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Characterization of Bone Morphogenetic Protein 7 in Breast Cancer

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 1,
Biokatu 6, Tampere, on February 8th, 2008, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
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Tampere Graduate School in Biomedicine and Biotechnology (TGSBB)
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www.uta.fi/taju
http://granum.uta.fi

Cover design by
Juha Siro

Acta Universitatis Tamperensis 1290
ISBN 978-951-44-7210-7 (print)
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 693
ISBN 978-951-44-7211-4 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2008

TIIVISTELMÄ

Luun morfogeneettiset proteiinit (BMP) ovat solunulkoisia signaalimolekyylejä, jotka säätelevät laajasti ja monimuotoisesti yksilön kehitysvaiheita ja luun muodostusta. Monimuotoisuudestaan johtuen BMP:t ovat myös olleet viimeisen kymmenen vuoden aikana kasvavan kiinnostuksen kohteena syöpätutkimuksessa. Tämän tutkimuksen tarkoitus oli karakterisoida luun morfogeneettisen proteiini 7:n (BMP7) aktivaatiota, ilmentymistä, kliinistä merkitystä ja tehtäviä rintasyövässä.

Aikaisemmat mikrosirupohjaiset tutkimukset rintasyöpäsolulinjoissa viittasivat siihen, että *BMP7* voisi olla monistuman kohdegeeni. *BMP7* geenikopiolukua ja ilmentymistä tutkittiin 22 rintasyöpäsolulinjasta ja 146 primaarisessa rintasyövän kasvainnäytteestä. *BMP7* geenikopioluvut analysoitiin FISH tekniikalla. Lähetti-RNA:n ilmentymistasot määritettiin joko RT-PCR:n avulla solulinjoista tai kvantitatiivisella RT-PCR metodilla 44 kasvainnäytteen osajoukosta. BMP7 proteiinin ilmentyminen selvitettiin immunohistokemialla solulinjoista, primaarisista kasvainnäytteistä ja 10 normaalista rintarauhasnäytteestä. *BMP7* geenikopioluku oli noussut puolessa solulinjoista ja 16 %:ssa kasvainnäytteistä. Lähetti-RNA:n ilmentymistasot vaihtelivat hyvin paljon sekä solulinjoissa ja kasvainnäytteissä. Vaikka varsin korkeita ilmentymistasoja havaittiin nimenomaan näytteissä, joissa myös geenikopioluku oli lisääntynyt, tilastollisesti merkittävää yhteyttä ei ollut *BMP7*:n kohonneen kopioluvun ja kohonneen ilmentymistason välillä. BMP7 proteiini kuitenkin ilmentyi voimakkaasti yli 70 %:ssa kasvainnäytteistä verrattuna normaalinäytteisiin, mikä viittaa syövälle spesifiseen yli-ilmentymiseen.

Systemaattinen kartoitus lähetti-RNA:n ilmentymisestä suoritettiin seitsemälle BMP ligandille (*BMP2-BMP8*) ja kuudelle BMP solukalvoreseptorille, jotka pystyvät välittämään BMP signaaleja. Ilmentymistasot määritettiin semikvantitatiivisella RT-PCR metodilla 22 rintasyöpäsolulinjasta, 39 rintasyövän kasvainnäytteestä sekä normaalista rintarauhasen epiteelisolulinjasta ja normaalista rintarauhasen kudosnäytteestä. Ilmentymisprofiilit solulinjoissa ja kasvainnäytteissä olivat yleisesti ottaen hyvin samansuuntaisia. Ligandien ilmentymisfrekvenssit ja –tasot vaihtelivat hyvin paljon ligandista toiseen. *BMP7*:n lisäksi *BMP4* ilmeni laajasti, vaihtelevalla tasolla ja syövälle spesifisesti. BMP reseptorit ilmentyivät ligandeihin verrattuna melkein kaikissa tutkituissa näytteissä, mikä viittaa siihen, että rintasyövässä BMP signalointi on mahdollista.

BMP7 yli-ilmentymisen kliinistä merkitystä tutkittiin rintasyöpäaineistossa, jossa oli 483 rintasyöpäpotilaan täydelliset kliinispatologiset tiedot ja jopa 15 vuotta kattavat seurantatiedot. Kasvainnäytteet sisälsivät 241 lobulaarista karsinoomaa, 242 duktaalista karsinoomaa ja 40 näiden potilaiden paikallisesti uusiutunutta kasvainnäytettä. BMP7 proteiinin ilmeneminen määritettiin immunohistokemian avulla ja BMP7 positiivisia kasvaimia oli 47 %. Proteiinin ilmeneminen oli kasvaintyypistä riippuvaista, koska sitä havaittiin useammin

lobulaarisissa (57 %) kuin duktaalisissa (37 %) rintasyövissä. Yllättävää kyllä, vain 13 % paikallisesti uusiutuneista kasvaimista oli BMP7 positiivisia. BMP7 ei vaikuttanut potilaiden eloonjäämiseen, mutta selvästi ja tilastollisesti merkittävästi nopeutti metastaasien muodostumista luuhun. Monimuuttuja-analyysin perusteella BMP7 itsenäisesti ennustaa aikaista luumetastaasin muodostumista rintasyövässä.

Viimeiseksi BMP7 yli-ilmentymisen vaikutusta rintasyöpäsolulinjojen ilmiasuun selvitettiin kaksisuuntaisella lähestymistavalla. *BMP7* ilmentyminen hiljennettin RNA interferenssitekniikkaa hyödyntäen kolmessa solulinjassa (BT-474, MCF7, SK-BR-3), joissa *BMP7* ilmentymistaso oli korkea. BMP7 rekombinattiproteiinia puolestaan annettiin kahdelle solulinjalle (MDA-MB-231, T-47D), joissa *BMP7* ei ilmene. Näiden manipulaatioiden seurauksia solujen kasvuun, solusykliin, apoptoosiin, migraatioon ja invaasioon määritettiin eri funtionaalisilla analyyseillä. BMP7 vaikutti kasvuun kahdessa solulinjassa. *BMP7* hiljentäminen vähensi BT-474 solujen kasvua, joka johtui solusyklin pysähtymisestä G1 vaiheeseen. BMP7 käsittely lisäsi MDA-MB-231 solujen kasvua, mikä puolestaan johtui vähentyneestä apoptoottisten solujen määrästä. Siten näissä kahdessa solulinjassa BMP7 stimuloi kasvua joko säätemällä solusykliä tai apoptoosia. Lisäksi BMP7 käsittely lisäsi merkittävästi MDA-MB-231 solujen migraatiota ja vielä dramaattisemmin näiden solujen invaasiota. BMP7 siis selvästi vaikuttaa rintasyöpäsolujen ilmiasuun ja tämä vaikutus on hyvin solutyyppikohtaista.

Yhteenvetona voidaan todeta, että BMP7 yli-ilmenee laajasti rintasyövässä ja tällä on vaikutusta rintasyöpäsolujen toimintaan. Kliininen data osoittaa myös, että BMP7 on osallinen luumetastaasien muodostumiseen rintasyövässä.

CONTENTS

List of original communications

This thesis is based on the following communications referred in the text by their Roman numerals:

I. Alarmo E-L, Rauta J, Kauraniemi P, Karhu R, Kuukasjärvi T, and Kallioniemi A (2006): Bone morphogenetic protein 7 is widely overexpressed in primary breast cancer. Genes Chromosomes Cancer 45:411-419.

II. Alarmo E-L, Kuukasjärvi T, Karhu R and Kallioniemi A (2007): A comprehensive expression survey of bone morphogenetic proteins in breast cancer highlights the importance of *BMP4* and *BMP7*. Breast Cancer Res Treat 103:239-246.

III. Alarmo E-L, Korhonen T, Kuukasjärvi T, Huhtala H, Holli K and Kallioniemi A (2007): Bone morphogenetic protein 7 expression associates with bone metastasis in breast carcinomas. Ann Oncol (in press).

IV. Alarmo E-L, Pärssinen J, Karhu R and Kallioniemi A (2007): BMP7 influences proliferation, migration and invasion of breast cancer cells. Submitted for publication.

Abbreviations

ACVR1	Activin A receptor type I
ACVR2A	Activin A receptor type IIA
ACVR2B	Activin A receptor type IIB
BAC	Bacterial artificial chromosome
BMP	Bone morphogenetic protein
BMP7	Bone morphogenetic protein 7
BMPR1A	BMP receptor type IA
BMPR1B	BMP receptor type IB
BMPR2	BMP receptor type II
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
GDF	Growth and differentiation factor
HMEC	Human mammary epithelial cells
HMG	Human mammary gland
HRT	Hormone replacement therapy
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
MAPK	Mitogen activated protein kinase
PAC	P1 artificial chromosome
PI	Propidium iodide
pM	Pathological metastasis stage
pN	Pathological lymph node stage
PR	Progesterone receptor
pT	Pathological tumor stage
PTEN	Phosphatase and tensin homolog
rhBMP7	Recombinant human BMP7
RNAi	RNA interference
RT-PCR	Reverse transcriptase PCR
siRNA	Short interfearing RNA
SMAD	Sma- and Mad-related protein
SMURF	Smad ubiquination regulatory factor
TBP	TATA-box binding protein

TGFβ	Transforming growth factor β
TMA	Tissue microarray

Abstract

Bone morphogenetic proteins (BMP) are extracellular signaling molecules that regulate vertebrate development and bone formation. Due to their pleiotropic actions, they have been an object of growing interest in cancer research in the last decade. The purpose of this study was to characterize the activation, expression, clinical relevance and function of bone morphogenetic protein 7 (BMP7) in breast cancer.

A previous report indicated that *BMP7* may be a putative amplification target gene based on a microarray study conducted on breast cancer cell lines. *BMP7* gene copy number and expression were explored in a large panel of 22 breast cancer cell lines, 146 primary breast tumors, and in normal mammary gland tissue. *BMP7* copy numbers were analyzed using FISH. *BMP7* mRNA and protein expression levels were determined using quantitative RT-PCR and immunohistochemistry. Increased *BMP7* copy number was detected in half of the cell lines and in 16% of the primary tumors. Variable *BMP7* expression was seen in both cell lines and primary tumors. Although the highest expression levels were detected in specimens with increased copy number, there was no significant association between *BMP7* copy number increase and elevated mRNA expression. However, strong BMP7 protein expression was observed in over 70% of primary breast tumors compared to the normal samples indicating cancer specific overexpression.

Systematic expression survey was performed for seven BMP ligands (*BMP2-BMP8*) and six BMP transmembrane receptors capable of transmitting BMP signals. Expression levels were determined using semiquantitative RT-PCR in 22 breast cancer cell lines and 39 primary breast tumors as well as in normal samples of mammary epithelial cell line and mammary gland tissue. In general the expression patterns in the cell lines were comparable to the patterns obtained from the primary tumors. The expression frequencies and levels differed considerably from one ligand to another and in addition to *BMP7*, *BMP4* had a wide, variable, and cancer-specific expression profile. BMP specific receptors were ubiquitously expressed suggesting that breast cancer can receive BMP signals.

The clinical relevance of BMP7 overexpression in breast cancer was studied in a group of 483 breast cancer patients with complete clinicopathological information and up to 15 years of follow-up. Samples contained 241 lobular carcinomas, 242 ductal carcinomas and 40 corresponding local recurrent tumors. BMP7 protein was expressed in 47% of the primary tumors determined by immunohistochemistry. BMP7 expression was tumor subtype dependent, since it was detected more often in lobular (57%) than in ductal (37%) tumors.

Interestingly, BMP7 expression was observed in only 13% of local recurrent tumors. BMP7 expression did not affect overall survival but was clearly and significantly associated with accelerated rate of metastasis formation in bone. A multivariant analysis confirmed that BMP7 was indeed an independent prognostic factor for early bone metastasis.

Finally, the possible contribution of BMP7 overexpression to breast cancer cell line phenotypes was examined using a bidirectional approach. *BMP7* expression was silenced using RNA interference in three cell lines (BT-474, MCF7, SK-BR-3) with high endogenous expression and exogenous BMP7 was given to two cell lines (MDA-MB-231, T-47D) with no expression. The consequences of the manipulations were determined using functional assays for proliferation, cell cycle, apoptosis, migration, and invasion. BMP7 influenced the growth of two breast cancer cell lines. *BMP7* silencing reduced growth in BT-474 cells that was caused by G1 arrest. Exogenous BMP7 treatment increased MDA-MB-231 growth instead by reducing the number of apoptotic cells. Thus in these two cell lines BMP7 stimulated proliferation either by regulating cell cycle or apoptosis. BMP7 treatment also significantly induced migration and even more drastically invasion of MDA-MB-231 cells. BMP7 does clearly have an impact on breast cancer cell phenotype and this is evidently dependent on the cellular context.

Taken together, BMP7 is widely overexpressed in breast cancer and has an impact on breast cancer cell behavior. The clinical data furthermore implies that BMP7 is involved in the bone metastasis process in breast cancer.

Introduction

Cancer is a disease of malignant cell overgrowth and is dependent on age, environment and genes (reviewed by Breivik 2005). Cancer incidence increases with age and cancer development is associated with exposure to environmental factors such as smoking, diet, and radiation. The most important factor is genes, which are also the mediators for age and environment related effects. Cancer is in essence a genetic disease in which an accumulation of genetic alterations leads to tumor formation. Three types of genes are ultimately involved in tumorigenesis, oncogenes, tumor suppressor genes and genes that contribute to the maintenance of genetic stability (reviewed by Ponder 2001, Balmain et al. 2003, Vogelstein and Kinzler 2004). In normal tissue unaffected (wild-type) counterparts of oncogenes and tumor suppressor genes regulate cell growth. In cancer, mutations occurring in oncogenes make them constitutively active or active in a context where they would normally be inactive, and thus their actions accelerate cell growth. The genetic events that activate oncogenes are intragenic mutations, chromosomal translocations, and gene amplification i.e. increase in the gene copy number. Tumor suppressor genes instead suffer from inactivating mutations and are thereby unable to prevent accelerated cell growth. These mutations include missense mutations, truncating mutations, insertions and deletions, and epigenetic silencing, most commonly methylation of gene promoter regions that prevent transcription. A single mutated allele is sufficient for the activation of an oncogene whereas mutation or loss of both alleles is required for tumor suppressor inactivation. Genes responsible for genomic integrity are called ‘caretakers’. These caretaker genes do not affect cell growth, but loss of their function leads to an increased mutation rate, thus accelerating tumorigenesis.

However, there are genes that do not fit well into these classical definitions. They do not harbor mutations but are still aberrantly expressed at elevated or diminished levels in cancer (reviewed by Vogelstein and Kinzler 2004). The same gene can even act as an oncogene or tumor suppressor gene depending on the context. One such bidirectional gene is the transforming growth factor β (TGFβ) that can inhibit cancer cell growth in the initial phases of tumorigenesis but subsequently lose this ability and instead promote metastasis (reviewed by Massague and Gomis 2006). TGFβ is a signaling molecule that belongs to a large superfamily of cytokines also including bone morphogenetic proteins. The purpose of this study was to explore the possible activation, expression, clinical relevance, and functional role of bone morphogenetic protein 7 in breast cancer.

Review of the literature

1. Breast cancer

Breast cancer is the most common cancer among women in western countries. In Finland, over 4000 women were diagnosed with breast cancer in 2005 and this number has increased steadily since the 1950s (Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi updated on 18.9.2007). Breast cancer cases represent roughly one third of all cancer cases among women in Finland. Fortunately, breast cancer is not the most aggressive cancer, since five years after diagnosis 89% of breast cancer patients are still alive. Yet, due to the high incidence rate, breast cancer also has the highest mortality rate among women in Finland. Breast cancer risk factors include age, geographical status, family history, lifestyle risk factors (alcohol, diet, obesity), and lifelong exposure to estrogens (reviewed by Dumitrescu and Cotarla 2005).

The normal mammary gland ductal system consists of luminal epithelial cells lining the inner lumen of the ducts, surrounded by myoepithelial cells and basement membrane (reviewed by Anderson 2002). Breast cancer originates from the epithelial cells in the terminal ductal lobular units (reviewed by Allred et al. 2001, Polyak 2001). On the basis of histopathological and clinical features breast cancers are conventionally divided into subtypes: special type and non-special type (reviewed by Hanby 2005). Non-special type breast cancer is commonly called ductal carcinoma and approximately 80% of breast cancers belong to this group. Breast cancers of special type include lobular as well as tubular, mucinous, medullary and other rare types of breast carcinomas. Lobular carcinomas represent the second largest group of breast cancers accounting for 5-10% of all cases and recent reports indicate an increased incidence in recent decades (Li et al. 2000, Verkooijen et al. 2003). Lobular carcinomas can be identified according to their distinctive morphological growth pattern, where cancer cells grow as narrow cords and form a diffuse, swirling pattern (reviewed by Hanby 2005). Compared to ductal carcinomas, lobular carcinomas tend more often to be hormone receptor positive and ERBB2 negative. They proliferate more slowly, and in general also contain fewer genetic changes (Korhonen et al. 2004, reviewed by Lacroix et al. 2004, Simpson et al. 2005). However, there are other ways of classifying breast tumors, such as basal type tumors that are ER (estrogen receptor), PR (progesterone receptor), ERBB2 negative and cytokeratin positive and luminal type tumors that are ER positive (Sims et al. 2007). Recent gene expression analyses have also provided a means of classification through distinct gene expression profiles that segregate tumors into

normal-like, luminal A and luminal B, ERBB2, and basal tumors (Perou et al. 2000, Sorlie et al. 2001, reviewed by Chang et al. 2005). All these subtype classifications aim at a better grouping of breast cancer patients in such a way that the optimal therapy response is achieved.

Upon diagnosis of breast cancer several prognostic and predictive markers are determined which in turn guide the therapy options: surgery, radiotherapy, adjuvant chemotherapy, and adjuvant endocrine treatment (reviewed by Sainsbury et al. 2000). In addition to the histological tumor subtype discussed above, these markers include axillary node status, tumor size, nuclear grade, estrogen and progesterone receptor status, measures of proliferation, and ERBB2 status (reviewed by Clark 2001, Chang et al. 2005). The nuclear grade reflects the degree of differentiation and combines the histological evaluation of nuclear pleomorphism, mitotic activity, and tubule formation (reviewed by Lacroix et al. 2004). Low-grade tumors are often well differentiated and have a more favorable prognosis than high-grade poorly differentiated tumors. Estrogen receptor status is another well known prognostic and predictive factor since ER positive tumors respond well to endocrine therapy (reviewed by Esteva and Hortobagyi 2004, Lacroix et al. 2004). Typically low-grade tumors express ER whereas high-grade tumors are ER negative. Positive axillary lymph node status and positive ERBB2 expression are in turn both markers for poor prognosis (reviewed by Esteva and Hortobagyi 2004).

