# Interactions between estrogen and bone morphogenetic proteins in breast cancer cells

Master's Thesis

Heidi-Marja Virtanen
BioMediTech (BMT)
University of Tampere

May 9th 2016

# Acknowledgements

This thesis was carried out in the Cancer Genomics research group in BioMediTech, University of Tampere. Firstly, I want to thank the group leader Anne Kallioniemi for giving me the opportunity to do my Master's thesis in her group. She has been, despite her myriad of other occupations, supportive and advisable since the beginning. I'm also grateful to Emma-Leena Alarmo, who guided me through my laboratory studies, and helped me in many things.

I want to thank Minna Ampuja for her advice, and overall, for being such a positive influence in our office. Thanks to Kati Rouhento for guiding me with the practical work. I also thank everyone else in our group.

Thanks to my fellow students for your friendship and the good memories. I also want to thank my family for all their support. You mean everything to me.

Tampere, May 2016

Heidi-Marja Virtanen

# PRO GRADU -TUTKIELMA

Paikka: TAMPEREEN YLIOPISTO,
BioMediTech (BMT)
Tekijä: VIRTANEN, HEIDI-MARJA ANNELI
Otsikko: Estrogeenin ja BMP:iden interaktiot rintasyöpäsolulinjoissa
Sivumäärä: 48 sivua
Ohjaajat: Professori Anne Kallioniemi ja FT Emma-Leena Alarmo
Tarkastajat: Apulaisprofessori Heli Skottman ja Professori Anne Kallioniemi
Päiväys: Toukokuu 2016

---

## Tiivistelmä

**Tutkimuksen tausta ja tavoitteet:** Rintasyövän riskitekijöinä ovat usein erinäiset hormonaaliset tekijät, ja erityisesti estrogeenilla on todettu olevan edistävä vaikutus rintasyöpäkasvaimen syntyyn ja kasvuun. Luun morfogeneettiset proteiinit (BMP) ovat ryhmä kasvutekijöitä, jotka on liitetty erilaisiin syöpiin, kuten rintasyöpään. BMP:illä on havaittu olevan erilaisia vaikutuksia rintasyöpäsoluihin, vähentäen joidenkin kasvua, ja puolestaan lisäten joidenkin liikkumista ja levittäytymistä. Estrogeeni- ja BMP-signalointireittien on todettu olevan yhteydessä toisiinsa, ja ne voivat vaikuttaa toistensa toimintaan. Tässä tutkimuksessa tavoitteena on selvittää estrogeenin vaikutusta BMP:iden geenieksprессioon kuudessa eri rintasyöpäsolulinjassa, sekä tutkia estrogeenin ja BMP4:n vaikutusta solukasvuun kahdessa rintasyöpäsolulinjassa.

**Tutkimusmenetelmät:** Rintasyöpäsolulinjoja (estrogeenireseptoripositiiviset BT-474, MCF-7, MDA-MB-361, T-47D ja ZR-75–30, sekä estrogeenireseptorinegatiivinen MDA-MB-231) kasvatettiin estrogeenivapaissa olosuhteissa kolmen päivän ajan, jonka jälkeen ne käsiteltiin 17β-estradiolilla (E2, 100 nM) tai vehikkelikontrollilla. *BMP4*:n ja *BMP7*:n, sekä positiivikontrolligeenien *GREB1* ja *TFF1* ilmenemistasot määritettiin 24h ja 48h altistuksen jälkeen qRT-PCR:lla käyttäen SYBR Green I -väriin pohjautuvaa menetelmää. Solukasvun tutkimista varten BT-474 ja T-47D-soluja kasvatettiin kolmen päivän ajan estrogeenivapaassa mediumissa, jonka jälkeen ne käsiteltiin E2:lla (100 nM), ihmisen rekombinantti BMP4:llä (100 ng/ml), molemmilla näistä, tai vehikkelikontrollilla. Kasvua seurattiin mikroskopoimalla ja kuvaamalla soluja, lisäksi solumäärä laskettiin kuusi (T-47D) tai seitsemän (BT-474) päivää altistuksien jälkeen.

**Tutkimustulokset:** Positiivikontrolligeenien *GREB1* ja *TFF1* ekspressio lisääntyi odotetusti suurimmassa osassa estrogeenireseptoripositiivisia solulinjoja estrogeenikäsittelyn jälkeen. E2-käsittely vaikutti eri solulinjojen *BMP4*- ja *BMP7*-ekspressioon eri tavoin. *BMP4*:n ekspressio laski merkittävästi BT-474-, MCF-7- ja MDA-MB-361-solulinjoissa, mutta muissa ei näkynyt merkittävää muutosta E2-käsittelyn jälkeen. *BMP7*-ekspressio laski BT-474-, T-47D- ja ZR-75-30-solulinjoissa, muissa ei jälleen havaittu merkittävää muutosta. Solukasvueksperimentissä estrogeeni lisäsi odotusten mukaisesti solukasvua sekä BT-474- että T-47D-solulinjassa. E2- ja BMP4-käsiteltyjen solujen kasvu oli huomattavasti vähäisempää kuin pelkällä estrogeenilla käsiteltyjen solujen.

**Johtopäätökset:** Estrogeeni vaikuttaa eri tavoin *BMP4*- ja *BMP7*-ekspressioon riippuen solulinjasta. Osassa solulinjoja se laskee ekspressiota, ja osan ekspressioon se ei vaikuta merkittävästi. BMP4 kykeni vähentämään estrogeenin aiheuttamaa rintasyöpäsolujen kasvun kiihtymistä. Tämä löydös on erittäin mielenkiintoinen, ja sillä saattaa olla kliinistä merkitystä rintasyöpäpotilaiden hoidossa.

