out why BMP7 is able to both increase and inhibit growth. Interestingly, it was previously shown that in the case of BMP4 endogenous levels don't matter since BMP4 reduces growth of breast cancer cells regardless of the endogenous level (Ketolainen et al., 2010).

The cell lines of this study originate from different sites. Two of the cell lines (BT-474 and HCC1954) were derived from primary tumors and two (SK-BR-3 and MDA-MB-231) from pleural effusions (ATCC). MDA-MB-361, in contrast, was derived from a brain metastasis. However, there is no correlation in the behavior of the two cell lines derived from primary tumors, as in HCC1954 cell line BMP7 inhibited the growth of cells and had no effect on migration, whereas in BT-474 cell line no effect was seen in growth but BMP7 increased migration. Similar discrepancies in phenotypic responses were evident in SK-BR-3, MDA-MB-361 and MDA-MB-231, which were derived from distant sites. Thus, the site of acquisition doesn't seem to correlate with the effect BMP7 has on cells.

It has been suggested that the p53 status of the cells may have an effect on the response of the cells to BMP7. Yan et al. (2007) showed that silencing BMP7 expression inhibited the proliferation of an MCF-7 cell line with a p53 knockdown and the cell line MDA-MB-231, which is naturally p53-deficient. They found sufficient BMP7 expression in MDA-MB-231 to merit BMP7 silencing treatment in the cell line in order to examine proliferation effects. However, this is contradictory to some previous studies, which found no evidence of BMP7 in MDA-MB-231 (Arnold et al., 1999; Alarmo et al., 2006). All the cell lines here were p53-deficient (Berglind et al., 2008). Despite the p53-status, in this study growth increase was only seen in MDA-MB-231 cells. However, the fact that BMP7 silencing leads to growth inhibition does no necessarily mean that addition of BMP7 will induce growth.

In conclusion, the effects of BMP7 in breast cancer are small and they do not seem to depend on the endogenous BMP7 level, except in proliferation, or on the site of origin of the cell line. More experiments are required in order to fully uncover the effect BMP7 has on breast cancer cells.

7 Conclusions

Despite rigorous efforts to find better treatment options and diagnostic tools, breast cancer is still the most common cancer in women and a leading cause of death. The heterogeneous nature of breast cancer and the multitude of factors found to be associated with breast cancer progression complicate the research. A class of growth factors, the bone morphogenetic proteins, appears to be involved in many types of cancer, including melanoma, breast, prostate and lung cancer. However, their exact role in carcinogenesis is presently unclear.

In this study, the effects of BMP7 on the growth and migration of breast cancer cells were examined using five breast cancer cell lines. The cell lines had various responses to BMP7. Both growth-promoting and growth-inhibiting effects were seen after BMP7 treatment. In addition, in some cell lines BMP7 did not affect growth. Migration increased in four cell lines and SMAD or MAPK signaling pathways were activated in all cell lines. Thus BMP7 influences every cell line, but the outcomes are different and presumably depend on other differences between the cell lines. Use of multiple cell lines is consequently warranted in these types of studies. However, conclusive results concerning the role of BMPs in breast cancer remain elusive.

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