2. Basic aspects of tumorigenesis

2.1 Clonal evolution of cancer

Cancer is thought to arise through the sequential accumulation of genetic defects (reviewed by Ponder 2001, Nowell 2002). A mutation in a single cell leads to a growth advantage of its progeny, which then further gains additional defects conferring neoplastic phenotype and allows the clonal expansion of cancer cells. Multistep accumulation of genetic defects was first illustrated in colon carcinoma, where specific gene alterations have been linked to morphologically distinct stages of tumorigenesis (reviewed by Kinzler and Vogelstein 1996).

Breast cancer is also thought to follow a multistep cascade through atypical hyperplasia, carcinoma *in situ*, invasive carcinoma to metastatic cancer (reviewed by Allred et al. 2001, Polyak 2001). However, in the case of breast cancer, this should not be interpreted purely as a linear model of progressive molecular changes. Molecular analysis has shown that many cancer specific alterations in invasive cancers are already found in the carcinoma *in situ* lesions and that degree of differentiation, the grade already detectable in carcinoma *in situ*, separates breast cancers into well-differentiated, low-grade tumors and poorly differentiated, high-grade tumors (reviewed by Reis-Filho and Lakhani

2003, Lacroix et al. 2004, Simpson et al. 2005). Thus high grade tumors do not necessarily evolve from low grade tumors.

It has been suggested that the initial cancer mutation or mutations in breast cancer could target breast tissue specific stem cells instead of terminally differentiated epithelial cells (reviewed by Polyak and Hahn 2006). Tissue specific stem cells harbor properties similar to those of a cancer cell, including the ability to self-renewal and migration to distant parts of the body. On the other hand, it is equally possible that mutations target the progeny of stem cells or differentiated epithelial cells that then dedifferentiate through a process called epithelial-mesenchymal transition (EMT).

Either way, it has been estimated that in solid tumors four to seven genetic defects are needed for the development of cancer (reviewed by Ponder 2001). These defects are translated into diverse abilities to overcome the restrictions in the normal tissue environment. All cancer cells are thought to acquire six alterations in cell physiology that are 1) self-sufficiency in growth signals, 2) insensitivity to growth inhibitory signals, 3) evasion of apoptosis, 4) unlimited replicative potential, 5) sustained angiogenesis, and 6) tissue invasion and metastasis (reviewed by Hanahan and Weinberg 2000).

2.2 Tumor metastasis

Cancer deaths are mainly caused by metastatic outgrowths of the primary tumor that are difficult targets for therapy (reviewed by Steeg 2006). In order to metastasize a cancer cell must invade surrounding tissues. It is thought that epithelial-mesenchymal transition (EMT), where epithelial cells lose their polarization and adhesive properties and acquire mesenchymal properties, contributes to the dissemination of cancer cells from the primary tumor (reviewed by Thiery 2002). In the next steps, cancer cells have to enter the circulation, survive in the circulation, migrate to distant tissues, exit circulation into target organ parenchyma, and finally grow in a new environment (reviewed by Chambers et al. 2002, Nguyen and Massague 2007). Cancer cells can enter the circulation (a process called intravasation) either through lymphatic or blood vessels, and the fact that the tumor vasculature is often imperfect and leaky, can enhance this process. In the bloodstream cancer cells have to tolerate the stress of blood pressure, lack of substratum and the presence of immune cells (reviewed by Steeg 2006). In the process of extravasation, the exit from circulation, cancer cells must first arrest in the circulatory system. This is caused mainly through mechanistic barriers, since the capillaries are simply too small to allow the flow of cancer cells, but adhesive molecules may also play a role (reviewed by Chambers et al. 2002, Nguyen and Massague 2007). Cancer cells then invade the target organ parenchyma either by mechanistically breaking through the capillary wall or by remodeling the capillary wall in a way that allows transmigration of the cancer cells. The final challenge is the colonization of the target organ, growth in a new microenvironment that can occur immediately or after a period of dormancy, even decades after primary tumor

treatment. As a whole metastasis is a very inefficient process and likely requires genetic defects that are quite different from those detected in the primary tumor (reviewed by Steeg 2006, Nguyen and Massague 2007).

Interestingly, primary cancers exhibit a preference for secondary growth sites of cancer cells called organ tropism (reviewed by Chambers et al. 2002, Nguyen and Massague 2007). Breast and prostate cancers metastasize most often to bone, whereas colon carcinomas prefer liver. Already a century ago Stephen Paget suggested a "seed and soil" theory where seeds (cancer cells) spread in all directions, but are able to grow only in congenial soil (target organ) (reviewed by Chambers et al. 2002). Later on circulation patterns were acknowledged to influence the locations where cancer cells first travel. Since cancer progression is seen as an evolutionary process, it has been suggested that metastasis depends on genetic alterations separate from the primary tumor progression (reviewed by Nguyen and Massague 2007). Recently metastasis-specific gene expression signatures have been identified in primary tumors, suggesting that a predisposition to metastasis is already present in primary tumor cells. Most likely all the above mentioned aspects affect the growth of cancer cells in a given target organ.

Breast cancer metastasizes most often to bone, 80% of patients with advanced disease suffer from skeletal metastases that are incurable (reviewed by Kozlow and Guise 2005). Bone metastases can be either osteolytic (destruction of bone) or osteoblastic (overgrowth of bone) and in both cases the normal homeostasis of bone resorption by osteoclasts and bone formation by osteoblasts is disturbed (reviewed by Mundy 2002, Roodman 2004). Breast cancer bone metastases are typically characterized as osteolytic, but at least 15-20% are osteoblastic or mixed lesions (reviewed by Roodman 2004, Kozlow and Guise 2005). The invasion by cancer cells of the endosteal bone allows interaction between the tumor and bone derived cells (reviewed by Mundy 2002, Kakonen and Mundy 2003, Roodman 2004, Kozlow and Guise 2005). Tumor cells secrete factors that indirectly through osteoblasts stimulate osteoclasts which in turn begin the resorption of bone. Since bone is a reservoir of multiple growth factors, their release by osteoclast action in turn stimulates the growth of tumor cells. As a result a loop of deleterious functions, a "vicious cycle", increases the tumor cell growth and bone destruction. The precise mechanisms leading to osteoblastic metastasis in breast cancer have been studied less, but it is likely to involve growth factors secreted by the tumor cells that induce osteoblasts to form bone (reviewed by Mundy 2002, Logothetis and Lin 2005).

3. Bone morphogenetic proteins

3.1 Structure and function

Bone morphogenetic proteins (BMP) belong to the transforming growth factor β (TGFβ) superfamily of signaling molecules that is consisting of BMPs, activins/inhibins, TGFβs and other individual members (reviewed by Kawabata et al. 1998, Chang et al. 2002). Over 20 BMPs have been identified so far in humans and they comprise the largest subfamily of TGFβ family (reviewed by Ye et al. 2007a). Some members of the BMP family are also called growth and differentiation factors (GDF) (reviewed by Ducy and Karsenty 2000). Based on sequence similarity, BMPs can be further divided into subgroups (Newfeld et al. 1999, reviewed by Kawabata et al. 1998, Botchkarev 2003).

A mature BMP molecule is dimeric, composed of two monomers linked by a disulfide bond (Griffith et al. 1996, reviewed by Kingsley 1994, Reddi 1998, Sebald et al. 2004). BMPs are synthesized as large precursor proteins that contain signal peptide, prodomain and mature monomer domain (reviewed by Kingsley 1994, Sebald et al. 2004). In the secretory process prodomains are subsequently proteolytically cleaved at consensus site RXXR and the mature dimeric protein is produced (reviewed by Ducy and Karsenty 2000). Prodomains are thought to be involved in the regulation of BMP activity and availability (Constam and Robertson 1999, Gregory et al. 2005, Sopory et al. 2006). BMPs also exist as heterodimers that can be biologically more active than homodimers (Aono et al. 1995, Israel et al. 1996, Zhu et al. 2006).

BMPs were originally identified according to their ability to form bone at extraskeletal sites (reviewed by Reddi 1997, Wozney 2002). BMPs are capable of inducing endochondral bone formation (reviewed by Wozney and Rosen 1998). They induce the differentiation of mesenchymal cells into chondroblasts and osteoblasts, enhance their actions and subsequent new bone formation. They regulate bone and cartilage formation during embryogenesis, organogenesis, and in adult tissues during the bone repair process. BMPs are now known to have many more functions beyond bone formation. They play critical roles during development all the way from the very early steps of embryogenesis, formation of left-right asymmetry, neural and skeletal patterning, limb formation, to organogenesis (reviewed by Hogan 1996b, Zhao 2003). Homozygous mutant mouse phenotypes reveal that certain BMPs are actually vital for development since *Bmp2* and *Bmp4* null mutant mice die during embryogenesis (reviewed by Hogan 1996a). Bmp2 and Bmp4 have been proposed to regulate the development of mouse mammary gland (Phippard et al. 1996, Cho et al. 2006) but overall very little is known of BMP functions during breast development. However, receptors specific for BMP signaling are expressed in developing mammary gland implicating a role for these ligands (reviewed by Wakefield et al. 2001).

Human bone morphogenetic protein 7 (BMP7, also known as osteogenic protein 1, OP-1) cDNA was identified in 1990 (Celeste et al. 1990, Ozkaynak et

al. 1990). Based on amino acid sequence similarity it is closely related to BMP5, BMP6, BMP8A, and BMP8B (Celeste et al. 1990, Griffith et al. 1996, reviewed by Chang et al. 2002) thus forming a distinct subgroup within BMPs. BMP7 can induce new bone formation (Sampath et al. 1992, reviewed by Groeneveld and Burger 2000) and possesses one of the prominent osteogenic activities among the BMP family members (Luu et al. 2007). Due to this ability, BMP7 has been used in clinical applications in orthopedics and has been approved by FDA (Food and Drug Administration) for treatment of long-bone fractures and spinal fusions in USA (reviewed by Luo et al. 2005, Brown et al. 2006). Studies using *Bmp7* null mice revealed that Bmp7 is also required during development. Without Bmp7 mice died shortly after birth due to renal failure and defects were also detected in eye and skeleton formation (Dudley et al. 1995, Luo et al. 1995, Jena et al. 1997). Bmp7 is thus particularly important for kidney development (Vukicevic et al. 1996, reviewed by Simic and Vukicevic 2005) and is also expressed in fetal heart (Helder et al. 1995). The double loss of Bmp7 and Bmp6 leads to severe heart defects (reviewed by Zhao 2003). Bmp7 is known also to be expressed in adult kidney and to have a role in kidney homeostasis and has therefore been implicated in various renal injuries (reviewed by Patel and Dressler 2005, Simic and Vukicevic 2005).

3.2 Signaling pathway

In addition to their common structure, BMPs and other members of the family share a common signaling pathway. The rough backbone of the TGFβ superfamily signaling pathway is currently fairly well characterized and several excellent reviews cover the main steps in general (Heldin et al. 1997, Massague 1998, Miyazono et al. 2001, Shi and Massague 2003, ten Dijke and Hill 2004, Massague and Gomis 2006) and the BMP specific features (Kawabata et al. 1998, ten Dijke et al. 2003, Nohe et al. 2004, Miyazono et al. 2005). Ligand dimer initiates the signal by binding to two transmembrane receptors on the cell surface, namely type II and type I receptors. Ligand binding causes type II receptor to phosphorylate and thus activate the type I receptor. Upon receptor activation the signal is transferred to cytosolic Smad proteins. Type I receptor phosphorylates and in turn activates receptor regulated Smads, R-Smads. Phosphorylated R-Smads form a complex with common-Smad, Co-Smad. This complex translocates to nucleus and together with other nuclear cofactors regulates transcription of target genes (Figure 1). There are up to 42 members in the TGFβ family, but only five type II receptors, seven type I receptors, and 5 different R-Smads (reviewed by Feng and Derynck 2005). Therefore to obtain a specific response substantial signaling regulation is required. This can be achieved by regulating the signal extracellularly, on the membrane, in the cytosol and in the nucleus. The BMP specific features of the signaling pathway and its regulation are discussed below.

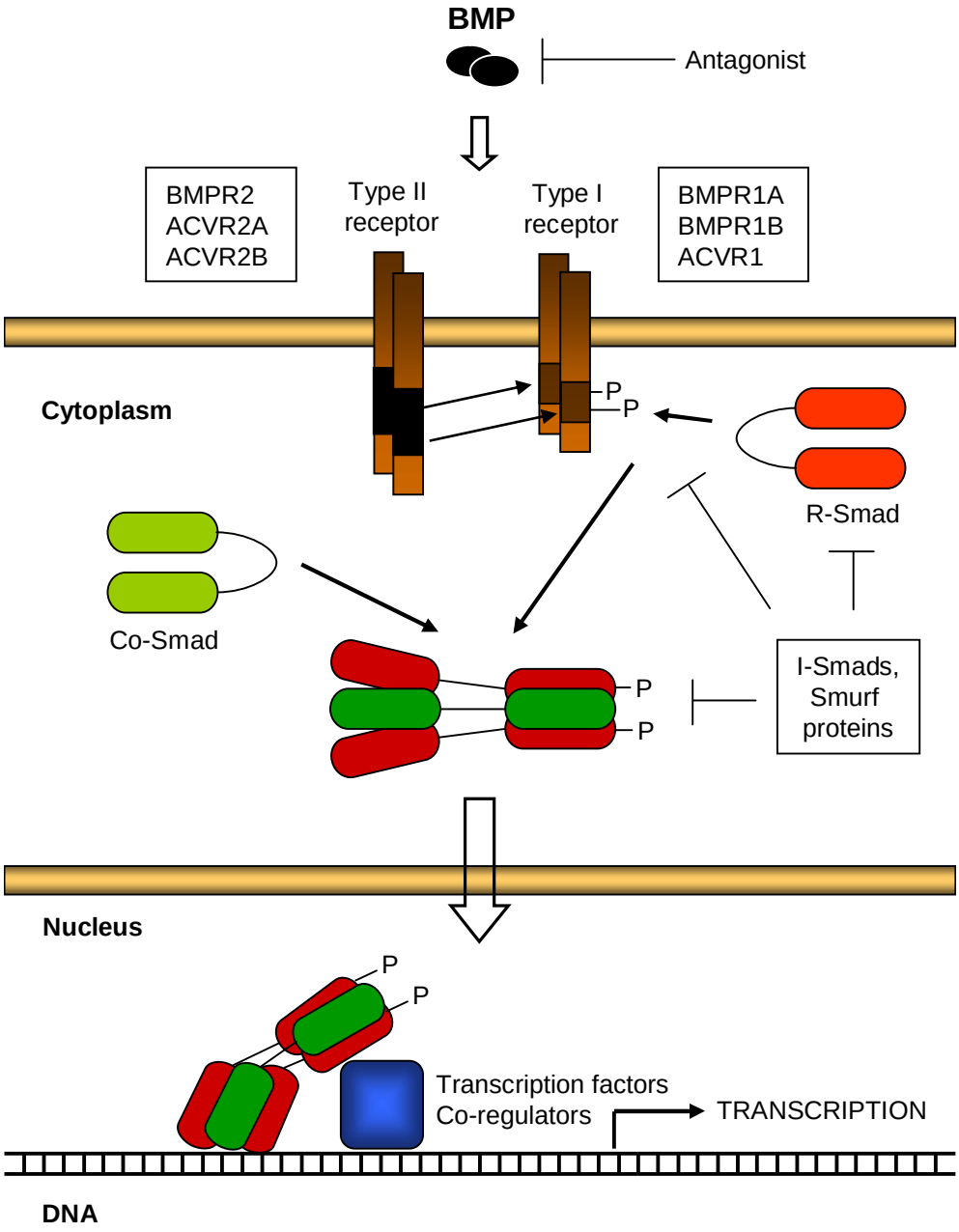


***Figure 1**. Schematic presentation of BMP signaling pathway.*

3.2.1 Receptors

There are three type I and three type II receptors that are able to bind and convey the signals of different BMP ligands (reviewed by Kawabata et al. 1998, ten Dijke et al. 2003, Nohe et al. 2004, Miyazono et al. 2005). Type II receptors include BMP receptor type II (BMPR2), activin A receptor type IIA (ACVR2A, also known as ActR-II), and activin A receptor type IIB (ACVR2B or ActR-IIB). Type I receptors consist of BMP receptor type IA (BMPR1A, also known as

ALK-3), BMP receptor type IB (BMPR1B or ALK-6), and activin A receptor type I (ACVR1 or ALK-2). Type I and II receptors are both serine/threonine kinase receptors and consist of an aminoterminal extracellular ligand binding domain, a single transmembrane domain and carboxyterminal intracellular kinase domain (reviewed by de Caestecker 2004). Type II receptors are thought to be constitutively active and their sole role is to activate type I receptors. However, BMPR2 for example has two splice variants with short and long C-terminal tail (Hassel et al. 2004, reviewed by Kawabata et al. 1998). This C-terminal tail can interact with numerous regulatory proteins and thus can have other important functions than the activation of type I receptors (Hassel et al. 2004, reviewed by Miyazono et al. 2005). Type I receptors contain a characteristic sequence of repeated glycines and serines, called GS domain separate from the kinase domain (reviewed by Shi and Massague 2003, de Caestecker 2004).

The mechanism of activation is similar for all type I and II receptors (reviewed by Feng and Derynck 2005). Upon ligand binding a stable receptor complex is formed consisting of two receptors from each type. Type II receptors then phosphorylate the GS domain of type I receptors. Activated type I receptor can now in turn phosphorylate and activate downstream Smad proteins. There is considerable variation in receptor activation caused by diverse binding affinities for different ligands, homo- or heteromeric receptor dimer formation, and mode of ligand binding either to preformed receptor complexes or complex formation after ligand binding. In general, the ligand binding affinity of type II receptor is lower than that of type I receptor (Rosenzweig et al. 1995, reviewed by ten Dijke et al. 2003). Even though all three type I receptors are able to bind different BMPs, the affinities to different members vary (reviewed by de Caestecker 2004). BMP2 is more prone to bind BMPR1A than BMPR1B, BMP4 binds both with similar affinity whereas BMP7 prefers ACVR1 and BMPR1B to BMPR1A (Macias-Silva et al. 1998, reviewed by Kawabata et al. 1998, ten Dijke et al. 2003, Sebald et al. 2004). There are also different modes of receptor heterocomplex formation (reviewed by de Caestecker 2004). BMP7 and BMP6 interact first with type II receptor and then recruit type I receptors. In contrast, BMP2 and BMP4 bind first to type I receptor and then recruit type II receptors.