# MASTER'S THESIS

Place: UNIVERSITY OF TAMPERE
BioMediTech (BMT)
Author: VIRTANEN, HEIDI-MARJA ANNELI
Title: Estrogen and BMP interaction in breast cancer cell lines
Pages: 48 pages
Supervisors: Prof. Anne Kallioniemi and Dr. Emma-Leena Alarmo
Reviewers: Associate Prof. Heli Skottman and Prof. Anne Kallioniemi
Date: May 2016

---

## Abstract

**Background and aims:** Breast cancer is the second most common cancer in the world after lung cancer, and the most common cancer among women. The risk factors for breast cancer include different hormonal factors and consequently, estrogen has been found to promote breast cancer pathogenesis. Bone morphogenetic proteins (BMP) are a group of growth factors that have been connected to different cancers, including breast cancer. BMPs have been noted to have different effects on breast cancer cells, decreasing proliferation in some, and in contrast, increasing migration and invasion in others. The estrogen and BMP signaling pathways have been shown to have a connection, and they can influence each other's functions. In this study, the objectives are to examine the effect of estrogen on BMP gene expression in six breast cancer cell lines, and to study the effects of estrogen and BMP4 on cell proliferation in two breast cancer cell lines.

**Methods:** The breast cancer cell lines (estrogen receptor positive BT-474, MCF-7, MDA-MB-361, T-47D and ZR-75-30, and the estrogen receptor negative MDA-MB-231) were cultured in estrogen free medium for three days before treatment with 17β-estradiol (E2, 100 nM) or vehicle control for 24 and 48 hours. The gene expression of BMP4 and BMP7, and the positive control genes GREB1 and TFF1 was assessed with qRT-PCR by using a SYBR Green I dye based assay. For examining the cell proliferation, BT-474 and T-47D cells were cultured for three days in estrogen free conditions, and treated with E2 (100 nM), human recombinant BMP4 (100 ng/ml), both, or vehicle control. The cell proliferation was followed by microscopy and imaging, and finally by counting the cells at six (T-47D) and seven (BT-474) days after the treatments.

**Results:** The expression of positive control genes *GREB1* and *TFF1* increased after estrogen treatment in most estrogen receptor positive cell lines, as expected. Treatment with E2 had a variable effect on *BMP4* and *BMP7* expression depending on the cell line. *BMP4* expression was notably decreased in BT-474, MCF-7 and MDA-MB-361 cell lines, but not in others. The expression of *BMP7* was notably decreased in BT-474, T-47D and ZR-75-30, but again, there was no significant change in the others. The cell proliferation experiment showed that as expected, E2 increased cell growth both in BT-474 and T-47D cell line, and that BMP4 was able to inhibit the effect of E2 on cell growth.

**Conclusions:** Estrogen has different effects on *BMP4* and *BMP7* expression depending on the cell line. In some it decreases BMP expression, and in others it has no significant effect. BMP4 was able to diminish the estrogen induced cell proliferation. This is an important finding that may have clinical applicability for the treatment of breast cancer patients.

# Contents

# Abbreviations

| | |
|---|---|
| ACVR | Activin receptor |
| BMP | Bone morphogenetic protein |
| BMPR | Bone morphogenetic protein receptor |
| BRAM1 | BMP receptor associated molecule 1 |
| Co-SMAD | Common mediator SMAD |
| CYP450 | Cytochrome P450 |
| DCIS | Ductal carcinoma in situ |
| E1 | Estrone |
| E2 | 17β-estradiol |
| E3 | Estriol |
| EMT | Epithelial-mesenchymal transition |
| ERα/β | Estrogen receptor α/β |
| ERE | Estrogen response element |
| ERK | Extracellular signal-related kinase |
| HER2 | Human epidermal growth factor receptor 2 |
| I-SMAD | Inhibitory SMAD |
| MAPK | Mitogen-activated protein kinase |
| PR | Progesterone receptor |
| R-SMAD | Regulatory SMAD |
| SERM | Selective estrogen receptor modulator |
| SMAD | Sma- and Mad-related protein |
| SMURF | SMAD ubiquitin regulatory factor |
| TAK1 | TGF-b activated kinase 1 |
| TGF-β | Transforming growth factor beta |
| XIAP | X-linked inhibitor of apoptosis protein |

# 1. Introduction

Breast cancer is the second most common cancer in the world after lung cancer, and the most common in women with 1.67 million new cases in 2012 (Ferlay *et al.* 2015). In Finland, breast cancer is the most common cancer with 5000 new cases per year in 2014, around 15 % of all cancer cases (Finnish Cancer Registry 2016). Less than 1% of breast cancers occur in men (Korde *et al.* 2009). Breast cancer is only the fifth cause of death from cancer worldwide, and more women survive breast cancer in more developed countries, where lung cancer is now number one cause of cancer death in women (Ferlay *et al.* 2015). There is large variation in breast cancer mortality: in high income countries, breast cancer survival rates are much higher (80% 5-year-survival) than in low income countries (below 40% 5-year-survival) (Shah *et al.* 2014). In Finland, due to its high prevalence, breast cancer still remains first cause of death from cancer in women (Finnish Cancer Registry 2016).

Breast cancer risks include various hormonal factors such as early menarche, late menopause, nulliparity or late first birth, all of which increase the lifetime exposure to estrogen (Santen *et al.* 2009). The effects of estrogen are mediated through the estrogen receptors ERα and ERβ, and the ER status of a breast cancer patient is a notable prognostic indicator (Ye *et al.* 2009).

BMPs are extracellular signaling molecules belonging to the TGF-β superfamily that can control e.g. proliferation, differentiation, migration and apoptosis by regulating gene transcription (Alarmo and Kallioniemi 2010). BMPs are known to have a role in breast cancer, functioning either as pro-metastatic agents, promoting migration and invasion or as anti-metastatic, growth inhibiting factors (Ehata *et al.* 2013).