Interestingly, it has been shown that BMP receptors (BMPR2, BMPR1A, and BMPR1B) can virtually form oligomeric complexes in any combination thus adding to the flexibility of BMP signaling (Gilboa et al. 2000, reviewed by Sebald et al. 2004). Since BMPs are also known to form heterodimers, further complexity of receptor combinations can be expected. Finally, there is evidence that BMP specific receptors can form preformed heterocomplexes on the cell surface and depending on whether BMP binds to a preformed complex or complex formed after ligand binding either Smad pathway or p38 MAP kinase pathway is subsequently activated (Nohe et al. 2002, reviewed by Nohe et al. 2004). Taken together, basically straightforward receptor activation has multiple points of variation that lead to diverse downstream responses.

3.2.2 Smad pathway

The major pathway for BMP induced signals utilizes intracellular Smad proteins. There are three receptor regulated R-Smads (Smad1, Smad5, and Smad8) and one common-Smad (Smad4) that are activated specifically in response to BMP binding to its related receptor (reviewed by Heldin et al. 1997, Attisano and Wrana 2000, Itoh et al. 2000, Derynck and Zhang 2003, Massague et al. 2005, Miyazono et al. 2005). R-Smads and Smad4 share a similar structure of two conserved, globular domains (MH, Mad homology domains) connected by a non-conserved variable linker domain. The two MH domains are responsible for DNA binding, interaction with type I receptors (only R-Smads), R-Smad/co-Smad complex formation and transcriptional activation. The flexible linker region has binding sites for Smurf ubiquitin ligase and phosphorylation sites for MAP kinases. It has been reported that BMP4 and GDF5 activate all three R-Smads, whereas BMP6 and BMP7 activate only Smad1 and Smad5 (Aoki et al. 2001).

Activated type I receptors are able to bind cytosolic R-Smads (reviewed by Feng and Derynck 2005, Massague et al. 2005). Following this interaction, the SXS motif in the R-Smad is phosphorylated by the receptor and subsequently R-Smad is released. Activated R-Smads form heteromeric complexes with Smad4 that can be either trimeric or dimeric, and these complexes are then translocated into the nucleus (reviewed by Derynck and Zhang 2003, Feng and Derynck 2005).

BMP induced Smads themselves bind to DNA in distinct sequences called SBE (Smad binding element) or BRE (BMP response elements that are GC-rich sequences), but the interaction has actually quite low affinity and thus several cofactors are required to obtain high affinity and target selectivity (reviewed by Ten Dijke et al. 2002, Feng and Derynck 2005, Massague et al. 2005). Known transcription factors that bind to adjacent promoters and thus co-operate with BMP Smad regulated transcription activation include the Runx family of transcription factors, Menin, Hoxc-8, zinc finger proteins OAZ and YY1, and estrogen receptor to mention some (Hata et al. 2000, reviewed by Zwijsen et al. 2003, Feng and Derynck 2005, Miyazono et al. 2005). These can either function in transcriptional activation (such as Runx family) or repression (such as YY1). Furthermore, several nuclear coactivators and corepressors amplify and specify the signal response. Known coactivators p300/CBP and GCN5 act as histone acetyl transferases, making chromatin more accessible to Smads, whereas c-Ski and Sno act in reverse fashion as deacetylases (reviewed by Feng and Derynck 2005, Miyazono et al. 2005). Other nuclear repressors known to regulate BMP transcription are Tob, SIP1, and Evi-1 (reviewed by von Bubnoff and Cho 2001, Zwijsen et al. 2003, Feng and Derynck 2005). Through these cofactors signaling specificity is achieved by relatively few Smad proteins.

3.2.3 Other pathways

Members of the TGFβ superfamily are also known to Smad-independently activate the family of mitogen activated protein kinases (MAPK) Erk, JNK, and p38, but the exact mechanisms of activation are not clear (reviewed by Derynck and Zhang 2003, Nohe et al. 2004, Javelaud and Mauviel 2005). Erk MAPK is activated through Ras, whereas JNK and p38 MAP kinases are activated through TAK1 (TGFβ-activated kinase). Work done in the *xenopus* indicated that BMP signaling might lead to interaction between BMP receptor and XIAP (X-linked inhibitor of apoptosis) which in turn interacts with TAK1 and TAB1 (Tak binding protein) resulting in MAPK activation (Shibuya et al. 1998, Yamaguchi et al. 1999, reviewed by Herpin and Cunningham 2007). Recent work shows that BMP2 and BMP4 can activate p38 and Erk MAPK but not JNK (Kimura et al. 2000, Nohe et al. 2002, Jin et al. 2006, Otani et al. 2007, Yang et al. 2007, reviewed by Nohe et al. 2004). During renal epithelial morphogenesis BMP7 has been shown to activate p38 MAPK through integrin linked kinase (Piscione et al. 2001, Hu et al. 2004, Leung-Hagesteijn et al. 2005). In addition, it has been reported that BMP2 can activate protein kinase C and PI3 kinase pathways (Hay et al. 2001, Ghosh-Choudhury et al. 2002). Finally, LIM kinase 1 (LIMK1) that regulates actin polymerization has been shown to interact directly with the C-terminal tail of BMPR2, providing a new avenue for Smad-independent BMP signaling (Foletta et al. 2003, reviewed by de Caestecker 2004).

In addition to direct activation of MAP kinases by BMPs, MAPK can also modulate Smad activation. As noted earlier, the Smad linker region contains phosphorylation sites for MAP kinases. Erk MAPK that is activated by epidermal growth factor (EGF) or hepatocyte growth factor (HGF) can phosphorylate the Smad1 linker region (Kretzschmar et al. 1997, Massague 2003, Sapkota et al. 2007, reviewed by Derynck and Zhang 2003). This leads either to cytoplasmic retention of Smad1 or its degradation and subsequent attenuation of BMP signal.

BMP-Smad pathway is known to cross-talk with many other major pathways adding to the complex regulation of this family of cytokines. Quite obviously, BMP pathway interacts with other related pathways of the TGFβ superfamily (reviewed by von Bubnoff and Cho 2001, Herpin and Cunningham 2007). This is exemplified by the shared use of type I and type II receptors by both BMPs and activins. Moreover, Smad4 is used by all the TGFβ members and can result in intracellular competition for this common Smad. Regulatory molecules (discussed in detail below) such as inhibitory Smads and BAMBI are induced by BMPs and in addition to regulating the BMP itself they can affect TGFβ/Activin signaling. BMPs have been shown to have a cooperative effect with Wnt induced signaling on several target genes during embryogenesis (reviewed by Attisano and Labbe 2004). The cofactor p300 provides a physical bridge to synergistic signaling of both BMP and STAT pathways in astrocyte differentiation, and interferon induced STAT1 regulates BMPs by interacting with I-Smad and Runx transcription factors (reviewed by von Bubnoff and Cho 2001, Nohe et al. 2004, Miyazono et al. 2005). Finally, both synergism and antagonism has been

detected between BMP and Notch pathways (reviewed by Miyazono et al. 2005, Herpin and Cunningham 2007). Thus BMP signaling would appear to form a network of signaling pathways rather than act in strictly linear fashion.

3.3 Regulation of signaling pathway

The BMP signaling pathway is heavily regulated at multiple levels. In addition to the diversity obtained by heteromeric ligands, receptors, Smads and their coregulators as well as other pathways discussed above, BMP signaling is modified by extracellular antagonists, accessory receptors on the membrane, and intracellular control of Smad activity.

Several antagonists are known to specifically bind and control different BMPs with different affinities. They include noggin, the chordin family members, twisted gastrulation, and DAN family members (such as gremlin, cerberus, dan, sclerostin) (reviewed by Reddi 2001, Balemans and Van Hul 2002, Ebara and Nakayama 2002, Canalis et al. 2003, Gazzerro and Canalis 2006). Antagonists have characteristic cystein-rich domains that form cystein-ring structures. These peptides bind the BMP ligand and prevent ligand interaction with the membrane receptors as seen with noggin, which blocks the receptor interacting epitopes of BMP7 (Groppe et al. 2002). By contrast, another known antagonist follistatin binds the functional epitopes of receptors thus inhibiting the BMP receptor interaction (reviewed by Balemans and Van Hul 2002, Canalis et al. 2003). Many of the antagonists themselves are also target genes of BMPs, thus creating a feedback loop and resulting in correct amounts of BMP outside the cell (reviewed by Miyazono 2000, Gazzerro and Canalis 2006). A recent study showed that antagonistic function can also take place intracellularly. CRIM1 is a transmembrane protein that containing chordin like cysteine-rich domains. It has been shown that CRIM1 can prevent BMP7 and BMP4 actions already in the Golgi compartment. It affected the BMP preprotein processing, reduced secretion of the mature form, and tethered BMP on the cell membrane (Wilkinson et al. 2003).

Pseudoreceptor BAMBI on the cell membrane can inhibit BMP signaling by interfering with the receptor complex (Onichtchouk et al. 1999, reviewed by Canalis et al. 2003). Membrane anchored proteins DRAGON and RGMAa (repulsive guidance molecule) on the other hand have been shown to enhance BMP signaling (Samad et al. 2005, reviewed by Gazzerro and Canalis 2006). However neither BAMBI nor DRAGON has been shown to interact with BMP7 whereas endoglin, a transmembrane glycoprotein, binds and enhances BMP7 actions (Barbara et al. 1999, Scherner et al. 2007).

Intracellularly BMP signaling is regulated by inhibitory Smads, I-Smads (reviewed by Ten Dijke et al. 2002, Canalis et al. 2003, Massague et al. 2005). There are two I-Smads, Smad6 and Smad7 that resemble the R-Smads, but lack the DNA-binding domain and also the C-terminal SXS motif that in the R-Smads is phosphorylated by the receptor. I-Smads were originally identified on the basis of their ability to compete with R-Smads in the interaction of activated type I

receptors. Smad6 also competes with Smad4 for the complex formation with Smad1 thus attenuating the BMP signal. Smad6 represses transcription by binding to Hoxc-8 repressor that normally would dissociate from DNA by Smad1. I-Smad expression is induced by BMP signaling, so they form yet another feedback mechanism in order to preserve the correct quantity of BMP signals. Crosstalk with other pathways is seen in upregulation of I-Smads through JAK-STAT and NF-κB pathways.

Smurf1 and Smurf2 (Smad ubiquination regulatory factor) are E3 ubiquitin ligases that specifically recognize target proteins, Smads, and direct them to degradation by proteasome machinery (reviewed by von Bubnoff and Cho 2001, ten Dijke et al. 2002, Massague et al. 2005). Smurf1 targets Smad1 and Smad5, whereas Smurf2 targets Smad1, both by interacting with the R-Smad linker region. Smurf mediated degradation occurs both at basal and activated state of BMP signaling thus keeping the responses at optimal level. In addition, Smurf1 has been shown to target BMP type I receptors as well as Smad1 and Smad5 for degradation through interaction with I-Smads (Murakami et al. 2003).

BMP7 specific features of signaling are summarized in Figure 2.

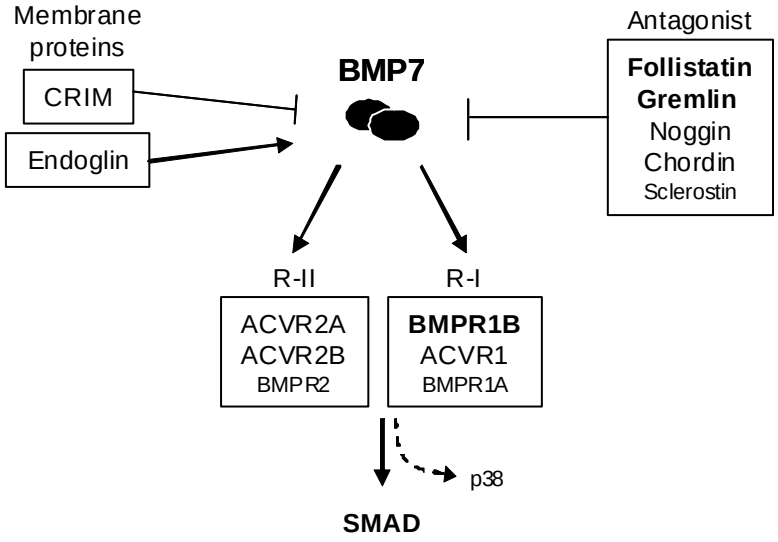


Figure 2. *Components on the signaling pathway known to interact with BMP7.*

3.4 Target genes

Some of the BMP target genes in the developmental phases and during bone induction are well known, including transcription factors Id (inhibitor of differentiation), Runx2, MFH-1, Vent2 as well as homeobox genes Msx2 and Dlx5 (reviewed by Canalis et al. 2003). Recent studies utilizing microarray platforms have significantly increased the knowledge in this field. Microarray approach has been used to determine the genes activated during differentiation of

osteoblastic cells. Analyses show that at least two hundred genes are either upregulated or downregulated in the osteoblastic differentiation process (Korchynskyi et al. 2003, Peng et al. 2003, de Jong et al. 2004). These include already known target genes such as Id and Runx2, Dlx homeobox genes as well as new candidates such as transcription factors Hey1 and Tcf7. Balint and colleagues (2003) have shown that over a period of 24 hours altogether 1800 genes were responsive in BMP2 induced osteogenic differentiation. They further showed that these responses could be divided into separate waves of expression; initial expression of nuclear proteins and developmental factors; followed by genes responsible for cell morphology and growth as well as basement membrane formation; and eventually genes involved in the synthesis and assembly of the bone phenotype.

During embryonic development certain genes are expressed in a spatiotemporally coordinated manner. These synchronous genes form a so-called synexpression group. Such a group has been identified in *xenopus* where BMP4 induced expression of BMP4, BMP7, Tsg, BAMBI, Smad6, Smad7, BMPR2, and transcription factor Vent2, all common players in BMP signaling (Karaulanov et al. 2004). BMPs can therefore induce themselves and also regulate their own signaling as discussed earlier. Target gene activation can thus occur in quite a complex and stepwise fashion. In order to identify new potential target genes, von Bubnoff and colleagues (2005) studied *xenopus* Id3 and vent2 promoter regions, compared them to human and mouse sequences, and determined conserved sequence elements responsive to BMPs. Consequently, these promoter sequences were used to identify 100 putative target genes *in silico*.

Microarray platforms have also been used to study BMP7 regulated gene expression. It was shown that BMP7 can also initiate osteogenic differentiation, with nearly 900 genes that were either down- or upregulated including Runx2 (Gu et al. 2004). In the renal proximal tubule epithelial cells BMP7 regulated the expression of several genes, both well-known (Id1-3) as well as novel chemochine and cytokine target genes (Gould et al. 2002).

Taken together, a given BMP functions in different tissues at various phases of development, a feature that probably contributes to the abundance of target genes. Some of these target genes are known, but most likely several are still waiting to be discovered.

4. BMPs and cancer

Powerful developmental pathways, such as BMP signaling, are often disrupted in cancer (Kelleher et al. 2006) and for the last ten years bone morphogenetic proteins have been increasingly studied in carcinogenesis. BMPs have been explored in various cancer types originating from different tissues such as breast, prostate, bone, skin, lung, pancreas, colon, intestine, brain, and ovaries using both *in vitro* and *in vivo* exprerimental methods (Kleeff et al. 1999, Langenfeld

et al. 2006, Piccirillo et al. 2006, Bleuming et al. 2007, Deng et al. 2007, Theriault et al. 2007, reviewed by Yoshikawa et al. 2004, Hsu et al. 2005, Kim and Kim 2006, Ye et al. 2007a). However, the number of studies is still very limited and results are somewhat contradictory. A simplified conclusion is that BMPs are involved both in the promotion and inhibition of cancer progression. This has been more clearly observed with the superfamily member TGFβ that possesses bidirectional functions in tumorigenesis (reviewed by Derynck et al. 2001, Siegel and Massague 2003, Massague and Gomis 2006). TGFβ inhibits normal epithelial cell proliferation through a well characterized cytostatic program. However in cancer this growth inhibition is often lost, even though the signaling pathway components remain intact. Moreover, TGFβ induces EMT as well as proangiogenic and immunosuppressive effects, of which all promote tumorigenesis. It is also strongly involved in the metastatic process. Whether similar dual mode of action could be true for bone morphogenetic proteins is currently unknown.

An indication of tumor suppressor like activity of BMPs is the notion that BMP signaling is disturbed in some inheritable cancer predisposition syndromes. Germline mutations of *BMPR1A* (20-25%) and *SMAD4* (15-20%) are associated with juvenile polyposis syndrome with increased risk of colorectal, gastric, small intestinal, and pancreatic cancers (reviewed by Waite and Eng 2003a). Mutations in *BMPR1A* are also associated with some cases of Cowden syndrome with elevated risk of breast, thyroid and endometrial cancers (reviewed by Harradine and Akhurst 2006). In addition, somatic mutations in *SMAD4* have been detected in half of all pancreatic cancers and in one third of metastatic colon cancers (reviewed by Massague et al. 2000) and *SMAD8* was lost by epigenetic silencing in one third of breast cancers (Cheng et al. 2004). On the other hand, tumor suppressor activity is not the only activity linked to BMPs, since they have been proposed to play a role in bone metastasis in prostate cancer (reviewed by Keller et al. 2001, Logothetis and Lin 2005, Vessella and Corey 2006).

4.1 BMP signaling in breast cancer

4.1.1 Expression profiles of BMPs and their receptors

Although the expression profiles of only few BMPs have been comprehensively evaluated in breast cancer, this data indicates diverse cancer specific patterns that vary from one ligand to another. *BMP2* transcripts have been detected at variable levels in breast cancer cell lines and in primary tumors (Arnold et al. 1999, Clement et al. 2000, Schwaninger et al. 2007). When *BMP2* expression was compared between normal mammary gland tissue and tumor specimens, significantly lower levels were detected in both non-invasive and invasive breast tumors and also in liver metastatic tumor tissues than in normal samples, indicating that *BMP2* expression is downregulated in breast cancer (Reinholz et

al. 2002). *BMP6* mRNA has been detected in a few breast cancer cell lines (Arnold et al. 1999, Clement et al. 1999, Schwaninger et al. 2007). Variable *BMP6* levels have also been detected in tumor samples, where overexpression was seen in only a minority of samples when compared to normal appearing cells in the tumor resection margin (Clement et al. 1999). However, non-cancerous tissues adjacent to the tumor cells may contain cancer specific alterations and thus do not necessarily reflect the normal expression status. BMP6 protein has been detected in breast cancer skeletal metastases (Autzen et al. 1998), but in another tumor set no protein expression was detected in primary breast cancers with established skeletal metastases (Bobinac et al. 2005). Zhang and colleagues (2007) have proposed that epigenetic mechanisms regulate *BMP6* transcript levels, since demethylation of *BMP6* promoter increased *BMP6* levels in breast cancer cells. In addition, transcripts of *BMP3*, *BMP4*, *BMP5*, and *BMP8* as well as BMP specific receptors have been reported in a few breast cancer cell lines (Arnold et al. 1999, Clement et al. 2000, Schwaninger et al. 2007). GDF9a and BMP15 protein levels were downregulated in breast tumors compared to normal samples (Hanavadi et al. 2007). BMP7 protein expression has been detected at variable levels in primary breast tumors (Schwalbe et al. 2003, Buijs et al. 2007a) and its role will be discussed in more detail in the following chapters.