Functional cross-talk between estrogen and BMPs has been detected in some studies. BMPs have been reported to have inhibitory effects on breast cancer cell proliferation induced by estrogen, and estrogen has been found to be able to alter the SMAD signaling of BMPs by downregulating BMP receptor expression (Takahashi *et al.* 2008). Estrogen and BMPs may also have an effect on each other's function via interactions between their receptor and downstream signaling (Ye *et al.* 2009). However, the interplay between estrogen and BMPs has been studied in very few cell lines, and information on the subject is still scarce. In this study, the effect of estrogen on BMP4 and BMP7 gene expression, and the effect of estrogen and BMP4 treatment on cell proliferation were assessed. The gene expression studies were conducted with five estrogen receptor positive breast cancer cell lines, and an estrogen receptor negative control breast cancer cell line. Two cell lines were treated

with estrogen and BMP4 in order to analyze their effects on cell proliferation with both cell counting and microscopy.

# 2. Literature Review

## 2.1. Breast cancer

Cancer is the result of a combination of many factors, such as inherited mutations or polymorphism of cancer susceptibility genes, environmental agents influencing somatic genetic changes and several other systemic and local factors (Polyak 2001). About 5-10% of breast cancers are hereditary, thus the affected person has inherited a gene mutation exposing them to breast cancer (http://www.cancer.gov/types/breast/patient/breast-treatment-pdq, accessed 9.3.2016). BRCA1 and BRCA2 are well-known genes with mutations that increase breast and ovarian cancer risk, and possibly the risk of other cancers, too (http://www.cancer.gov/types/breast/patient/breast-treatment-pdq, accessed 9.3.2016). In addition to mutations in high-penetrance cancer susceptibility genes BRCA1, BRCA2, and TP53, several moderate- and low-penetrance allele variants and SNPs in FGFR2 (fibroblast growth factor receptor 2), TNRC9 (thymocyte selection-associated high mobility group box 9), MAP3K1 (mitogen-activated kinase kinase kinase 1), LSP1 (lymphocyte specific protein), CASP8 (caspase 8), and TGF-β1 have been noted to be associated with breast cancer risk (Polyak 2007).

Inherited gene mutations are risk factors only in a small portion of breast cancers. Most cases are sporadic, occurring due to exposure to risk factors. Breast cancer risk is greater when breast tissue is prolongedly exposed to estrogen because of early menarche, nulliparity or older age at first birth, late menopause, or hormone replacement therapy for symptoms of menopause (Shah *et al.* 2014). Other risk factors include older age, family history of breast cancer, radiation therapy to chest or breast area, alcohol consumption, and obesity (http://www.cancer.gov/types/breast/patient/breast-treatment-pdq, accessed 9.3.2016, (Shah *et al.* 2014). The molecular basis of breast cancers not caused by inherited genetic changes is still quite poorly characterized (Polyak 2001).

Breasts consist of glandular, fibrous, and adipose tissue, and the glandular tissue contains 15 to 20 lobes (Figure 1) (Springhouse 2002). Within the lobes there are clustered acini: small, saclike duct terminals that secrete milk when lactating (Springhouse 2002). Lactiferous ducts and sinuses drain the lobules, and store the milk during lactation (Springhouse 2002). Ductal carcinoma, which originates in the ductal cells, is the most common breast cancer type (http://www.cancer.gov/types/breast/patient/breast-treatment-pdq, accessed 9.3.2016). Other breast

cancer types include lobular carcinoma, and the uncommon inflammatory breast cancer (http://www.cancer.gov/types/breast/patient/breast-treatment-pdq, accessed 9.3.2016).

In addition to histology, other factors are also assessed in diagnostics to help determine the prognosis and treatment options of a patient. The estrogen receptor (ER) and progesterone receptor (PR) status of a breast cancer patient is determined to predict response to endocrine therapy (Shah *et al.* 2014). HER2 (human epidermal growth factor receptor 2) status is a prognostic factor, and it can be used to predict response to therapies targeting the HER-2/neu receptor, such as trastuzumab (Shah *et al.* 2014). Gene expression profiles are used nowadays to classify breast cancers to luminal A ($ER\alpha^+$, $PR^+$, low proliferation rate), luminal B ($ER\alpha^-$, $PR^+$, high proliferation rate), HER2-overexpressing, and triple-negative carcinoma (shortened as TNC; $ER\alpha^-$, $PR^-$, $HER2^-$) (Huang *et al.* 2015). Polyak (2007) divides the molecular subtypes in five different groups: basal-like, luminal A, luminal B, $HER2^+/ER^-$, and normal-breast-like. The different subtypes have different clinical outcomes and treatment responses as a result of the molecular differences (Polyak 2007).

The different subtypes are seen already in premalignant DCIS (ductal carcinoma in situ) stage, and they are conserved in different ethnic groups, which indicates that each tumor type has a specific tumor progression pathway (Polyak 2007). This leads to the possible explanation that a distinct cell

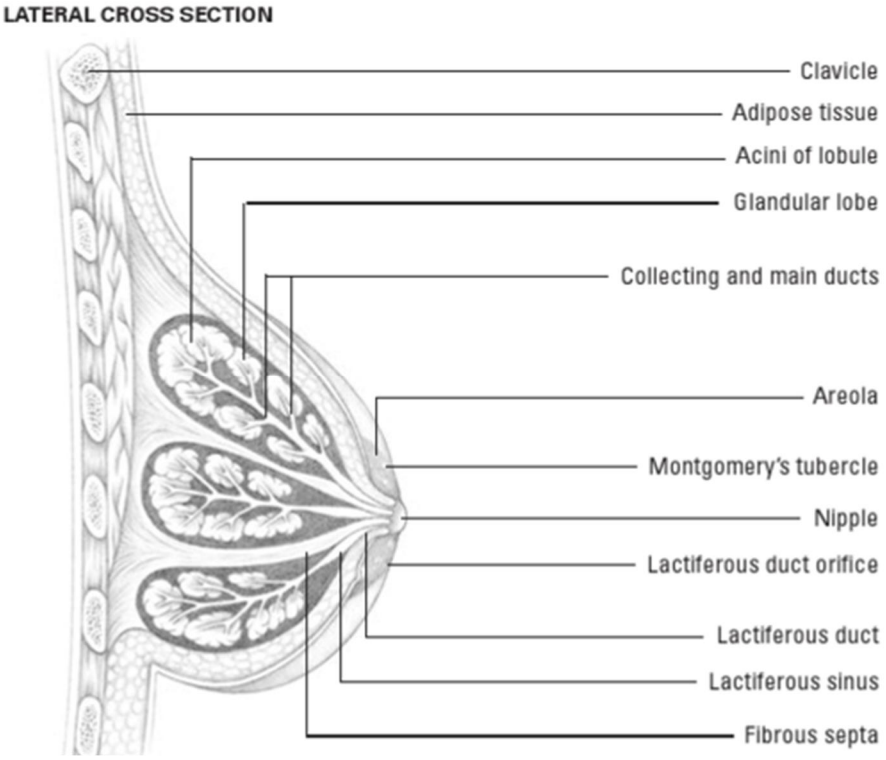



**Figure 1**: Anatomy of the female breast. Image acquired from Springhouse (2002)

of origin (for example cancer stem cells) and tumor subtype-specific genetic and epigenetic changes are either both or the other responsible for the tumor heterogeneity (Polyak 2007).

## 2.2. Estrogen and breast cancer

Since the notion that bilateral oophorectomy resulted in breast cancer remission in premenopausal women over a 100 years ago, the connection between breast cancer and estrogen has been recognized (Clemons and Goss 2001). Both endogenous and exogenous estrogens have been associated with breast cancer pathogenesis (Clemons and Goss 2001). Early menarche is a risk for both pre- and postmenopausal women, and it has been shown that women who had early menarche (≤ 13 years), had an almost twofold increase in risk of developing hormone receptor positive tumors (Shah *et al.* 2014). Overall, high endogenous sex hormone levels increase breast cancer risk (Shah *et al.* 2014). Postmenopausal women with breast cancer have been shown to be exposed to more endogenous estrogen than women without breast cancer (Feigelson and Henderson 1996). Combined estrogen and progesterone hormone replacement therapy (HRT) for menopause symptoms is a greater risk than oral contraceptives, since HRT is used at an older age when breast cancer risk is higher (Clemons and Goss 2001; ESHRE Capri Workshop Group 2004). Breastfeeding has been noted to have a protective effect (ESHRE Capri Workshop Group 2004; Feigelson and Henderson 1996; Shah *et al.* 2014). In postmenopausal women, obesity increases breast cancer risk while obese premenopausal women are surprisingly at a smaller risk of breast cancer (Clemons and Goss 2001). This is due to the effects of excessive adipose tissue in premenopausal women: obese women often have longer menstrual cycles and more anovulatory cycles, and are thus exposed to less estrogen in total (Clemons and Goss 2001). In contrast, more estrogen is produced by aromatase activity in the excessive adipose tissue in postmenopausal women, and there is a lower serum concentration of the sex-hormone binding globulin, which is why they have higher bioavailable serum estrogen levels (Clemons and Goss 2001).

### 2.2.1. Normal functions of estrogen in the body

Estrogen is the primary hormone associated with the female sex: it determines the development and regulation of female reproductive system and secondary sex characteristics. Ovaries, controlled by the cyclically produced gonadotropins, produce most of the serum estrogen in premenopausal women as seen in Figure 2 (Clemons and Goss 2001). In postmenopausal women, however, aromatization of adrenal and ovarian androgens produces small amounts of estrogen in other tissues than the gonads, e.g. liver, muscle, and fat tissue (Figure 2) (Clemons and Goss 2001). Estrogens are synthesized from

androgens by the catalyzing action of the cytochrome P450 (CYP450) aromatase enzyme, also called CYP19, and it has been speculated that its genetic variations may be associated with increased breast cancer risk (Clemons and Goss 2001; Tsuchiya *et al.* 2005). The 17ɓ-hydroxysteroid dehydrogenase (17ɓ-HSD) converts estrone (E1) to potent estradiol (E2) (Tsuchiya *et al.* 2005).

Estrogens play a role in several different tissue types and affect both female and male physiology (Diel 2002). Generally, tissues with reproductive functions, such as mammary glands, ovaries, vagina and uterus, where estrogen stimulates cell proliferation and the biosynthesis of progesterone receptors (PR), have the highest numbers of estrogen receptors (ER) (Diel 2002). However, estrogen also acts in non-classical target tissues i.e. brain, bones, cardiovascular system, kidneys, immune system and liver (Diel 2002). Development of secondary sexual characteristics, regulation of gonadotropin secretion for ovulation, maintenance of bone mass, regulation of lipoprotein synthesis and insulin responsiveness, and maintenance of cognitive function are examples of estrogen's functions in the female body (Nelson and Bulun 2001). The development of mammary glands starts during embryogenesis, but only after the onset of puberty does the initiation of the ovarian steroid hormone production induce the outgrowth of the ducts (Hynes and Watson 2010). The repeated estrous cycles cause the ductal network to grow increasingly complex (Hynes and Watson 2010). Functionally,