Only few studies have explored the possible consequences of BMP signaling on patient outcome. BMPR1B receptor expression was shown to be associated with poor prognosis for breast cancer patients (Helms et al. 2005). In the same study, BMPR1B expression in ER positive breast cancer specimens correlated with high tumor grade, high tumor proliferation index, and cytogenetic instability, thus linking active BMP signaling to tumor progression. Further analysis showed that BMPR1B expression was accompanied by Smad1/5/8 phosphorylation as well as elevated expression of antiapoptotic proteins XIAP and IAP-2 (Helms et al. 2005). Two studies have examined the clinical significance of individual ligands. Hanavadi and colleagues (2007) showed that decreased expression of GDF9a and BMP15 was associated with poor prognosis. In another study BMP2/4 expression did not correlate with survival or clinicopathological parameters (Raida et al. 2005a). However, the antibody used in this study recognized both ligands and therefore the individual ligand expression profiles might have resulted in different predictions.

4.1.2 Possible function of BMPs

Functional roles of BMPs in breast cancer have mainly been explored in the case of BMP2. Supporting the fact that BMP2 expression is downregulated in breast cancer, it has been demonstrated that BMP2 inhibits breast cancer cell proliferation (Ghosh-Choudhury et al. 2000a, Ghosh-Choudhury et al. 2000b, Pouliot and Labrie 2002). BMP2 increased the level of cyclin kinase inhibitor p21 and thus induced p21 association with cyclin D1 and E and subsequent inhibition of CDK (cyclin dependent kinase) activity. BMP2 induced hypophosphorylation of the retinoblastoma protein (pRB), a key regulator of the

cell cycle and resulted in G1 arrest of breast cancer cells. This growth arrest required both cytoplasmic signal transducers Smad1 and Smad4. Furthermore, BMP2 increased the tumor suppressor PTEN levels in breast cancer cells by inhibiting PTEN degradation (Waite and Eng 2003b). However, the function of BMP2 is not so straightforward. BMP2 was able to protect breast cancer cells from hypoxia induced apoptosis (Raida et al. 2005a) and it also induced migration and invasion of breast cancer cell lines (Clement et al. 2005). Moreover, BMP2 was shown to promote tumor angiogenesis by increasing the endothelial cell tube formation *in vitro* and *in vivo* (Raida et al. 2005b). In *in vivo* xenograft mouse model, BMP2 overexpressing MCF7 breast cancer cells formed tumors with pronounced vascularization, but no tumor formation was detected with BMP2 negative MCF7 cells (Clement et al. 2005, Raida et al. 2005b).

Other BMP family ligands and their functions have hardly been studied at all in breast cancer. One study shows that forced expression of GDF9a and BMP15 that were also downregulated in cancer specimens led to growth reduction in one breast cancer cell line (Hanavadi et al. 2007). By contrast, inhibition of BMP signaling using a dominant negative form of BMPR2 receptor led to growth reduction of breast cancer cells, implying that active BMP signaling could induce proliferation (Pouliot et al. 2003). A dual function was observed in MDA-MB-231 breast cancer cell line in response to BMP6 when it reduced proliferation as well as protected cells from apoptosis (Du et al. 2007). BMP4 in turn disturbed the lumen formation of mammary epithelial cells resulting in the promotion of invasive behavior of these cells also thus suggesting BMP4 involvement in breast cancer progression (Montesano 2007).

Based on the variable data on the expression and functions of various BMPs, other contributing factors have been considered in their regulation, such as estrogen, vitamin D, and EGF (epidermal growth factor). One major risk factor for breast cancer is increased lifelong exposure to estrogen and dysregulation of ER (estrogen receptor) is linked to cancer cell proliferation in mammary tumorigenesis (Anderson 2002). BMP6 expression was induced by EGF and estrogen in some breast cancer cell lines, but estrogen has also been shown to suppress BMP2 activity (Clement et al. 1999, Yamamoto et al. 2002, Zhang et al. 2005). A more complex connection between estrogen and BMPs was suggested by Zhang et al. (2007) when they demonstrated that *BMP6* promoter hypermethylation was detected in ER negative breast cancer cell line and primary tumors, but not in ER positive cell lines and tumors. Vitamin D is known to inhibit the growth of breast cancer cells (Welsh 2007). BMP2 and BMP6 were found to be upregulated and inhibitory Smad6 downregulated upon vitamin D analogue induced growth reduction (Lee et al. 2006a, Lee et al. 2006b). Thus vitamin D could mediate growth inhibitory effects through active BMP signaling in breast cancer.

Alternative pathway activation and involvement of tumor stromal cells could also have an impact in the variable functions of BMPs. MAP kinase activation was involved in BMP4 induced disruption of mammary epithelial lumen and BMP2 induced endothelial cell activation and possibly in consequent tumor angiogenesis (Raida et al. 2005b, Montesano 2007). BMP6 protected breast

cancer cells from apoptosis by activating both Smad and p38 MAPK pathways (Du et al. 2007). Tumor tissue microenvironment is an important factor in carcinogenesis. Sneddon et al. (2006) detected elevated levels of GREMLIN 1 in breast tumor stroma and proposed a model where BMP antagonist produced by tumor stroma maintains the tumor cell expansion analogous to the stem cell expansion in the normal tissues. In another study of mammary carcinoma BMP2 induced expression of Tenascin-W, an extracellular matrix regulator found in the tumor stroma (Scherberich et al. 2005).

As a whole, despite the fact that most of studies on BMPs in cancer have concentrated on breast carcinoma, a systematic view of BMP actions in breast cancer is still emerging. These studies illustrate that the pleiotropy BMPs exert in normal tissues is also possible in cancer.

4.1.3 BMPs and bone metastasis

Breast and prostate cancers are known to metastasize particularly to the bone and thus the possible role of BMPs in this process has been a natural focus for research. Recent studies on prostate cancer suggest that BMP signaling has an active role in the bone metastasis process. Overexpression of BMP antagonist noggin significantly decreased the formation of both osteoblastic and osteolytic bone metastases *in vivo* (Feeley et al. 2005, Feeley et al. 2006). Schwaninger et al. (2007) showed that noggin was not expressed in osteoinductive (osteoblastic bone metastases forming) prostate and breast cancer cell lines, but was detected in osteolytic cell lines. An *in vivo* model using an osteoinductive prostate cancer cell line showed that forced expression of noggin did not affect the growth of the tumors but rather diminished the osteoblastic response in bone (Schwaninger et al. 2007). This could equally apply to osteoinductive breast cancer cell lines.

Some implications of BMPs contribution to bone metastases have also been reported in breast cancer, but this field has not been adequately studied. Bone morphogenetic proteins secreted from breast cancer cell lines resulted in upregulation of bone sialoprotein (BSP) expression in preosteoblast cells (Bunyaratavej et al. 2000). BSP is involved in new bone formation and could thus provide a possible link between the bone metastatic process and breast cancer. Another link might be Runx2 that is a known target gene and a cofactor for BMP signaling. Recently, it was demonstrated that intact Runx2 is required for the formation of breast cancer osteolytic metastases in bone (Barnes et al. 2004, Javed et al. 2005).

4.2 BMP7 in cancer

4.2.1 Expression profile of BMP7

BMP7 expression has been studied in various types of cancer. The first indication of cancer specific expression came from tumor samples of breast, prostate, osteosarcoma, and chondrosarcoma where *BMP7* mRNA was expressed at elevated levels compared to normal tissues (Weber et al. 1998). *BMP7* mRNA expression has also been detected in a subset of ovarian cancer cells (Shepherd and Nachtigal 2003, Moll et al. 2006). Microarray based studies indicated that *BMP7* was upregulated in ovarian cancers (Hibbs et al. 2004, Sunde et al. 2006). Hibbs et al. (2004) detected BMP7 protein in only a minority of tumor samples and usually in stromal cells, but not at all in normal ovaries (Table 1). In osteosarcoma, BMP7 expression was observed in cell lines (Gobbi et al. 2002) and primary tumors but was not associated with overall survival (Sulzbacher et al. 2002). BMP7 expression and clinical correlation have also been studied in renal cell carcinoma. BMP7 was expressed in one third of the primary tumors studied, more often in female than male patients and the expression pattern also correlated with the histological subtype of renal cell carcinoma (Kwak et al. 2007). BMP7 expression associated with longer disease free time, but it was not an independent prognostic factor. Interestingly, upregulation of BMP7 protein expression has been detected in primary and metastatic melanomas compared to normal nevi (Rothhammer et al. 2005). In contrast to renal carcinomas, strong BMP7 expression was associated with shorter tumor recurrence time in melanoma (Rothhammer et al. 2007). In addition to melanoma, *BMP7* has also been detected more often and at elevated levels in the skeletal metastases of prostate cancer than in primary cancers (Masuda et al. 2003). In primary prostate cancers and local recurrences *BMP7* was found to be expressed less frequently and at lower levels than in normal prostate tissue (Masuda et al. 2004, Buijs et al. 2007b). A novel mechanism for BMP7 regulation was detected in cancer originating from brain (glioblastoma and astrocytoma), where it was found to be silenced by hypermethylation in cancer cell lines compared to non-methylated status in normal brain and primary tumors (Ordway et al. 2006).

Two studies have evaluated BMP7 expression in breast cancer. Schwalbe and colleagues (2003) observed BMP7 protein expression in three cancer cell lines and variable expression in altogether 170 primary breast tumors. In the normal mammary, BMP7 was detected in mammary gland end buds. In this patient series, BMP7 protein expression was associated with ER and PR expression status in the primary tumors. Later on, Buijs and colleagues (2007a) also reported variable *BMP7* expression in 67 primary breast cancers. Taken together, BMP7 expression has been detected in various cancer types and the expression pattern is cancer type specific. Moreover, the few studies that have explored the clinical significance of BMP7 expression in cancer came to antagonistic conclusions depending on the cancer type in question, thus linking BMP7 either to tumor promotion or suppression.

Table 1. *BMP7 expression in different tumor types.*

Reference	Tumor type	Primaries (n)	Metastases (n)	Comment
Rothhammer et al. 2005	Melanoma	Up (5)	Up (4)	Compared to normal nevus
Rothhammer et al. 2006		Up (72)	Up (96)	Compared to normal nevus
Masuda et al. 2003 [a]	Prostate		Up (7)	Bone metastases compared to primary prostate cancer and normal bone
Masuda et al. 2004 [a]		Down (28)	Down (23) [b]	Compared to normal prostate tissue
Buijs et al. 2007 [a]		Down (14)		Comparison between patient matched laser captured non-cancerous and cancer cells
Hibbs et al. 2004	Ovarian	Variable expression (30)		No expression in normal samples
Kwak et al. 2007	Renal cell carcinoma	34% positive (185)		No normal samples
Sulzbacher et al. 2001	Osteosarcoma	91% positive (47)		No normal samples
Schwalbe et al. 2003	Breast	100% positive (170)		No comparison with normal samples
Buijs et al. 2007 [a]		100% positive (67)		No normal samples

Up denotes upregulated and down downregulated expression compared to corresponding normal tissues. BMP7 expression determined using immunohistochemistry unless otherwise stated.

[a] mRNA levels

[b] local recurrent tumor

4.2.2 Possible function of BMP7

BMP7 function has been studied in some cancer cell line models. These studies illustrate a complicated role for BMP7 actions in cancer. Work done by Miyazaki et al. (2004) indicated that increasing exogenous doses of BMP7 inhibit the growth of prostate cancer cells. A similar but variable growth inhibitory effect was detected in four out of six anaplastic thyroid carcinoma cell lines (Franzen and Heldin 2001). In both cases, BMP7 induced G1 cell cycle arrest caused by hypophosphorylation of pRB, and upregulation of p21 and p27 CDK inhibitors. Another study showed that increasing doses of BMP7 variably also inhibited growth of myeloma cell lines. In a subset of these cell lines the growth inhibition was caused by increased apoptosis rather than cell cycle arrest as indicated above (Baade Ro et al. 2004). Moderate growth suppression has also been detected in a colon carcinoma cell line in response to BMP7 (Beck et al. 2006). Inhibition of BMP7 expression in one prostate cancer cell line in turn resulted in more invasive and motile phenotype (Ye et al. 2007b).

Yang and colleagues (2005) identified upregulated Bmp7 expression throughout the development of prostate adenocarcinomas in *pten* null mice. To study the functional significance of BMP7, they treated prostate cancer cell lines with BMP7 that led to different and highly cell type specific phenotypes (Yang et al. 2005). All cell lines showed activation of R-Smads despite diverse functional changes. BMP7 inhibited growth of non-neoplastic prostate cell line BPH1, but not the growth of cancer cell lines PC-3 and DU145. PC-3 cells responded to BMP7 with increased invasive potential as well as morphological change towards spindle-like shape, increased expression of myofibroblast marker SMA, and decreased expression of cell adhesion marker E-cadherin, indicating EMT phenotype. None of the other cancer cell lines responded in a similar manner. In LNCaP cancer cells and to a greater extent in C4-2B cancer cells BMP7 treatment protected cells from apoptosis and this was accompanied by sustained survivin levels in C4-2B cells but not in LNCaP cells (Yang et al. 2005). A subsequent study from the same group (Yang et al. 2006) demonstrated that in addition to Smad pathway, BMP7 protected C4-2B cells from apoptosis by also activating the JNK pathway. The results were further validated in *pten* null mice, where BMP7 upregulation was accompanied by upregulation of survivin, activated R-Smad, and activated JNK (Yang et al. 2006). Protumorigenic effects have also been observed in other studies. BMP7 was able to act as a chemoattractant and thus increased migration and invasion of some but not all prostate cancer cell lines (Feeley et al. 2005, Feeley et al. 2006). In colon carcinoma BMP7 induced proinvasive effects Smad independently by activation of other pathways, such as ERK and JNK MAP kinases (Grijelmo et al. 2007). Together these studies suggest a complicated functional role for BMP7 that seems to be highly context dependent.

BMP7 has been implicated in the epithelial-mesenchymal transition (EMT) process and bone metastasis. In contrast to the induction of EMT in prostate

cancer cells discussed above, BMP7 reversed the TGFβ induced EMT in esophageal adenocarcinoma cell line (Rees et al. 2006). In normal mammary epithelial cells BMP7 was not able to induce EMT and it was shown to counteract TGFβ induced EMT in mouse mammary epithelial cells by re-expression of E-cadherin (Zeisberg et al. 2003, Valcourt et al. 2005). Kowanetz and colleagues (2004) showed that counteraction was obtained through BMP7 induced upregulation of Id2 and Id3 since they alone could neutralize TGFβ inducible EMT. Id proteins are well known target genes for the BMP family that can stimulate proliferation and tumor neoangiogenesis (reviewed by Perk et al. 2005). Interestingly, with Id2-3 knockout, BMP7 actually stimulated EMT and clearly induced the SMA levels in mouse mammary epithelial cells (Kowanetz et al. 2004, Valcourt et al. 2005). Moreover, Id2-3 also neutralized the weak antiproliferative effect of BMP7 in epithelial cells (Kowanetz et al. 2004). Kowanetz et al. (2004) also reported that BMP7 stimulated both anti-proliferative (upregulation of p21 and downregulation of c-myc) and pro-proliferative (upregulation of Id2-3) effects, and together this could explain the variable effects in the proliferation response observed in different epithelial cells including carcinomas.

In prostate cancer, BMP7 has been linked to the promotion of osteosclerotic bone metastasis. BMP7 was able to promote VEGF expression in prostate cancer cell line that in turn could promote osteoblastic activity in osteoblast (Dai et al. 2004). Prostate cancer derived BMP7 also induced mineralization of osteoblast precursor cells (Dai et al. 2005). On the other hand, Buijs and colleagues (2007a, 2007b) recently showed that BMP7 treatment reduced bone metastasis formation and growth in mouse xenograft models of breast and prostate cancers. In primary breast tumors *BMP7* mRNA levels were lower with patients developing a bone metastasis than liver or lung metastasis (Buijs et al. 2007a). However, there were no significant differences in *BMP7* expression level between patients who did or did not develop a bone metastasis.

In breast cancer the data available on BMP7 function is truly limited. Variable BMP7 expression has been detected in primary tumors (Schwalbe et al. 2003, Buijs et al. 2007a) as previously discussed. Three studies have explored other contributing factors in BMP7 activity. Estrogen has previously been shown to suppress BMP7 expression in the chicken oviduct (Monroe et al. 2000) and in one breast cancer cell line BMP7 levels also decreased in response to estrogen (Kusumegi et al. 2004). Recently, it was shown in a breast cancer cell line that BMP7 is a novel target gene for the p53 family of proteins (Yan and Chen 2007). Moreover, they showed that reduction of BMP7 level in p53 mutant breast cancer cell line resulted in decreased proliferation, but not in the p53 wild-type breast cancer cell line (Yan and Chen 2007). BMP7 was identified as a target gene for LMO4 (Lim only protein 4) that is often overexpressed in breast cancer (especially in ER negative tumors) and associated with poor outcome (Wang et al. 2007). LMO4 upregulation led to the sequestering of histone deacetylase HDAC2 from the BMP7 promoter and subsequent BMP7 expression. Since at least part of the LMO4 inducible phenotypes were reversible with follistatin in mammary epithelial cells, it is likely that BMP7 mediates some LMO4 effects.

In light of these few examples, BMP7 is also influenced by other factors present in breast cancer.

In conclusion, BMP7 as well as other members of this family has diverse cancer specific expression patterns that furthermore differ between cancer types. BMPs are likely to play dual roles in cancer progression similarly to TGFβ. BMP inducible phenotypes in cancer seem to be dose-dependent and very clearly context dependent as can be expected given the heavily regulated signaling pathway. More detailed analysis on BMP signaling and its regulation as a whole is obviously needed to clarify the impact of BMPs in breast and other cancers.