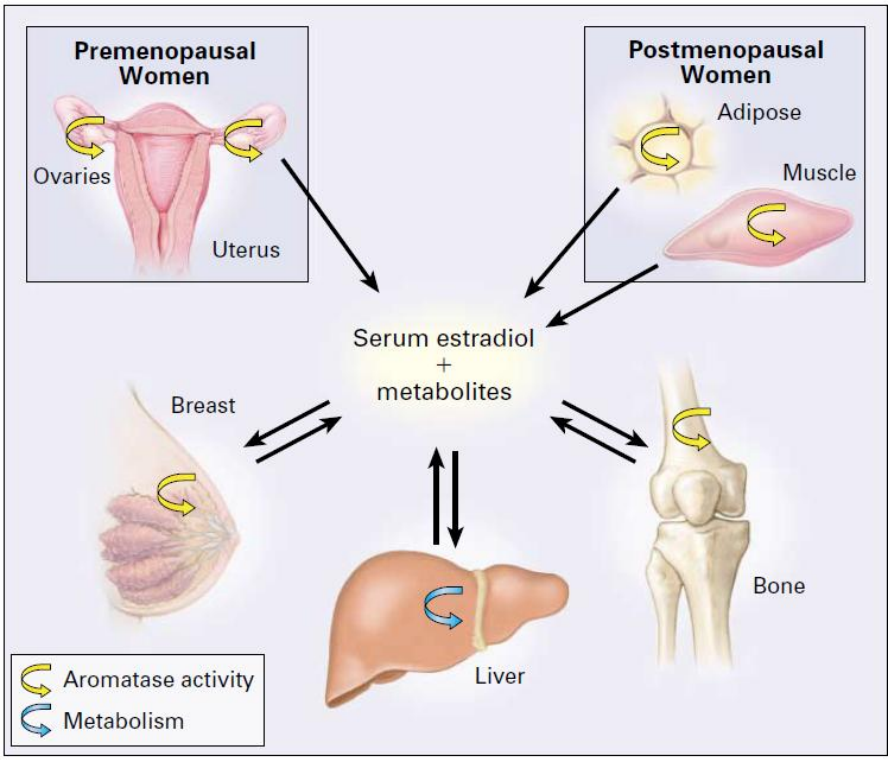



**Figure 2**: Effects of whole-body and locally synthesized estrogen on multiple organs. Arrows indicate sites where androgen is converted to estrogen. Figure acquired from Clemons and Goss (2001).

breasts mature during pregnancy, when the milk-producing structures are formed (Hynes and Watson 2010).

Estradiol (E2) is a steroid hormone, and the primary of the three major naturally occurring estrogens, estrone (E1) and estriol (E3) being the other two. E2 applies its effect after binding to estrogen receptors α (ERα) and ERβ, after which the ER dimerizes and binds to nuclear estrogen response elements (EREs), initiating transcription, or tethering to other transcription factors, such as AP1 (activation protein 1) and SP1 (specificity protein 1) (Figure 3) (Band and Laiho 2011; Simpson and Santen 2015). ERα regulates gene transcription via formation of a multiprotein complex, which contains general transcription factors, co-activator, co-repressors, co-integrators, histone acetyltransferases (HAT) and histone deacetylases (HDAC), providing tissue- and context-specific activation of ERα signaling (Band and Laiho 2011). Their activity not only limited to the nucleus, ERs in the plasma membrane and mitochondria also have a role in cell signaling (Simpson and Santen 2015). Contrary to the classic model of estrogen response, non-genomic estrogen activation pathways with rapid E2 effects have been described (Figure 3) (Hewitt *et al.* 2016; Simpson and Santen 2015). The membrane-associated mediators of these events are not yet fully known, but GPER (G protein coupled ER) is suspected to be a potential mediator of the rapid membrane localized hormone

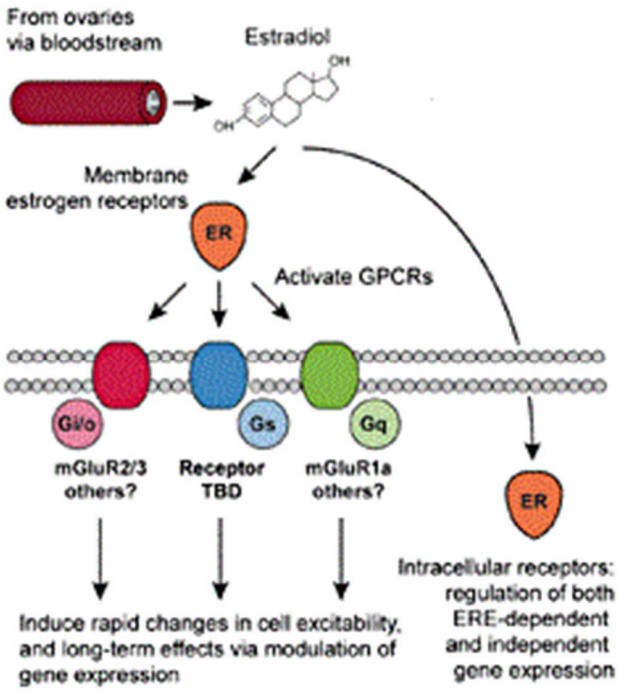



**Figure 3**: Classical and non-classical mechanisms of estrogen action. In the classical pathway, estrogen regulates gene transcription via the nuclear ERs by tethering to EREs, initiating transcription. The non-classical routes induce rapid changes in cells, exerted by estrogen. Image acquired and edited from Boulware and Mermelstein (2005)

response (Hewitt *et al.* 2016). However, the rapid estrogen response signaling pathways are complex, and differ between cell types (Simpson and Santen 2015).

The localization and distribution of ERa and ERb varies in pre- and postmenopausal women: ERa is found mostly in the inner layer of epithelial cells that line the acini and intralobular ducts, and in the myoepithelial cells of the external layer of interlobular ducts, while ERb is localized more widely in epithelial and stromal cells in premenopausal women (Huang *et al.* 2015). Less than 10% of normal mammary epithelial cells express ERa, and more than 50% express ERb in postmenopausal women (Huang *et al.* 2015). Corresponding to premenopausal women, ERb is expressed in stromal cells in postmenopausal women, but not ERa (Huang *et al.* 2015).