Aims of the study

For the last decade an increasing number of studies have shown that bone morphogenetic proteins are involved in cancer progression, but the data is still rather limited. The significance of BMPs, especially BMP7, in breast cancer is unclear and the main aim of this study was to characterize the role of BMP7 in breast cancer with the following specific aims.

1) Analyze *BMP7* copy number changes and BMP7 expression in breast cancer cell lines and in primary breast tumors.

2) Determine the expression profiles of six other BMP family members and six BMP specific receptors in breast cancer.

3) Explore the clinical relevance of BMP7 expression.

4) Study the BMP7 inducible phenotypes in breast cancer cell lines.

Materials and methods

1. Breast cancer cell lines and normal mammary epithelial cells (I-IV)

A total of 23 breast cancer cell lines were included in this study. BT-474, CAMA-1, DU4475, HCC38, HCC1419, HCC1954, MCF7, MDA-MB-134, MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-436, MDA-MB-453, SK-BR-3, T-47D, UACC732, UACC-812, UACC-893, UACC3133, ZR-75-1, and ZR-75-30 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and MPE600 cells were originally provided by Dr. Helene Smith. Normal human mammary epithelial cells (HMEC) were obtained from Clonetics (Walkersville, MD). All cells were cultured under recommended conditions.

2. Clinical tumor and normal tissue samples (I-III)

Two sets of patients were included in this study. In Study I, primary tumor samples from 146 breast cancer patients diagnosed between the years 2000 and 2003 were obtained from the Department of Pathology, Tampere University Hospital. Study III consisted of primary tumors from 483 breast cancer patients diagnosed between the years 1990 and 1999 in the area served by Tampere University Hospital and 40 histologically verified recurrence samples from these patients. All tumor samples were formalin-fixed and embedded in paraffin. The use of these samples was approved by the Ethics Committee of the Pirkanmaa Hospital District and the National Board of Medicolegal Affairs.

Study I contained 104 samples of invasive ductal carcinomas, 31 invasive lobular carcinomas, six mucinous carcinomas, two ductal carcinomas *in situ* and three other carcinoma subtypes. In Study III, patients were part of an earlier study in which patient identification and data collection had been described (Korhonen et al. 2004). This cohort included 242 invasive ductal carcinomas and 241 invasive lobular carcinomas. The clinicopathological parameters of tumors used in this study are presented in Table 2.

In Study III complete patient information also included data on hormone replacement therapy (HRT), primary treatments, recurrences and survival. Altogether, 22% of the patients had received HRT for at least 2 years at the time

Table 2. *Clinicopathological characteristics of primary tumor samples used in Studies I and III.*

Variable	Study I (n=146)	Study III (n=483)
pT stage		
pT1	70	307
pT2	35	136
pT3 - pT4	38	37
pTis	2	
n.a.	1	3
Lymph node metastasis		
Negative	75	279
Positive	60	166
n.a.	11	38
Histological/ nuclear grade		
I	13	123
II	42	161
III	49	68
n.a.	42	131
ER		
Positive	124	395
Negative	22	75
n.a.		13
PR		
Positive	95	319
Negative	51	151
n.a.		13
ERBB2		
Positive	24	58
Negative	121	399
n.a.	1	26

of diagnosis, 19% had a record of prior HRT use whereas 59% had never received HRT. In 79 cases the HRT information was not available. All patients had their primary tumor surgically removed with axilla clearance. Post-operative radiotherapy with adjuvant medication (endocrine treatment and/or chemotherapy) was given for 33% of the patients, 30% received only radiotherapy, 7% only adjuvant medication, 30% of patients were not given any adjuvant treatment and in 7 cases primary treatment data was not available. There were 50 patients who developed a local recurrence and 112 developed a distant metastasis. The survival data was obtained from the Finnish Cancer Registry, the Finnish Population Register Centre, and the patient records. The mean follow-up period was 7.3 years (maximum 15.2 years).

In addition to paraffin-embedded samples, 44 freshly frozen tumor specimens (a subset of the 146 patients in Study I) were obtained from the Department of

Pathology, Tampere University Hospital and used in Studies I and II. Normal mammary gland sections and normal kidney sections were also obtained from the Department of Pathology. Human Mammary Gland (HMG) cDNA was from BD Biosciences Clontech (Palo Alto, CA).

3. Tumor tissue microarray (I, III)

Tissue microarray (TMA) was used in fluorescence *in situ* hybridization and immunohistochemistry analyses. For the purposes of this study two different TMAs, one including the first set of patients (I) and one with second set of patients (III), were constructed as described (Kononen et al. 1998). A representative tumor area was selected from hematoxylin-eosin stained histological sections. A single core biopsy (with a diameter of 0.6 or 1 mm) was taken from an analogous area of the paraffin-embedded tumor block. A single biopsy from each tumor was then transferred to a recipient paraffin block to a predefined array position. Five μm thick sections were cut from the completed TMA block for subsequent analyses.

4. Fluorescence *in situ* hybridization (I)

BMP7 copy number was determined from breast cancer cell lines and primary tumor samples on TMA using fluorescence *in situ* hybridization (FISH). Altogether three contiguous BAC/PAC clones specific for *BMP7* gene region (RP11-560A15, RP4-813D12, and RP3-481F12) were identified using the Map Viewer (www.ncbi.nlm.nih.gov/mapview). Chromosome 20-specific alpha satellite centromere reference probe was a kindly provided by Dr. Mariano Rocchi. *BMP7* specific BAC/PAC DNAs were labeled with SpectrumOrange-dUTP (Vysis, Downers Grove, IL) and reference probe with SpectrumGreen-dUTP by random priming.

To verify that the *BMP7* probes recognized a single copy target on chromosome 20, control hybridizations were done to normal metaphase chromosomes. FISH to breast cancer cell lines was done with one *BMP7* BAC probe (RP11-560A15) as previously described (Kallioniemi et al. 1992). On the TMA, FISH was done with three contiguous *BMP7* BAC/PAC probes using the Paraffin Pretreatment -kit (Vysis) with slight modifications as described (Andersen et al. 2001). Hybridization signals were evaluated using an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a 60X oil-immersion objective.

For the breast cancer cell lines mean absolute copy numbers for both *BMP7* and reference probe were evaluated from 50 intact nuclei and relative copy numbers (*BMP7* vs. 20 centromere) calculated. On the TMA, the entire tumor area was assessed. A specimen was considered to have an increased copy

number if the relative copy number was greater than 1.5 in at least 25% of the tumor cells.

5. mRNA expression analyses (I, II, IV)

5.1 RNA extraction and reverse transcription

Total RNA was extracted from the breast cancer cell lines and from HMECs using TRIZOL Reagent (Invitrogen, Carlsbad, CA) or RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. In the case of tumor samples, a representative tumor area was first selected based on hematoxylin-eosin stained tumor tissue section and a core-biopsy (diameter 2 mm) from a corresponding freshly frozen tumor specimen was then obtained for RNA extraction. Tumor specimens were homogenized with a syringe and a needle (20G, Ø 0.9 mm) and total RNA was extracted with RNeasy Mini Kit. Total RNAs from cell lines and tissue samples were quantitated using a spectrophotometer.

Total RNA from cell lines and primary breast tumors was reverse transcribed using SuperScript™ First-Strand Synthesis System (Invitrogen). Briefly, 0.2-3 μg of cell line RNA or 0.16-1.6 μg of tumor sample RNA was first denatured at 65°C for 5 min together with 50 ng of random hexamers and 1 mM dNTP mix adjusted to 10 μl with DEPC water. The cDNA synthesis reaction contained denatured RNA/primer mix and 20mM Tris-HCl (pH 8.4), 50 nM KCl, 5 mM $MgCl_2$, 10 mM DTT, 40 units of RNaseOUT and 200 units of Superscript III RT in a total volume of 20 μl. Random hexamers were first annealed at 25°C for 10 min, followed by cDNA synthesis step at 50°C for 50 min and the reaction was terminated at 85°C for 5 min. A 1:3 or 1:10 dilution was prepared from the cell line or tumor cDNA and used for all subsequent PCR reactions.

5.2 Semiquantitative RT-PCR (I, II)

Semiquantitative RT-PCR was used to determine the mRNA levels of seven BMP ligands (*BMP2-BMP8*) and six receptors (*ACVR1, BMPR1A, BMPR1B, BMPR2, ACVR2A, ACVR2B*). *TBP* (TATA box binding protein, a housekeeping gene) mRNA expression levels were used as a reference. Gene specific primer sequences are presented in Table 3. The PCR reaction contained 1 x PCR Gold buffer (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs each, 0.2-0.4 mM gene specific primers, 1.5-2.5 units of Amplitaq Gold DNA polymerase (Applied Biosystems), and 1 μl cDNA template adjusted to 50 μl with sterile H_2O. The PCR program began with initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94-95°C for 15-30 s, annealing at 50-60°C for 30-60 s and elongation at 68-72°C for 60-120 s, with final

elongation at 68-72°C for 10 min. PCR products were run on a 1.5% agarose gel and quantitated using Typhoon Trio gel imaging system and ImageQuant TL software according to manufacturer's instructions (Amersham Biosciences, Uppsala, Sweden). Relative expression levels were obtained by dividing ligand or receptor specific expression levels by the *TBP* transcript level and multiplied by a hundred.

Table 3. *Primers used in semiquantitative RT-PCR.*

	Forward 5'-3'	Reverse 5'-3'
BMP2	GTCCTGAGCGAGTTCGAGTT	GCATCTTGCATCTGTTCTCG
BMP3	TGCAGATATTGGCTGGAGTG	GGTACACAGCAAGGCTCAGG
BMP4	AGGAAGAGCAGATCCACAGC	TCGTGTCCAGTAGTCGTGTGA
BMP5	ATACAAGGACCGGAGCAACA	GCTCTCACGGATCGAAGAAG
BMP6	CCGCATCTACAAGGACTGTG	AGATTGCTAGTGGCCGTGAT
BMP7	CCAACGTGGCAGAGAACAG	GGTGGCGTTCATGTAGGAGT
BMP8[a]	GCTCTTCATGCTGGACCTGT	ACCACCTGGAACATGCTGAC
ACVR1[b]	ATGTCTTTTAGCCTGCCTGCTG	ATCAAGCTGATTGGTGCTCTGG
BMPR1A[b]	TGATTTGGAACAGGATGAAGC	TGTAGCACATTTCAGGAAGTC
BMPR1B[b]	GCAGCACAGACGGATATTGT	TTTCATGCCTCATCAACACT
ACVR2A[b]	GCAAAATGAATACGAAGTCTA	GCACCCTCTAATACCTCTGGA
ACVR2B[b]	ACACGGGAGTGCATCTACTACAACG	TTCATGAGCTGGGCCTTCCAGACAC
BMPR2[b, c]	CTGCACAGTGTGCTGAGGAAAG	TGAACTGCCCTGTTACTGCCA
TBP	CATGACTCCCATGACCC	TGGTTCGTGGCTCTCTTA

[a] Identifies both ligands BMP8A and BMP8B highly similar to each other.

[b] Primer sequence from the work of Miyazaki et al. (2004).

[c] Detects two splice variants, the short (BMPR2-SF) and long form (BMPR2-LF).

5.3 Real-time quantitative RT-PCR (I, IV)

BMP7 and *TBP* expression levels were analyzed by real-time quantitative RT-PCR using the LightCycler equipment (Roche, Mannheim, Germany) from primary breast tumors and cell lines. *TBP* was used as a reference. Quantitation was either by hybridization probe (*BMP7* and *TBP*) or by SYBR Green (*BMP7*) method. Two sets of hybridization probes and primers, Molecular Beacon Probe set from Gorilla Genomics (Alameda, CA) and probes and primers from TIB MOLBIOL (Berlin, Germany) were used. The primer and probe sequences are presented in Table 4.

The PCR reaction for the Gorilla Genomics probes contained 1 x PCR buffer, 1 x probe mix, 1 x TITANIUM Taq DNA polymerase (Clontech Laboratories, Inc., Palo Alto, CA), and 1 µl cDNA or gene specific DNA standard, adjusted to 20 µl with sterile H_2O. PCR program included initial denaturation at 95°C for 2

min, 45 cycles of amplification (95°C for 10 s, 55°C for 10 s, 72°C for 6 s), and final elongation at 40°C for 30 s.

Alternatively, *TBP* or *BMP7* transcript levels were determined using gene specific primers and hybridization probes from TIB MOLBIOL and LightCycler FastStart DNA Master Hybridization probe kit (Roche, Mannheim Germany). PCR reaction contained 2 µl LightCycler FastStart DNA Master Hybridization probe reaction mix, 4 mM $MgCl_2$, 0.8 µM of each primer, 0.2 µM FL probe, 0.2-0.4 µM LC Red640 probe and 1.5 µl cDNA adjusted to 20 µl with sterile H_2O. The PCR program included denaturation at 95°C for 10 min, 45 cycles of amplification (95°C for 10 s, 55°C for 10 s, 72°C for 11-12 s), and elongation at 40°C for 30 s.

The SYBR green method was used to trace *BMP7* levels after siRNA treatment. Transcript levels were determined using DyNAmo™ Capillary SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). The PCR reaction contained 1x Dynamo master mix and 0.5 µM of each primer and 1 µl cDNA adjusted to 20 µl with sterile H_2O. The PCR program included denaturation at 95°C for 10 min, 45 cycles of amplification (95°C for 10 s, 55°C for 15 s, 72°C for 10 s), followed by 40°C for 30 s. Quantitative analysis of hybridization probes and SYBR Green method was performed using the LightCycler software according to the fit-point method as described (Kauraniemi et al. 2003). *BMP7* expression levels were normalized against *TBP* levels.

Table 4. *Primer and probe sequences used in real-time quantitative RT-PCR.*

Primers	**Forward (5'-3')**	**Reverse (5'-3')**
Gorilla		
BMP7	GCAGCATCCAATGAACAAGA	GAGACTTCCCAGCCAATGAC
TBP	TGCGGTAATCATGAGGATAAGA	CCAACTTCTGTACAACTCTAGCATA
TIB MOLBIOL		
BMP7	GCTTCGACAATGAGACGTTC	TGGACCTCCGTGGCCTT
TBP	GGAGAGTTCTGGGATTGTAC	TGCCAGTCTGGACTGTT
SYBR Green		
BMP7	GCACTTGGGCAGGGAAT	CTTGAAGAAAGCCACCATGAA
Probes [a]	**FL probe (5'-3')**	**LC Red640 probe (5'-3')**
BMP7	GGCACAACCTGGGCCTGC	GCTCTCGGTGGAGACGCTGGA
TBP	GTGCAATGGTCTTTAGGTCAAGT	TACAACCAAGATTCACTGTGGATACA

[a] Only the sequences for the TIB MOLBIOL were reported by the manufacturer.

6. Protein expression analysis by immunohistochemistry (I, III)

Immunohistochemistry was performed with enzyme-mediated detection using immunoperoxidase and diaminobenzidine as a chromogen for the breast cancer

cell lines and primary tumors as well as for normal samples from mammary gland and kidney. A cell line array was constructed from 11 cell lines (BT-474, HCC1419, MCF7, MDA-MB-361, MDA-MB-453, SK-BR-3, T-47D, UACC-812, UACC-893, ZR-75-1, and ZR-75-30). Cells were fixed with 10% formalin, embedded in paraffin, and five μm sections cut for immuhistochemical analysis. A normal human kidney section was used as a positive (medullary rays) and a negative (glomeruli) control to verify BMP7 antibody specificity (Wang et al. 2001). The slides were de-paraffinized and rehydrated, followed by antigen retrieval in a microwave oven (850W, 10mM Tris HCl, 1mM EDTA, pH 9.0, 2 x 7 min). BMP7 goat polyclonal antibody (sc-9305, Santa Cruz Biotechnology, Heidelberg, Germany) was used as primary antibody (1:150 or 1:180 dilution, 25 min RT). Polyclonal Rabbit Anti-Goat Immunoglobulin (1:200 dilution, DakoCytomation, Glostrup, Denmark) and ChemMate DAKO EnVision/HRP, Rabbit/Mouse (DakoCytomation) with diaminobenzidine were used for visualization. Staining was done either manually or with automated DakoCytomation Techmate 500 plus. The slides were counterstained with hematoxylin-eosin. The BMP7 staining intensity was evaluated from the entire cell line or tumor core biopsy area. For the cell lines, the staining was classified as either positive or negative. For the tumor samples, nonhomogenous and very weak staining or no staining at all was classified as negative. Homogeneous staining of the epithelial cell area across the entire tissue specimen was classified as positive. In Study I positive samples were further divided into either strong or moderate staining groups.

7. siRNA treatment (IV)

To study BMP7 function in breast cancer RNA interference technique was used for *BMP7* silencing in three breast cancer cell lines (BT-474, MCF7, and SK-BR-3). For all three cell lines 7.5 x 10^4 (24-well) or 37.5 x 10^4 (6-well) cells were first incubated in DMEM medium without antibiotics for 24h. siRNA oligonucleotides (100 or 125 nM) were transfected using Oligofectamine™ Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Two siRNA duplexes were obtained from Proligo (Paris, France): BMP7 specific siRNA (sense 5'-CCTCGTGGAACATGACAAGtt-3',) and a nonsilencing control scramble siRNA (sense 5'-ACACTGTCCCCACCATAACtt-3'). The *BMP7* silencing was verified using qRT-PCR. Functional assays were done at the time points indicated.

8. rhBMP7 treatment (IV)

BMP7 function was also studied by adding recombinant human BMP7 (R&D Systems, Minneapolis, MN) to the growth medium of two breast cancer cell lines

MDA-MD-231 and T-47D. To better distinguish possible BMP7 specific phenotype changes in the cell line, reduced serum conditions were used in the functional assays. Medium containing DMEM, 1% FBS, 2mM glutamine, and 1% penicillin/streptomycin was used for MDA-MB-231 cells. T-47D cells were grown in standard medium containing 5% FBS, since they are more sensitive to reduced serum conditions. First, 2.5 x 10^4 cells (24-well) or 12.5 x 10^4 cells (6-well) were seeded and after 24 h medium was replaced with medium containing BMP7 (50 ng/ml, unless otherwise stated) or an equivalent volume of vehicle (4 mM HCl, 0.1% BSA), used as a control. Fresh medium with BMP7 or vehicle was added every other day and functional assays were done at the time points indicated.

9. Functional assays (IV)

9.1 Proliferation assay

To study the possible contribution of BMP7 in breast cancer cell proliferation, cells were counted after siRNA or ligand treatment. Proliferation assays were performed on 24-well plates. Cells were counted using Z2 Coulter Counter (Beckman Coulter, Fullerton, CA) 24, 48, and 72 hours after siRNA transfection or two, four, and seven days after first addition of BMP7 or vehicle. Each assay contained six replicates and was repeated at least twice.