### 2.2.2. Effects of estrogen on breast cancer development and growth

Estrogen and ER signaling is important both in normal mammary gland development and breast carcinogenesis. Experimental data strongly indicates that estrogens have a role in the development and growth of breast cancer (Clemons and Goss 2001). ERα is responsible for the proliferative effect of estrogen, and recent studies have shown that as normal breast tissue transitions to DCIS, the number of ERα-positive cells increases, whereas ERβ-positive cell number decreases (Huang *et al.* 2015). The correlation between ERα and ERβ status and breast cancer survival outcome has been demonstrated in several studies (Huang *et al.* 2015). ERα is generally regarded as a good indicator for endocrine therapy and breast cancer survival, and its loss indicates invasiveness and poor prognosis (Huang *et al.* 2015).

High levels of estrogen-receptors have been directly correlated with elevated risk of breast cancer (Clemons and Goss 2001). Estrogen-receptor levels are generally low in normal mammary tissue, but they vary between women, usually increasing with age, especially in white women compared to Japanese or black women (Clemons and Goss 2001). The increase of estrogen receptors may be a consequence of down-regulation failure, caused possibly by the loss of a tumor-suppressor gene function (Clemons and Goss 2001).

Santen *et al.* (2009) suggest a combination of two theories of the involvement of estrogen in breast cancer carcinogenesis: (i) estrogens cause proliferation, and the increase in cell divisions exposes the mammary cells to more mutations, which, if unrepaired, enable the cells to become cancerous and (ii) estrogens may be metabolized into genotoxic metabolites, or create reactive oxygen species by redox cycling, which can damage DNA, causing mutations. They also concluded that apparently some of estrogen's effects on breast tumorigenesis occur independently of ER's, which implies that

blocking estradiol metabolite formation without lowering the actual estradiol levels might reduce breast cancer risk (Santen *et al.* 2009). High levels of estrogen metabolite 4-hydroxyestradiol, which generates DNA damaging free radicals from reductive oxidative-cycling, have been detected in benign and malignant mammary tumors (Tsuchiya *et al.* 2005). CYP1B1, catalyzing 4-hydroxylation of estrogens, is mainly expressed in estrogen target tissues, and its expression levels have been noted to be elevated in tumor tissues (Tsuchiya *et al.* 2005). Thus, local formation of 4-hydroxyestradiol by CYP1B1 is implied to play a role in breast carcinogenesis, with CYP1B1 as a notable influence (Tsuchiya *et al.* 2005).

As mentioned, higher serum estrogen levels have been associated with a higher breast cancer risk in postmenopausal women (Clemons and Goss 2001). Breast density, along with bone density are indicators of high serum estrogen levels (Clemons and Goss 2001). Women with denser breast tissue on mammography had higher serum estrogen concentration compared to women with less dense breasts, while hormone replacement therapy was noted to increase breast density in postmenopausal women (Clemons and Goss 2001).

### 2.2.3. Endocrine therapies for breast cancer

Before the discovery of antiestrogens, e.g. the selective estrogen receptor modulator (SERM) tamoxifen, radical operations, such as oophorectomy for premenopausal women, adrenalectomy for postmenopausal, and hypophysectomy for both, were used to treat estrogen-responsive breast cancer (Simpson and Santen 2015). About 70% of breast cancers are ER positive and considered hormone sensitive, and thus are responsive to SERM treatment (Shah *et al.* 2014). SERMs, such as tamoxifen and raloxifene, bind to the ER, and act either as agonists or antagonists in estrogen target tissues (ESHRE Capri Workshop Group 2004). Differences in ER expression, receptor conformation on ligand binding, and the expression of co-regulating proteins are factors that influence the SERM's agonist or antagonist activity in a given target tissue or circumstance (ESHRE Capri Workshop Group 2004).

Tamoxifen is the most used SERM in breast cancer treatment, and tamoxifen and raloxifene are both used in breast cancer prevention (ESHRE Capri Workshop Group 2004). Both drugs interfere with ER binding to DNA structures by recruiting co-repressors interacting with the ERs at the EREs in target genes, and thus exert anti-estrogenic activity (ESHRE Capri Workshop Group 2004). Tamoxifen and raloxifene use as preventative measure reduces breast cancer incidence by significantly reducing ER-positive breast cancers (Chlebowski 2014). Tamoxifen better reduces invasive breast cancers than raloxifene, and only tamoxifen reduces DCIS (Chlebowski 2014).

Aromatase inhibitors, such as anastrozole, have been shown to be potent blockers of estradiol production, and can be used in postmenopausal women, whose estrogen is produced solely by aromatase (Simpson and Santen 2015). In breast cancer prevention perspective, SERMs and aromatase inhibitors have not been directly compared, but it appears, when compared with placebo, that aromatase inhibitors better reduce breast cancer incidence compared to tamoxifen and raloxifene (Chlebowski 2014).

## 2.3. Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are a group of extracellular signaling molecules that belong to the TGF-b superfamily (Alarmo and Kallioniemi 2010; Carreira *et al.* 2014). BMPs have mitogenic, differentiating, chemotactic, and osteolytic activities, and they mediate cell proliferation and mesenchymal stem cell (MSC) differentiation (Carreira *et al.* 2014). Although originally identified by their bone forming abilities at ectopic sites, BMPs are now known to play a critical part in different developmental phases (Alarmo and Kallioniemi 2010; Wang *et al.* 2014). In many cancer types, different BMP ligands appear to be aberrantly expressed (Ehata *et al.* 2013). BMPs influence different types of cancer cells, regulating their proliferation and invasiveness, and also affect tumor microenvironments and regulate tumor angiogenesis (Ehata *et al.* 2013).

BMPs constitute the largest group in the TGF-b superfamily, and around 20 BMP family members have been characterized and divided into subgroups depending on their known functions and similarity in sequences (Bragdon *et al.* 2011; Carreira *et al.* 2014). Not all BMPs are truly osteogenic, for example BMP1 is in reality metalloprotease, and thus does not belong to the TGF-b superfamily (Carreira *et al.* 2014). Some BMPs are also called growth and differentiation factors (GDF) (Alarmo and Kallioniemi 2010). The known members of the BMP family include BMP2-7, BMP8A/B, BMP10, BMP15, GDF1-3, GDF5-7, myostatin, GDF9-11 and GDF15 (Alarmo and Kallioniemi 2010). BMPs are dimeric molecules composed of two disulfide bond -linked identical monomers that include seven conserved cysteine residues, six of which are highly conserved, forming a “cysteine knot” motif linked by three intramolecular disulfide bonds (Alarmo and Kallioniemi 2010; Carreira *et al.* 2014).

BMPs are synthesized as large, inactive precursors, containing a signal peptide guiding the protein to the secretory pathway, a pro-domain mediating proper folding, and a C-terminal mature peptide (Alarmo and Kallioniemi 2010; Bragdon *et al.* 2011; Carreira *et al.* 2014). The pro-domain is proteolytically cleaved at the consensus site Arg-X-X-Arg to generate the mature and active

homodimers or heterodimers of BMPs (Bragdon *et al.* 2011; Carreira *et al.* 2014). The pro-domain is also thought to regulate BMP activity and availability, because it may remain non-covalently bonded to the mature dimeric BMP secreted out of the cell (Alarmo and Kallioniemi 2010; Bragdon *et al.* 2011). BMPs are active both as homodimers and heterodimers, but BMP heterodimer's, such as BMP4/7, BMP2/6 and BMP2/7, increased potency compared to heterodimers has been shown is some studies (Bragdon *et al.* 2011; Carreira *et al.* 2014)

BMPs have a unique ability to initiate bone formation (Alarmo and Kallioniemi 2010). Osteoprogenitor cells, osteoblasts, chondrocytes, platelets and endothelial cells produce BMPs in bones (Carreira *et al.* 2014). BMPs stimulate the differentiation of mesenchymal cells to chondroblasts and osteoblasts, and the subsequent new bone construction during embryogenesis and also during bone repair processes in adult tissues (Alarmo and Kallioniemi 2010; Carreira *et al.* 2014). Possibly the most important BMP function is their role in in different developmental phases, from regulation of the primal stages of embryogenesis, mesoderm formation, cardiac development, formation of the left-right asymmetry, neural and skeletal patterning and limb formation to organogenesis (Alarmo and Kallioniemi 2010; Wang *et al.* 2014). BMPs act in connection with other growth factors, in a complex cell signaling system (Carreira *et al.* 2014)

### 2.3.1. BMP signaling

BMPs and other members of the TGF-b family share a common signaling pathway, in addition to their common structure (Alarmo and Kallioniemi 2010; Brazil *et al.* 2015). BMPs activate SMAD-dependent and various SMAD-independent signaling pathways, directly affecting gene transcription (Bragdon *et al.* 2011).

The BMP receptor complex includes the BMPRI and BMPRII serine-threonine kinase receptors, containing an N-terminal extracellular ligand binding domain, a transmembrane domain, and an intracellular region (Carreira *et al.* 2014). BMP ligands bind to BMP type I receptors (BMPRI) or to activin-like kinase (ALK) 2, 3, or 6 (Figure 4) (Brazil *et al.* 2015). The complex then binds to the BMP type II receptor (BMPRII), phosphorylating the type I receptor, which in turn activates, and phosphorylates a set of SMADs, called the receptor SMADs (R-SMAD1/5/8), which bind to SMAD4, the nuclear common SMAD (co-SMAD) shared by the TGF-b/activin/nodal and BMP signaling pathways (Brazil *et al.* 2015; Katsuno *et al.* 2008). R-SMADs form complexes with the co-SMAD, and these active SMAD complexes regulate transcription of target genes in the nucleus (Alarmo and Kallioniemi 2010). SMAD-response elements are present in BMP target genes like inhibitor or

differentiation (Id1-3) genes, SnoN, and inhibitory SMAD6, which convey many of the downstream effects of BMP signaling (Brazil *et al.* 2015).

In addition to the SMAD pathway, other, non-SMAD, intracellular pathways are activated in response to BMP ligands (Figure 4) (Alarmo and Kallioniemi 2010). These include the MAP kinases (MAPK) ERK, JNK and p38, phosphatidyl inositol-3 kinase (PI3K/AKT) and small GTPase pathways, which cooperate with SMAD signaling pathways and regulate various cellular responses in target cells (Ehata *et al.* 2013). It has been suggested that the pathway activation is accomplished via the protein-protein interactions of BRAM1 (bone morphogenetic protein receptor associated molecule 1) or XIAP (X-linked inhibitor of apoptosis protein), and downstream signaling molecules TAK1 (TGF-b-activated kinase 1) and TAB1 (TAK1 binding protein) with BMPR-IA (Bragdon *et al.* 2011).

BMP signaling is widely regulated on multiple levels in cells, including intracellularly by inhibitory SMADs 6 and 7 (I-SMAD), SMURF ubiquitin ligases, miRNAs, methylation, and also extracellularly by pseudoreceptors such as BMP and BAMBI (activin membrane bound inhibitor) and BMP