9.2 Cell cycle and apoptosis assays

Cell cycle and apoptosis analyses were done 24, 48, and 72 hours after siRNA transfection or four and seven days after the first addition of BMP7 or vehicle on a 6-well plate. Each assay was done in triplicate and repeated at least twice. Both floating and adherent cells were collected and half of the cells in each well were directed to the cell cycle and half to the apoptosis analysis.

For cell cycle analysis, the cell pellet was suspended in hypotonic staining buffer (0.1 mg/ml sodium citrate, 0.03% Triton X-100, 50 μg/ml propidium iodide (PI), 2 μg/ml Ribonuclease A) and cells were allowed to stain for 30-60 min on ice. Apoptosis assay was done using Annexin V FITC apoptosis detection kit (Calbiochem, La Jolla, CA) according to manufacturer's instructions. Briefly, the cell pellet was suspended in 0.5 ml cold 1x binding buffer and allowed to stain with 0.5 μg/ml Annexin V-FITC for 15 min at RT in the dark. Cells were centrifuged and supernatant discarded, the pellet was suspended with 0.5 ml cold 1x binding buffer and stained with 0.6 μg/ml of PI. Cell cycle and apoptosis assays were quantitated with flow cytometer (EPICS XL-CML, Beckman Coulter). Cell cycle distribution was analyzed with Cylchred program

(http://www.cardiff.ac.uk/medicine/haematology/cytonetuk/documents/software.htm) and apoptosis with EPICS XL-CML flow cytometer software.

9.3 Migration and invasion assays

Two cell lines (SK-BR-3 and MDA-MB-231) that are inherently capable of migrating *in vitro* were used for the migration chamber assay. SK-BR-3 cells were collected 48 hours after siRNA transfection (6-well plate) and 10 x 10^4 cells were transferred in 350 µl of serum free medium to the upper chamber of a Thincerts™ cell culture insert (8.0 µm pore size, Greiner bio-one, Frickenhausen, Germany). In the lower chamber, 750 µl of standard cell culture medium was added and cells were allowed to migrate through the PET (polyethylene terephthalate) membrane 22 h at 37°C. MDA-MB-231 cells were treated with BMP7 ligand or vehicle for six days (6-well plate). Cells were collected and 2.5 x 10^4 cells transferred to the upper chamber in 350 µl of culture medium containing 1% FBS. The lower chamber was filled with 750 µl DMEM, 1% FBS, and cells were allowed to migrate 22 h at 37°C. Equal number of treated cells were plated and counted with Z2 Coulter Counter after 22 h in order to evaluate the possible contribution of cell growth in the amount of migrating cells. Each assay was performed in six replicates and repeated twice.

Invasion through basement membrane was studied using BD Biocoat Matrigel Invasion Chambers with 8.0 µm pore size (BD Biosciences, Bedford, MA). MDA-MB-231 cells were treated with BMP7 ligand or vehicle for six days on a 6-well plate. Cells were collected and 5 x 10^4 cells transferred to the upper chamber in 350 µl of culture medium containing 1% FBS. A lower chamber was filled with 750 µl of DMEM, 10% FBS, and cells were allowed to invade through Matrigel 22 h at 37°C. Each assay was performed in six replicates and was repeated twice.

Cells that either migrated or invaded through the pores were fixed and stained similarly. Briefly, cells on the upper side of the insert were removed and cells on the lower side (migrated or invaded cells) fixed with 100% methanol for 2 min and stained with 1% toluidine blue in 1% borax for 2 min. The inserts were then rinsed twice with sterile water and allowed to air dry. PET membranes from the inserts were removed and embedded with immersion oil on an objective slide. Six 10x images were captured from each membrane with an Olympus BX51 microscope (Olympus, Hamburg, Germany), using cell^B software (Soft-imaging system, Münster, Germany). The total area of migrated or invaded cells (in pixels) on a single membrane was determined from the six images with ImageJ software (Rasband 1997-2007).

10. Statistical analyses (I-IV)

Fisher's exact test (two rows, two columns) or the chi-square test (larger contingency tables) was used to evaluate the relationship of (1) BMP7 relative copy number or expression status, and (2) expression statuses of other BMP ligands or BMP receptors with the clinicopathological parameters of the primary tumors. The Mann-Whitney U test was used to compare the median *BMP7* expression levels between the tumor groups with and without *BMP7* copy number increase as well as to compare the medians of test and control groups in functional assays.

In Study III, all statistical analyses were performed using SPSS program (version 11.01, SPSS Inc., Chicago, IL). Associations of BMP7 expression with standard clinicopathological parameters and recurrence sites were evaluated using Fisher's exact test. McNemar test was applied to studies of matched pairs of primary tumor and local relapse. The rate of local recurrence and first distant metastases, and of disease-free time and survival times was analyzed using the Kaplan-Meier method. The log-rank test was used to determine the significance of difference between BMP7 positive and negative tumor groups. Cox regression was used in the univariate and multivariate analyses.

Results

1. *BMP7* amplification in breast cancer cell lines and primary tumors (I)

BMP7 is located at the chromosomal region 20q13 that is commonly amplified in breast cancer. A previous microarray based study indicated that *BMP7* was a putative amplification target gene (Hyman et al. 2002). In order to validate and expand these results *BMP7* gene copy number was studied in breast cancer cell lines and in primary tumors.

FISH analysis of *BMP7* copy number was done in altogether 22 cell lines (BT-474, CAMA-1, DU4475, HCC38, HCC1419, HCC1954, MCF7, MDA-MB-134, MDA-MB-157, MDA-MB-361, MDA-MB-415, MDA-MB-436, MDA-MB-453, MPE600, SK-BR-3, T-47D, UACC732, UACC-812, UACC-893, UACC3133, ZR-75-1, and ZR-75-30). Half of the cell lines had at least two-fold increase in the relative *BMP7* copy number (*BMP7* vs. 20 chromosome centromere). The most prominent copy number increases were detected in MCF7 (13-fold), ZR-75-1 (seven-fold), and BT-474 (four-fold) cells. The three cell lines also showed extremely high level *BMP7* absolute copy number, more than 30 copies per cell. In addition, four cell lines (HCC1419, UACC732, UACC-812, and SK-BR-3) had a three-fold increase in *BMP7* relative copy number, two of them (HCC1419 and UACC732) with more than ten *BMP7* copies per cell.

BMP7 copy number analysis was also performed on clinical tumor specimens on TMA. A total of 146 primary breast tumors were analyzed using FISH. Absolute copy numbers of *BMP7* and 20 centromere could be evaluated from 125 samples (86% of cases). The uninformative cases were either lost samples or samples with unsuccessful hybridization (high background autofluorescence or low signal intensity). A total of 20 samples (16%) showed an increase in the relative copy number of *BMP7*. More specifically, 5.6% of the cases had high level *BMP7* copy number increase (relative copy number >3.0) and 10.4% moderate copy number increase (relative copy number 1.5-3.0). No extremely high absolute or relative copy number increases were seen in the primary tumors. Possible associations between *BMP7* copy number and standard clinicopathological parameters were statistically evaluated. A significant association was detected between increased *BMP7* copy number and high histological tumor grade ($p = 0.005$) as well as high proliferation activity as determined by routine Ki67 staining ($p = 0.044$). It is noteworthy that none of the lobular carcinomas had increased *BMP7* copy number ($p = 0.006$).

2. BMP7 mRNA and protein expression in breast cancer cell lines and primary tumors (I)

BMP7 mRNA expression was studied in the same set of cell lines as above with MDA-MB-231 instead of MDA-MB-157, and in normal mammary epithelial cells (HMEC) using semiquantitative RT-PCR. Variable *BMP7* mRNA expression was detected in 18 breast cancer cell lines using RT-PCR. Altogether 14 cell lines showed higher *BMP7* expression levels than the normal HMEC, and in four cell lines the expression levels were lower than in the normal sample (HMEC). No *BMP7* transcript was observed in the remaining four cell lines. Highest expression levels were detected in BT-474, DU4475, MCF7, MDA-MB-415, and SK-BR-3. High levels of *BMP7* expression were detected in both cell lines with *BMP7* copy number increase and cell lines with no copy number increase.

A more detailed analysis of *BMP7* expression levels was performed for 44 breast tumor specimens using real-time quantitative RT-PCR. Based on copy number status determined on TMA, 15 tumors with increased *BMP7* copy number and 29 tumors with no copy number change were selected and parallel freshly frozen tumor specimens were obtained for the expression analyses. To avoid any bias in tumor selection, the tumor groups were matched according to standard clinicopathological features so that they differed only in their copy number status. Variable *BMP7* expression levels were also detected in the primary tumors in both groups. A few tumors with increased *BMP7* copy number also showed the highest expression levels. However, no statistical difference was detected in the median expression level between the two tumor groups ($p = 0.47$). Since *BMP7* copy number status did not discriminate either cell lines or primary tumors, one can conclude that amplification is not the main mechanism for overexpression of *BMP7*.

BMP7 protein expression was examined in 11 breast cancer cell lines, 10 normal mammary gland tissue sections, and in TMA including 146 primary breast cancers. BMP7 protein was expressed exclusively in the cytoplasm in both cell lines and tissues. All of the 11 cell lines (BT-474, HCC1419, MCF7, MDA-MB-361, MDA-MB-453, SK-BR-3, T-47D, UACC-812, UACC-893, ZR-75-1, and ZR-75-30) examined showed distinct BMP7 staining with no clear differences in staining intensity and were thus regarded as BMP7 positive. Tissue samples showed an essentially homogeneous staining of epithelial cell compartment across the entire tissue specimen whereas adjacent stromal cells were always negative. BMP7 expression in the epithelial cells of the normal mammary gland was moderate in all 10 samples. BMP7 expression was evaluated altogether in 91 out of 146 of primary tumor samples. The remaining uninformative cases were due to either lost or unrepresentative samples. The majority of the evaluated samples (71.4%) showed strong BMP7 staining whereas moderate staining was observed in the rest of the cases (Figure 3). There was no one-to-one correlation between *BMP7* mRNA and protein expression

levels. Finally, no statistically significant association was detected between BMP7 mRNA or protein status and clinicopathological parameters.

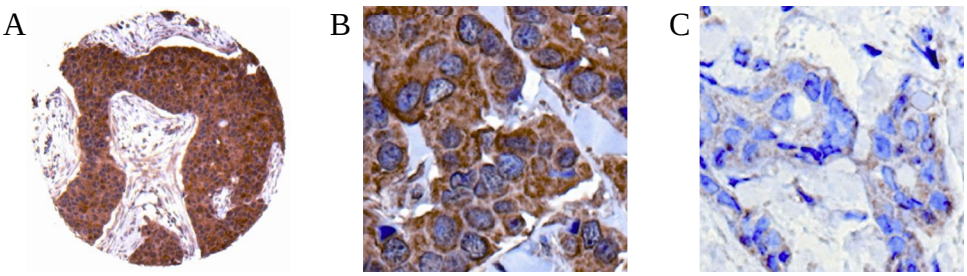


Figure 3. *A representative examples of strong BMP7 expression (A, tissue core biopsy, B, 20x objective) and moderate BMP7 expression (C, 20x objective).*

3. Expression profiles of *BMP2-BMP8* and six BMP specific receptors (II)

In Study II, mRNA expression levels of seven BMP ligands and six BMP specific receptors were explored in the same 22 breast cancer cell lines as above and in 39 primary breast tumors likewise in normal mammary epithelial cell line (HMEC) and normal mammary gland sample using semiquatitative RT-PCR. Although *BMP7* expression was already examined in Study I, BMP7 was included in this study too in order to better compare expression profiles between different ligands. The expression frequency, level and relation of cancer samples to normal samples were evaluated for each ligand and receptor.

Substantial differences were observed in the expression frequencies from one ligand to another (Figure 4). *BMP4*, *BMP7* and *BMP8* were expressed in the majority of cell lines and primary tumors. Different pattern was detected with *BMP2* and *BMP6* that were detected in most of the primary tumors but only in small subset of the cell lines. *BMP5* and *BMP3* were expressed at similar frequencies in both cell lines and tumors. *BMP5* transcript was observed in roughly half of the samples. *BMP3* was expressed least frequently of all the ligands.

Considerable variation was also detected in the expression levels of different BMPs in both cell lines and primary tumors. Generally, variations of expression levels were comparable between cell lines and primaries. *BMP4* had the most prominent profile, with a wide range of expression levels, and extremely elevated expression was detected particularly in a subset of primary tumors. Compared to other ligands *BMP7* also stood out due to the fairly elevated expression levels in both cell lines and tumors. A few cell lines had rather strong expression of *BMP2* and *BMP3*, compared to the levels seen in primary tumors. Comparison between the cancer and normal samples revealed that *BMP4* and

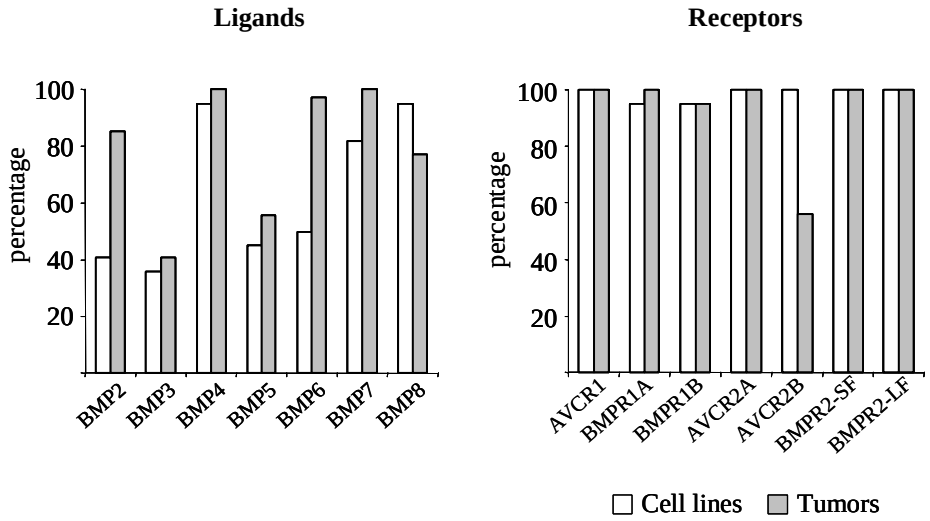


Figure 4. *Expression frequencies of BMP ligands and BMP specific receptors in breast cancer cell lines and primary tumors.*

BMP7 were detected at elevated levels in the majority of cancer samples. *BMP5* and *BMP8* transcripts were not detected in HMECs and *BMP5* expression in normal mammary was also at a very low level. By contrast, strong *BMP2* expression was observed in HMECs and *BMP6* expression was clearly elevated in both normal samples compared to the cancer samples.

In contrast to the ligands, all three type I and all three type II receptors were expressed in virtually all breast cancer cell lines and primary breast tumors (Figure 4). *ACVR2B* was the only exception, since it was detected only in 56% of the primaries. Variation was observed, however in the expression levels, which differed between different receptors. All three type I receptors showed wide variation in their expression levels in all samples and were also generally comparable between cell lines and primaries. Among the receptors *BMPR1A* had the most prominent profile with strong expression levels and the greatest variation. Interestingly, in the cell lines type II receptors had rather similar expression patterns but very low expression levels of *ACVR2A*, *ACVR2B*, and *BMPR2-LF* (splice variant *BMPR2* long form) were detected in primary breast tumors. Type I receptors and *ACVR2A* transcripts were seen at comparably high levels in HMEC among the cell lines but at lower levels in HMG among the primaries. *ACVR2B* and *BMPR2-LF* had distinctly low expression in HMECs likewise *BMPR1A* and *BMPR2-SF* (splice variant *BMPR2* short form) in normal mammary.

There were no apparent connections between cancer cell line characteristics and expression profiles of BMP ligands or BMP specific receptors. The expression profiles were also studied with respect to the clinicopathological parameters of primary tumors (such as histological tumor type, grade, tumor size, nodal status, etc.), but no obvious associations were found between specific expression pattern and tumor characteristic.

4. The clinical relevance of BMP7 (III)

The possible association between BMP7 expression and clinical characteristics was studied in a unique tumor material including 483 patients with invasive ductal (IDC) or lobular (ILC) carcinoma and complete patient records with up to 15 years of follow-up information. BMP7 expression was studied in 483 primary and 40 recurrent local tumors by immunohistochemistry and statistical analysis was used to evaluate the possible associations with clinicopathological parameters, primary treatments, recurrence sites, and survival. Normal kidney section was used to verify BMP7 antibody specificity. The distinction between moderate and strong BMP7 staining was not as evident as in Study I and thus primary tumor samples were classified as either BMP7 positive or negative.

In Study III, the BMP7 protein expression could be evaluated in 409 (85%) of the primary breast tumors and positive BMP7 expression was detected in altogether 47% of the tumor samples analyzed. The histological tumor subtype discriminated BMP7 expression. Of the lobular carcinomas 57% (118 out of 208 tumors analyzed) were BMP7 positive whereas BMP7 was expressed in only 37% (75 out of 201 tumors analyzed) of the ductal carcinomas ($p = 0.0001$). In light of this clear difference in BMP7 expression frequency the effect of BMP7 on clinicopathological parameters was explored separately in the tumor groups. BMP7 expression was associated with higher pathological T stage ($p = 0.034$) in the IDC group. The majority (70%) of BMP7 negative ductal tumors was classified as pT1 (< 20 mm of tumor diameter), whereas only 55% of BMP7 positive tumors belonged to this stage, indicating that BMP7 positive tumors have an increased size. No such association was observed among the ILC cases. In both groups, no statistically significant association was detected between BMP7 expression status and other tumor features (pN and pM status, hormone receptors, ERBB2 expression, proliferation activity, histological/nuclear grade, and DNA ploidy status). The possible contribution of hormone replacement therapy to BMP7 expression was evaluated in three patient groups stratified according to their HRT use (never users, prior users, and current users) but no association between these groups and BMP7 status could be found.

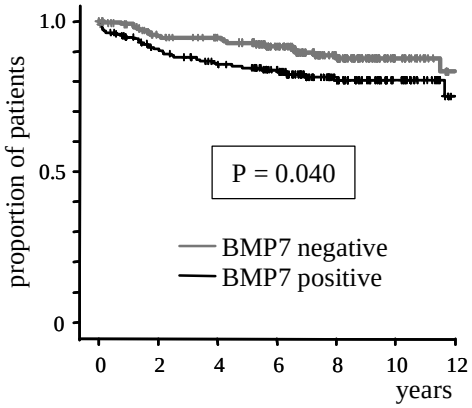
In addition to the primary tumor samples, BMP7 expression was determined in local recurrence tumors. From the 483 patients included, local recurrences were detected in a total of 50 patients. The recurrent tumor sample was available from 40 cases and BMP7 expression was evaluated in 38 (95%) cases. Only 13% of the local recurrences were BMP7 positive. Since the small number of relapse samples could affect the result, BMP7 expression patterns were next examined in a subset of 35 patients. In these cases BMP7 status had been evaluated from both the primary and the local recurrent tumor of the same patient. The primary tumors expressed BMP7 significantly more often than the corresponding local recurrences ($p = 0.004$). There were 18 patients with BMP7 positive primary tumor and only two of them had BMP7 expression in their local recurrence sample. BMP7 expression loss was not explained by the primary tumor subtype. One possibility was that the treatments these patients received after primary

tumor removal could have influenced BMP7 expression pattern in the local recurrence. In the subgroup of 35 patients, 15 patients did not receive any treatment, four patients received post-operative radiotherapy with adjuvant medication, 10 patients radiotherapy alone, four patients adjuvant medication alone, and for two patients no treatment information was available. However, post-operative radiotherapy or adjuvant therapy did not explain the BMP7 expression loss in the local recurrent tumor. The small number of patients in each treatment subgroup may have affected the results.

BMP7 status in the primary tumor seemed to be associated with the occurrence of local recurrence. ILC and IDC groups were studied separately. In ductal carcinomas, 17% of the patients in the BMP7 positive group and only 8% in the BMP7 negative group had local recurrences ($p = 0.065$). BMP7 expression was also linked to the rate of local relapse formation in patients with IDC. In the BMP7 positive group, 52 (70%) and 22 (30%) patients remained at risk 4 and 8 years after diagnosis respectively, whereas in the BMP7 negative group 101 (80%) and 52 (41%) remained at risk ($p = 0.054$). However, these differences did not quite reach statistical significance. No similar trend was detected with the lobular carcinomas.

Finally, the possible association between BMP7 expression and the occurrence of distant metastasis or patient survival was evaluated. Overall metastasis frequency among the 483 patients was 23% and bone was the most common site of metastasis in both ILC and IDC groups (16%). There was a slight tendency for patients with BMP7 positive tumor to develop distant metastases (26%) more often than patients with BMP7 negative tumor (21%). The overall metastasis frequencies appeared to reflect the frequencies detected in bone metastasis. Patients with BMP7 positive tumors seemed to develop particularly bone metastases more often (20% BMP7 pos. vs. 14% BMP7 neg.) whereas other metastasis sites (liver, lung, pleura, lymph nodes, skin, brain, and abdominal area) showed no marked tendencies. However, the frequency differences observed were not statistically significant. Interestingly, time to event analysis revealed that BMP7 expression in the primary tumor clearly and significantly accelerated the rate of bone metastases formation ($p = 0.040$, Figure 5). The two tumor groups were studied separately and the early bone metastasis formation was observed among the BMP7 positive ductal carcinomas ($p = 0.033$), but not among the lobular carcinomas ($p = 0.29$). A multivariate Cox regression analysis was performed to study whether well known prognostic factors (age, pN and pT stage, histological/nuclear grade, hormone receptor statuses, and ERBB2 status) have an impact on this finding. BMP7 expression in the primary tumor was found to be an independent prognostic factor for early bone metastasis formation with a risk factor of 2.14 (95% CI: 1.07-4.28, $p = 0.032$, Figure 5) in addition to positive lymph node status (risk factor 2.34, 95% CI: 1.14-4.79, $p = 0.020$), and high histological/nuclear grade (risk factor 3.69, 95% CI: 1.26-10.78, $p = 0.017$). Finally, no associations were detected in overall or breast cancer specific survival between patients with BMP7 positive or negative tumors in either ductal or lobular carcinoma groups.

Bone metastasis



Multivariant analysis

Variable	HR (95% CI)	P
pN stage	2.34 (1.14-4.79)	0.020
Grade		
II vs. I	-	
III vs. I	3.69 (1.26-10.78)	0.017
BMP7	2.14 (1.07-4.28)	0.032

Figure 5. *Kaplan-Meier plot of time to first bone metastasis in patients with BMP positive or negative primary tumor (left) and multivariate Cox regression analysis for bone metastasis (right).*

5. BMP7 function in breast cancer cell line models (IV)

The possible function of BMP7 in breast cancer was studied in cell line models. A bidirectional approach was used where *BMP7* expression was inhibited in cell lines with high endogenous level and exogenous BMP7 was given to cell lines with no *BMP7* expression. The consequences of these manipulations on proliferation, cell cycle distribution, apoptosis, migration and invasion were then explored. The results of Study I had indicated that *BMP7* is expressed at variable levels in breast cancer cell lines. Expression levels were first confirmed in five selected cell lines using real time quantitative RT-PCR. As expected, the highest *BMP7* expression levels were seen in BT-474, MCF7, and SK-BR-3 whereas in the T-47D and MDA-MB-231 cells virtually no *BMP7* mRNA was detected.

First the possible impact of *BMP7* overexpression on cancer cell behavior was examined by RNAi technique. *BMP7* expression was silenced in three cell lines with high endogenous expression, BT-474, MCF7, and SK-BR-3. *BMP7* expression levels between *BMP7* siRNA and non-silencing control siRNA treated cells were compared in order to determine the efficiency of *BMP7* silencing. The average decreases in the *BMP7* expression levels are presented in Table 5.

The consequences of *BMP7* reduction on cell proliferation were determined by cell counting. In the MCF7 or SK-BR-3, no difference was detected in the cell number between *BMP7* and control siRNA transfected cells. By contrast, *BMP7* siRNA treatment significantly decreased the cell number (up to 30% at 72 h, $p = 0.002$) in B7-474 cells. To further evaluate the changes detected in cell

Table 5. *Average BMP7 expression level decrease in BMP7 siRNA treated cells compared to control siRNA treated cells.*

Cell line	24h (%)	48h (%)	72h (%)
BT-474	62	87	87
MCF7	78	83	82
SK-BR-3	64	71	69

proliferation, cell cycle distribution and apoptosis were studied using flow cytometry. As expected, no alterations were observed in MCF7 or SK-BR-3 that did not show any change in proliferation. However, in the BT-474 cell line, *BMP7* siRNA treatment resulted in an increase in the G1 cell fraction at 48 h (74% vs. 65%, p = 0.002) and at 72 h (74% vs. 69%, p = 0.002) compared to control siRNA transfected cells. This was accompanied by a significant reduction of cells in the S phase in *BMP7* siRNA vs. control siRNA treated cells (48 h: 18% vs. 26%, p = 0.002 and 72 h: 19% vs. 24%, p = 0.041). There were no significant changes in the percentage of apoptotic cells between the *BMP7* and control siRNA transfected cells. Taken together, *BMP7* silencing in BT-474 cells led to reduced proliferation due to an increase of cells in the G1 phase and a concomitant decrease in the S phase of the cell cycle. Lastly, the possible consequences of *BMP7* silencing on migration phenotype were examined in SK-BR-3 cells that are capable of migrating *in vitro*. There were no significant differences in the total area of migrated cells between *BMP7* or control siRNA transfected cells.

Next exogenous BMP7 ligand was added to the growth medium of MDA-MD-231 and T-47D cells lacking endogenous *BMP7* expression in order to study cancer cell phenotypes possibly induced by an increased amount of BMP7. A BMP7 dose of 50 ng/ml was chosen as a starting point. In MDA-MB-231 cells, BMP7 clearly and significantly increased the cell number at four (57%, p = 0.002) and seven (122%, p = 0.002) days after ligand treatment compared to the vehicle treated cells at the same time point in the presence of 1% FBS. This was accompanied by BMP7 induced decrease in the amount of apoptotic cells after four (7.9% vs. 11.6%, p = 0.018) and seven days (2.3% vs. 3.9%) compared to the vehicle treated cells, but no changes were detected in cell cycle. MDA-MD-231 cells were also grown in culture medium containing 10% FBS and the growth induction was clearly smaller yet statistically significant (four days, 8%, p = 0.041, seven days, 5%, p = 0.015) indicating a presence of BMP7 regulatory factors in the serum. An opposite effect was observed in the T-47D cells where BMP7 treatment (50 ng/ml) decreased cell proliferation slightly. The cell number reduction was 14% (p = 0.009) after two days, 10% after four days (p = 0.015), and 16% (p = 0.004) after seven days of BMP7 treatment compared to the vehicle treated cells at the same time point. However, no changes were detected in either cell cycle or apoptosis. For both cell lines the influence of different BMP7 doses (10, 50, 250 ng/ml) were examined. The maximum effect was detected with 50 ng/ml and there was no additional growth alteration with

increasing doses. Taken together, exogenous BMP7 increased the growth of MDA-MB-231 cells and this was accompanied by a reduction of apoptotic cells. By contrast, BMP7 slightly decreased the growth of T-47D cells, but without any changes in either cell cycle or apoptosis.

Finally, MDA-MB-231 cells that are readily capable of migration and invasion *in vitro* were used to study the effect of BMP7 on these phenotypes. Cells were first incubated with BMP7 (50 ng/ml) or vehicle for six days, and the possible alterations in cell motility tested with migration and invasion chamber assays. BMP7 treatment significantly enhanced both cell migration and invasion and was not caused by increased proliferation. There was a 2.3-fold increase in the total area of migrated cells after BMP7 treatment compared to the vehicle treated cells ($p = 0.002$). Moreover, the invasion through Matrigel basement membrane matrix was even more effective, since the total area of invaded cells was 3.9-fold greater after BMP7 (50 ng/ml) treatment than vehicle treatment ($p = 0.002$). Taken together, exogenous BMP7 considerably enhanced both migration and invasion of MDA-MD-231 breast cancer cells.

Discussion

1. BMP7 is not an amplification target gene (I)

Gene amplification is a common mechanism for the activation of oncogenes in solid tumors. Copy number increase of a certain genomic region leads to increased expression of cognate genes that in turn promote growth of cancer cell (reviewed by Albertson et al. 2003, Albertson 2006). *BMP7* is located at the genomic region 20q13 that has previously been reported to be amplified in primary breast tumors (Kallioniemi et al. 1994, reviewed by Hodgson et al. 2003). Furthermore, amplified 20q13 region was associated with poor prognosis of breast cancer patients (Tanner et al. 1996). Several genes have been proposed to be the target genes contributing to the growth advantage. The complex nature of genomic aberrations in the 20q13 amplicon suggests that multiple genes may be involved in tumor progression (reviewed by Hodgson et al. 2003). Hyman and colleagues (2002) systematically searched for amplified and overexpressed genes in breast cancer cell lines using a microarray approach, and identified a putative novel target gene bone morphogenetic protein 7 at the 20q13 region.

In Study I, *BMP7* copy number increase and the possible impact on the mRNA and protein expression levels were further studied in a large panel of 22 breast cancer cell lines and 146 primary breast tumors. *BMP7* was indeed amplified in breast cancer cell lines and in primary breast tumors. In the cell lines, *BMP7* copy number was increased at least two-fold in half of the samples, and three cell lines showed exceptionally high levels of amplification. In primary tumors, at least moderate copy number increase was detected in 16% of the evaluated tumor samples and high-level increase was seen in altogether 5.6% of the cases. Observed frequencies were in good concordance with previous studies showing a copy number increase of the 20q13 region in 18% and high-level amplification in less than 10% of breast cancer cases (Tanner et al. 1996, Tirkkonen et al. 1998, Rennstam et al. 2003, Ginestier et al. 2006, Letessier et al. 2006).

The statistical evaluation showed that *BMP7* copy number increase was more often detected in tumors with high histological grade and high proliferation activity confirming similar observations done with the 20q13 region (reviewed by Hodgson et al. 2003). *BMP7* copy number increase was detected in invasive ductal carcinomas, but not at all in invasive lobular carcinomas, a phenomenon that has not previously been reported. In general, ILCs are known to harbor fewer genomic aberrations compared to IDC and lobular carcinomas actually resemble low-grade ductal tumors (reviewed by Simpson et al. 2005). Similarly,

BMP7 copy number increase was detected predominantly in high grade ductal tumors, suggesting that amplification of this locus may rather be associated with the grade of the tumors than the histological subtype.

BMP7 expression was detected widely at mRNA and protein levels, but no one to one correlation between increased copy number and elevated expression was observed, even though the highest mRNA levels were detected in cell line and tumor samples with high copy number increases. The previous study by Hyman and colleagues (2002) suggested a more straightforward connection between *BMP7* copy number increase and subsequent increase in the expression. Comparison of their cell line data with the data obtained in Study I actually revealed a good concordance in the cell lines shared by both studies. The connection between amplification and overexpression was more obvious in their smaller and different subset of breast cancer cell lines (Hyman et al. 2002). Taken together, the good concordance with the earlier reports on the amplification frequency, but the lack of cognate BMP7 expression increase suggests that *BMP7* is an innocent bystander and not an amplification target gene in the 20q13 region.

2. BMP7 is overexpressed in breast cancer (I)

Although amplification was not the activating mechanism, extensive BMP7 expression was detected at both mRNA and protein levels. A semiquantitative RT-PCR analysis in breast cancer cell lines showed that the majority of cell lines were positive for *BMP7* and altogether 64% of the cell lines showed variable and elevated expression compared to that detected in the normal mammary epithelial cell line. A more precise analysis using real-time quantitative RT-PCR also revealed a highly variable *BMP7* expression in a subset of 44 primary breast tumors with a few tumors exhibiting exceptionally strong expression. Similarly, a very recent study demonstrated variable *BMP7* mRNA expression levels in 67 primary breast tumors (Buijs et al. 2007a) thus confirming the data in Study I.

BMP7 protein was widely expressed in breast cancer cell lines and also in primary tumors. Eleven cell lines were positive for BMP7 expression and notably strong expression was detected in over 70% of the primary tumors, whereas only moderate expression was seen in ten samples of normal mammary gland. Wide and yet more variable BMP7 protein expression has been reported in another study including 170 primary ductal tumors (Schwalbe et al. 2003). In their study, a video-based computer system was used to analyze BMP7 immunoreactivity score in individual samples whereas in Study I the evaluation was performed for samples collected on a tissue microarray thus ensuring similar staining conditions from one sample to another. Schwalbe and colleagues (2003) also detected BMP7 protein expression in the normal mammary end buds, but did not compare it to the expression in primary tumors. BMP7 expression at the mRNA and protein level in Study I was not associated with any of the clinicopathological parameters of the primary tumors. By contrast, Schwalbe and

colleagues (2003) detected association between BMP7 status and hormone receptor statuses in their primary tumor set, although the article does not explicitly state whether the association was direct or inverse. Taken together, the results from the cell lines and the primary tumors in Study I indicate that BMP7 is widely expressed in breast cancer and strong BMP7 expression in particular is clearly breast cancer specific.

It is interesting notion that the *BMP7* mRNA was detected in varying levels whereas steadier overexpression was seen at the protein levels. This may simply be due to different analysis methods. Visual classification of BMP7 protein staining intensity cannot be directly compared to very accurate measuring of mRNA levels in real-time quantitative RT-PCR. Traditionally however, one would have expected a more straightforward dependence of protein levels on the mRNA levels. This is not necesserily always the case. Some studies have reported considerable variation between mRNA and protein expression (Gygi et al. 1999, Nie et al. 2006) and such variation has also been detected during oncogenesis (Rajasekhar and Holland 2004). Possible explanations for the observed distinction are mRNA and protein stability. BMP7 is known to have a very long 3'UTR (untranslated region), a characteristic linked to mRNA stability (Guhaniyogi and Brewer 2001) that could allow protein translation from limited a amount of mRNA. The prodomains of BMPs are also known to regulate mature protein stability (Constam and Robertson 1999) providing another mechanism for persistent protein expression. Either way, BMP7 is overexpressed in both mRNA and protein level in breast cancer.

3. Expression profiles of BMP ligands and BMP specific receptors (II)

In Study II a comprehensive expression survey of different BMP ligands and BMP specific receptors was performed in breast cancer cell lines and primary breast tumors using semiquantitative RT-PCR. Prior to this study there was a limited amount of information on BMPs in breast cancer and expression levels in particular had been examined usually in only few breast cancer cell lines. Likewise most studies had focused on one ligand at a time, thus making comparison between ligands difficult. Furthermore, BMP specific receptors had hardly been studied at all. The panel of expression patterns created in Study II clearly revealed distinct profiles of different BMP ligands suggesting that these are not regulated similarly and may even have diverse functions in breast cancer. The presence of BMP receptor transcripts in breast cancer further suggested that functional BMP signaling is possible in breast cancer. The expression profiles were described for the first time for ligands *BMP3*, *BMP4*, *BMP5*, *BMP8*, and all six BMP specific receptors.

The good concordance between the profiles in the cell lines and primary tumors indicated that cell lines are useful models in studies elucidating BMP function in breast cancer. However, there were a few exceptions to this rule.

BMP2 and *BMP6* were expressed less frequently in the cell lines than in the primary tumors. Type II receptors *ACVR2A*, *ACVR2B*, and *BMPR2-LF* in turn were detected in similar frequencies, but the expression levels were higher in the cell lines than in the primaries. Since breast cancer cell lines typically originate from cancer cells already disseminated from the tumor, they represent more advanced stage breast cancers than the primary tumors. These two ligands may be downregulated during breast cancer progression, whereas receptor expression is increased. Lastly, there were no noticeable implications in the expression profiles of certain cell line characteristics or clinicopathological parameters. However, this should not be interpreted to mean that no correlations exist, merely reflecting the fact that the somewhat limited number of tumor samples in Study II was not the most suitable for the identification of such dependencies.

BMP ligand expression frequency and levels varied considerably from one ligand to another. *BMP4* had the most prominent profile with high expression frequency and levels in both cell lines and primaries. High-level expression was also cancer specific, since normal samples showed relatively low-level expression. A similar profile was obtained for *BMP7*, thus confirming the results of cancer specific overexpression in Study I. *BMP2* transcript was detected at lower levels in cancer samples compared to normal samples and this study thus confirms earlier reports of *BMP2* downregulation in breast cancer (Reinholz et al. 2002). BMP2 has been shown to inhibit proliferation in some breast cancer cell lines (Ghosh-Choudhury et al. 2000a, Ghosh-Choudhury et al. 2000b, Pouliot and Labrie 2002) which might explain the less frequent *BMP2* expression in the cell lines. *BMP6* was also detected at lower levels in the cancer samples. A recent study suggested that epigenetic silencing, especially in the ER negative breast cancers, was responsible for reduced *BMP6* expression (Zhang et al. 2007). However the tumor material in Study I was mainly ER positive, and thus this phenomenon may not be restricted solely to ER negative tumors. Taken together, *BMP4* and *BMP7* were widely and strongly expressed in breast cancer implying a more deleterious role as compared to the other ligands studied.

All six receptors capable of transmitting BMP signals were expressed in both breast cancer cell lines and primary tumors. There is only one study adequately describing BMP receptor expression in breast cancer; Helms and colleagues (2005) identified BMPR1B in their search for differentially expressed genes during the progression of ER positive breast cancers. BMPR1B expression was reported in the majority of breast cancers, and was evident also in Study II. Helms and co-workers also demonstrated that BMPR1B expression was associated with advanced stage tumor characteristics (2005), but in the small tumor material in Study II, no such association was detected. Studies on prostate cancer showed that the expression of BMPR1A, BMPR1B, and BMPR2 is lost in more advanced stage cancers and the loss of BMPR2 especially is associated with poor prognosis (Kim et al. 2000, Kim et al. 2004). Such trends were not seen in breast cancer, since all BMP receptors were widely expressed in both cell lines and primaries. Most importantly, Study II showed that breast cancer is indisputably capable of BMP signaling.

4. BMP7 is a prognostic factor for early bone metastasis (III)

Previous studies have clearly indicated that BMP7 is overexpressed in breast cancer and in Study III the clinical relevance of BMP7 expression was examined in a large patient cohort (n = 483) with extensive patient information available. This included the clinicopathological parameters of primary tumors, primary treatments, recurrences and survival. The follow-up period was at maximum over 15 years.

BMP7 protein expression was evaluated in 409 of the primary tumors and of these 47% were BMP7 positive whereas no expression or very weak expression was detected in the remaining cases. In the BMP7 positive group no apparent staining intensity differences, as in Study I, were detected. Expression frequency was different from that observed in Study I where BMP7 protein expression was detectable in all 91 tumors evaluated. The primary tumor materials used in these two studies have some fundamental differences. In Study I the primary tumors were collected from patients diagnosed 2000-2003, whereas in Study III patients were diagnosed 1990-1999. The age of the tumor samples could have affected the staining intensity in general. The tumor material in Study I was also somewhat biased compared to the unbiased material in Study III. The primary tumors in Study I more often had higher pT stage (reflecting tumor diameter) and a higher histological/nuclear grade, characteristics of a more aggressive tumor phenotype. In Study III, BMP7 expression was indeed associated more often with high pT stage among the ductal tumors, which was also the major histological subtype in Study I. Finally, the number of specimens evaluated in Study III was over four times greater than in Study I, providing a better basis for analysis.

In Study III roughly half of the samples evaluated were of lobular type and half of ductal type. BMP7 expression was observed more often in the lobular carcinomas than in ductal carcinomas. Peculiarly, in renal cell carcinoma BMP7 expression was also dependent on histological subtype (Kwak et al. 2007), suggesting that BMP7 expression in general is affected by the cancer cell context. As stated above, BMP7 expression was associated with higher pT stage among the ductal tumors, indicating that BMP7 positive tumors are larger. No association was detected in the lobular carcinomas and which may be due to the higher overall frequency of BMP7 expression in these cases. As discussed earlier, it has previously been reported that BMP7 expression was associated with hormone receptor statuses (Schwalbe et al. 2003), but no such associations were found in either Study I or Study III.

Interestingly, examination of paired specimens from primary tumors and their corresponding local recurrences revealed that BMP7 expression was lost in the local recurrences. Of the 18 patients with BMP7 positive primary tumor, only two had BMP7 positive local recurrence. This event was not dependent on the tumor subtype and the primary treatments seemed to have had no effect. Study III unfortunately did not include any metastasis specimens to examine whether

BMP7 expression is also lost in distant recurrences. But the progression of local and of distant recurrences are diverse processes probably dependent on different tumor characteristics. As in Study III, in prostate cancer *BMP7* expression was also detected at lower levels in local recurrent tumors than in primary tumors (Masuda et al. 2004). However, BMP7 expression was downregulated in primary prostate cancers compared to normal prostate, which is the opposite to the pattern seen in breast cancer (Masuda et al. 2004, Buijs et al. 2007b). Clearly elevated BMP7 expression has been detected in metastatic lesions of prostate cancer and in melanoma (Masuda et al. 2003, Rothhammer et al. 2005, Rothhammer et al. 2007).

Bone metastasis was observed more often in patients with BMP7 positive primary tumor than BMP7 negative tumor although the difference was not statistically significant. A very recent study by Buijs et al. (2007a) did not detect such a trend but instead showed that *BMP7* mRNA levels in primary breast tumors did not differ between patients with or without bone metastasis. Comparison of their data to that presented in Study III is complicated by the different expression mode of BMP7 (mRNA vs. protein) evaluated.

Most interestingly, the time to event analysis in Study III indisputably showed that BMP7 expression in the primary tumor clearly and significantly accelerated the rate of bone metastasis formation. In addition, the multivariate testing confirmed that BMP7 was an independent prognostic factor for early bone metastasis, together with high grade and positive lymph node status. Strong BMP7 expression has also been shown to be associated with shorter tumor recurrence in patients with melanoma (Rothhammer et al. 2007).

Study III is one of the few studies to address the possible impact of BMP signaling on patient outcome in breast cancer. Helms and colleagues (2005) demonstrated that overexpression of BMPR1B was associated with shorter overall survival of breast cancer patients. By contrast, the expression of the individual ligands *GDF9a* and *BMP15* each correlated with better prognosis in breast cancer (Hanavadi et al. 2007), but it must be noted that their results were obtained using Mann-Whitney test instead of the more suitable Kaplan-Meier analysis. Functional studies have indicated that active BMP signaling is required for the formation of bone metastases in prostate cancer since BMP antagonist noggin decreased metastasis formation (Feeley et al. 2005, Feeley et al. 2006, Schwaninger et al. 2007). A well known target gene and cofactor of BMP7 signaling, Runx2, was also shown to be involved in osteolytic bone metastasis (Barnes et al. 2004, Pratap et al. 2006). On the other hand, Buijs and colleagues (2007a, 2007b) showed that BMP7 treatment actually reduced bone metastasis formation and growth in mouse xenograft models of breast and prostate cancers.

Since the association observed between BMP7 expression and accelerated bone metastasis in Study III was not extremely strong and since contradictory findings have been reported (Buijs et al. 2007a, Buijs et al. 2007b) it is likely that BMP7 is not the sole determinator of bone metastasis. Attention should be directed to the molecular milieu of BMP7 since this is likely to have a significant influence on cancer cell behavior. Nevertheless, these findings may eventually have clinical significance. Bone metastases are often difficult to discover due to

diagnostic limitations (reviewed Kozlow and Guise 2005). Reduction in bone metastasis progress has been seen in response to adjuvant treatment with biphosphanates (Powles et al. 2006). Thus determining BMP7 status in the primary tumors could help to identify patients at increased risk of early bone metastases who would benefit from biphosphanate treatment.

5. BMP7 stimulation leads to diverse phenotypic effects in breast cancer cells (IV)

The results of Studies I-III indicated that BMP7 was overexpressed in breast cancer cell lines and in primary tumors when compared to the expression levels in the normal mammary cells. Moreover, BMP7 expression in the primary tumor was associated with accelerated rate of bone metastasis occurrence and was an independent prognostic factor for early bone metastasis formation. In Study IV the possible impact of BMP7 overexpression on breast cancer phenotypes was explored in breast cancer cell line models. Few functional studies have addressed the issue whether different BMP ligands have an impact in breast cancer and so far the results have been contradictory. BMP ligands have been shown to both promote and inhibit breast cancer progression (Ghosh-Choudhury et al. 2000a, Ghosh-Choudhury et al. 2000b, Pouliot and Labrie 2002, Pouliot et al. 2003, Clement et al. 2005, Raida et al. 2005b, Buijs et al. 2007a). Often the functional studies from breast and other cancers have concentrated only on one cell line at a time or stimulated few cancer cell lines with exogenous ligand without paying attention to the endogenous expression levels, thus complicating the interpretation of the results. A bidirectional approach was chosen in Study IV that aimed to overcome these pitfalls, *BMP7* expression was silenced in three different breast cancer cell lines with high endogenous *BMP7* levels and exogenous BMP7 was given to two cell lines lacking *BMP7* expression. The possible phenotypic consequences of these manipulations were examined with functional assays for proliferation, cell cycle, apoptosis, migration, and invasion.

The manipulation of BMP7 levels led to changes in cell proliferation in three out of five cell lines studied. *BMP7* silencing reduced the cell number in BT-474 cell line caused by G1 arrest and parallel increase of S phase cells. In BT-474 cells BMP7 thus enhances cell proliferation through regulation of the cell cycle. Similarly, and even more pronounced increase in the cell number was detected in MDA-MB-231 cells in response to exogenous BMP7 treatment. This was caused by a reduction of apoptotic cells, so BMP7 seemed to protect MDA-MB-231 cells from apoptosis rather than regulate cell cycle. Interestingly, the growth reduction was notably smaller when MDA-MB-231 cells were incubated in the presence of 10% serum, suggesting that serum contains BMP7 regulatory factors. T-47D cells responded in an opposite way and a slight reduction in the cell number was detected after BMP7 treatment. This was not reflected as changes either in cell cycle or apoptosis. Together with the fact that the magnitude of cell number alteration was rather small compared to those seen in

MDA-MB-231 or BT-474 cells makes the contribution of BMP7 in T-47D cell proliferation unclear. In the remaining two cell lines, SK-BR-3 and MCF7, no alterations in the cell number were detected. Interestingly, in prostate, myeloma and anaplastic thyroid carcinoma cell lines, BMP7 treatment resulted in reduced growth either by changes in cell cycle or apoptosis (Franzen and Heldin 2001, Baade Ro et al. 2004, Miyazaki et al. 2004). However, according to the results of Study IV, BMP7 reduced growth only slightly and only in one cell line, suggesting that in cases where BMP7 does affect proliferation of breast cancer cells it is more likely to be growth promoting than inhibiting.

Previous studies on other cancer types, prostate, colon and kidney have implicated BMP7 in both the enhancement and inhibition of migratory and invasive properties (Feeley et al. 2005, Feeley et al. 2006, Grijelmo et al. 2007, Ye et al. 2007b). The possible impact of BMP7 on migration and invasion in breast cancer was studied in two cell lines that are capable of migrating *in vitro*. Whereas the *BMP7* silencing did not alter the migration of SK-BR-3 cells, BMP7 treatment significantly increased the migration and even more dramatically the invasion of MDA-MB-231 cells. Buijs and colleagues (2007a) proposed a somewhat opposite role for BMP7 in breast cancer since they saw reduced formation and progression of osteolytic metastases in mouse xenograft model using BMP7 overexpressing MDA-MB-231 cells. They also detected that BMP7 treatment inhibited the growth of MDA-MB-231 (without BMP7 overexpression) cells transplanted in the mouse mammary fat pad or in bone.

In light of the data in Study IV it is evident that BMP7 truly acts cell type specifically even within a particular cancer type. This has been shown previously with prostate cancer, where BMP7 induced different phenotypic responses strictly cell type specifically (Yang et al. 2005). Moreover, the other reports on BMP7 actions also indicate that no single phenotype can be expected. The results presented in Study IV suggest that more attention should be paid to the molecular milieu in which BMP7 functions. The fact that BMP signaling is extensively regulated at many levels may, for example, explain why BMP7 stimulates proliferation in two distinct ways, either by affecting cell cycle, as in BT-474 cells, or by altering apoptosis, as in MDA-MB-231 cells. Since all the breast cancer cell lines used here express BMP specific receptors the answer to diverse responses could be in the cancer specific disturbances of Smad binding proteins or perhaps in the activation of Smad independent signaling pathways reported in cancer (Yang et al. 2006, Grijelmo et al. 2007, reviewed by Massague and Gomis 2006). Nevertheless, this data shows that BMP7 has indeed an impact in breast cancer. Due to the diverse phenotypes it induces, more detailed analysis of the signaling network is clearly needed in order to fully understand and exploit this information.

Summary and conclusions

Bone morphogenetic proteins have been a target of increasing interest in cancer research for the last ten years. In breast cancer the number of studies was and is still limited and the role of BMP7 especially was merely introduced when this work began. The main aim of this study was to characterize the activation, expression, clinical relevance, and function of bone morphogenetic protein 7 in breast cancer.

Previous studies have indicated that BMP7 is a potential novel amplification target gene residing in the commonly amplified 20q13 region in breast cancer. Comprehensive evaluation of BMP7 copy number and expression in breast cancer cell lines and in primary breast tumors has established that increased copy number was not the mechanism behind BMP7 overexpression. Nevertheless BMP7 was indeed found at highly elevated levels compared to the normal mammary epithelial cell and tissue samples in 64% of the cancer cell lines and a large fraction of the primary tumors. The notable expression was further confirmed when the expression profiles of six other BMP ligands (*BMP2*, *BMP3*, *BMP4*, *BMP5*, *BMP6*, and *BMP8*) were determined in breast cancer cell lines and primary tumors. In addition to *BMP7*, *BMP4* exhibited wide, variable and cancer specific expression compared to the other ligands. The variable expression patterns of each ligand suggested that the BMP family members are not similarly regulated, nor are they likely to harbor similar functions in cancer cells. The wide expression of all six BMP specific receptors, however, allows functional BMP signaling in breast cancer. In the future it would be useful also to determine the protein levels of six other BMPs and the exact mechanism of BMP expression and secretion. Such a study would reveal whether disturbances in this process are significant in cancer.

The clinical relevance of BMP7 expression was studied in a group of 483 breast cancer patients with extensive patient information including clinicopathological parameters, primary treatments, recurrences, and survival. BMP7 was more often expressed in invasive lobular carcinomas than in invasive ductal carcinomas, but the expression in the ductal type was associated with increased tumor size. In the corresponding local recurrence tumors BMP7 expression was frequently lost. Most importantly, patients with BMP7 positive tumors developed bone metastases more rapidly than patients with BMP7 negative tumors and multivariant analysis confirmed that BMP7 was an independent prognostic factor for early bone metastasis. The biology behind this phenomenon remains a mystery, and sample material from tumor metastases would be very valuable in deciphering the biological role of BMP7 in bone metastasis formation.

Finally, the functional contribution of BMP7 in breast cancer was studied in five cancer cell lines. A few studies on other cancer types have proposed a growth inhibitory role for BMP7, but this seemed not to be the case in breast cancer. None of the cell lines responded to BMP7 with substantial growth reduction and in two cell lines BMP7 notably increased the cell number either by regulating cell cycle or apoptosis. BMP7 treatment also clearly induced migration and invasion of MDA-MB-231 cells. Similar strictly cell type specific responses to BMP7 have also been detected in prostate cancer cell lines. However, there are also opposite views since BMP7 has been shown to inhibit bone metastasis formation and progression in mouse models of breast and prostate cancer. The results in this dissertation illustrate the complex nature of BMP7 functions in breast cancer that are highly context dependent. The challenge is now to find this context that brings the worst out of BMP7. Evidently intimate knowledge behind the pleiotropy is needed before BMP7 can be fully exploited in breast cancer. Eventually the naturally occurring BMP inhibitors outside as well as inside the cell provide a plethora of potential therapeutical intervention points and thus might also allow the fine-tuning of therapy options in breast cancer (reviewed by Tsuchida et al. 2006, Gazzerro and Minetti 2007).

Acknowledgements

This study was carried out in the Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital during the years 2004-2007. Professor Olli Silvennoinen, M.D., Ph.D., the Head of the Institute of Medical Technology, and Docent Erkki Seppälä, M.D., Ph.D., Head of the Department of Clinical Chemistry, are acknowledged for providing the excellent research facilities. The former and present Heads of Tampere Graduate School in Biomedicine and Biotechnology, Professor Kalle Saksela, M.D., Ph.D., and Professor Anne Kallioniemi M.D., Ph.D., are sincerely thanked for providing me with the graduate school position that allowed me to carry out this work.

I wish to express my deepest gratitude to my brilliant supervisors. Professor Anne Kallioniemi M.D., Ph.D., gave me an opportunity to enter the fascinating world of science and her proficient guidance has taken me through these years to the point of the public defense of my dissertation. I feel truly priviledged to have worked under her caring and skillful supervision. I am likewise grateful for the guidance, encouragement, and positive attitude of Docent Ritva Karhu Ph.D., during this time. It has been a great pleasure to work with both of you.

I wish to thank my dissertation committee members Professor Olli Silvennoinen, M.D., Ph.D., and Docent Outi Monni Ph.D., for their help and valuable comments on this dissertation.

The official reviewers Professor Päivi Peltomäki, M.D., Ph.D., and Professor Johanna Ivaska Ph.D., are gratefully thanked for the evaluation of my manuscript and their helpful comments thereof. I wish to also thank Ms. Virginia Mattila, M.A., for her careful revision of the language of my manuscript.

I am very grateful to my co-authors for their professional help and invaluable contribution to this study. Jenita Pärssinen, Ph.D., is thanked especially for her wide participation in this study and Päivikki Hemmilä, Ph.D., is thanked for her practical guidance in the early steps of my dissertation. Tuula Kuukasjärvi, M.D., Ph.D., Professor Kaija Holli, M.D., Ph.D., Tarja Korhonen, M.D., have provided their expertise and knowledge of the clinical aspects for which I am very grateful. The statistical knowhow of Heini Huhtala, M.Sc., is greatly appreciated.

I express my sincere gratitude to Ms. Kati Rouhento for her skillful assistance in the laboratory work and her indispensable contribution to the completion of this thesis. The skillful techical assistance of Ms. Reija Randen and Ms. Jaana Leppikangas is also warmly acknowledged.

The past and present members of Anne's lab, Jenita, Päivikki, Ritva, Kati, Maarit, Riina, Eeva, Kimmo, Johanna, and Maria are cordially thanked for their help in and out of the laboratory. The laughter in lab meetings and the not-so-scientific discussions have made our lab a pleasant place to work. Jenita is warmly thanked for sharing this dissertation path with me, and her friendship has been important also during less dazzling days.

I wish to heartily thank my family and friends for their support and interest they took in my thesis. I am very grateful to my dear mother Raija for her love, strength, and faith in me. Finally, I wish to thank my darling husband Janne, his immense love and unquestioning support have carried me through the hard times and together we have enjoyed the good ones.

The financial support of the Medical Research Fund of Tampere University Hospital, the Academy of Finland, the Pirkanmaa Cultural Foundation, the Finnish Cancer Organisations, and Finnish Konkordia-liitto is also gratefully acknowledged.

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Original communications