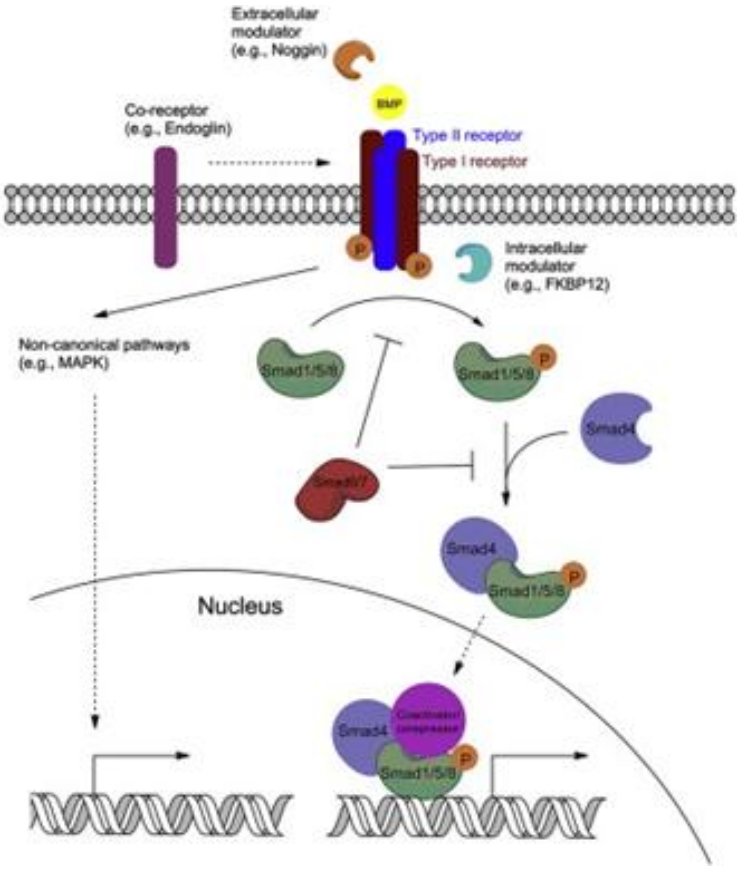



**Figure 4**: BMP signaling via the canonical, SMAD-dependent pathway or non-canonical pathways. In the canonical pathway, as BMP ligand binds the BMPRI, they bind to BMPRII, phosphorylating BMPRI, recruiting R-SMADs, and thus activating the SMAD pathway and regulating gene transcription. The non-canonical pathways include for example the MAPK pathway. Image acquired from Wang *et al.* (2014)

antagonists, including Grem1 (Alarmo and Kallioniemi 2010; Brazil *et al.* 2015). Extracellular or intracellular regulators, acting as antagonists of the BMP signaling pathway, may bind to the receptors or sequester BMP ligands, blocking signal transduction (Carreira *et al.* 2014). There are also some membrane-bound peptides that are able to enhance BMP signaling, including membrane-anchored proteins RGMa (repulsive guidance molecule) and DRAGON (RGMb) (Alarmo and Kallioniemi 2010).

### 2.3.2. BMPs in cancer

BMPs are members of the TGF-b superfamily, and currently, TGF-b is widely known to exhibit bi-directional functions in cancer progression (Band and Laiho 2011; Ehata *et al.* 2013). It has tumor suppressor properties, such as inhibiting the growth of various epithelial cells, endothelial cells, hematopoietic cells and immune cells, and inducing apoptosis of different epithelial cells (Ehata *et al.* 2013). Disturbance in TGF-b signaling leads to the development of certain types of cancer, e.g. colorectal and pancreatic cancer (Ehata *et al.* 2013). On the other hand, at later stages of carcinogenesis when cancer cells have developed the ability to bypass the tumor suppressive functions of TGF-b, they may use it for tumor progression (Band and Laiho 2011; Massagué and Gomis 2006). Breast cancer and glioblastoma cells often lose TGFb cytostatic responses, but the TGF-b receptors remain functional and SMADs active, leading to the utilization of the TGF-b system to induce gene responses promoting tumor growth, invasion, evasion of immune surveillance and metastasis (Massagué and Gomis 2006). TGF-b is also a potent inducer of epithelial-mesenchymal transition (EMT), contributing to tumor invasion (Band and Laiho 2011; Massagué 2008).

BMPs have been reported to regulate the progression of several different types of cancer, but unlike TGF-b, which is known to have bi-directional functions in cancer progression, the roles of BMPs in cancer are not yet fully acknowledged (Ehata *et al.* 2013). The effects of BMPs in cancer cells and tumor microenvironments are divergent: on the other hand, BMPs inhibit the proliferation of cancer cells, with some exceptions, and induce the differentiation of certain cancer stem cells, attenuating their aggressiveness, but they also play a critical role in tumor angiogenesis regulation and cancer cell metastasis (Ehata *et al.* 2013). In vitro studies that implicate BMPs as promoting tumorigenesis or metastasis have been reported in non-small cell lung cancer (NSCLC), renal cell carcinoma, medulloblastoma, osteosarcoma, ovarian cancer, and an extensive variety of breast and prostate cancer cell lines (Thawani *et al.* 2010). Other studies with different cell lines, however, show BMP induced inhibition of cell proliferation (Thawani *et al.* 2010).

### 2.3.3. BMPs in breast cancer

Not much is known of the role of BMPs in breast development, but since BMP-specific receptors are expressed in the developing mammary gland, active BMP signaling is probable (Alarmo and Kallioniemi 2010). Different BMP ligands have been observed to be aberrantly expressed in breast cancer (Alarmo and Kallioniemi 2010). Breast cancer commonly metastasizes to bones, and because of the role of BMPs in bone formation and repair, the significance of BMPs in bone metastasis has been studied considerably (Ye *et al.* 2009). The SMAD-dependent BMP signaling pathway has been shown to induce breast cancer cell invasion and bone metastasis (Katsuno *et al.* 2008).

BMP2, BMP6, and BMP7 are currently best known in breast cancer, and the importance of BMP4 has also been contemplated (Alarmo and Kallioniemi 2010; Kallioniemi 2012). Davies *et al.* (2008) demonstrated that BMP2 and BMP7 were expressed at significantly lower levels in patients with more aggressive breast tumors. BMP2 expression has been indicated in breast cancer cell lines, mainly at low levels (Alarmo and Kallioniemi 2010). BMP2 has been shown to inhibit E2-induced proliferation via the p21 up-regulation, which inhibits the E2-induced cyclin D1 –associated kinase activity (Ye *et al.* 2009). However, BMP2 also has beneficial effects for the cancer cells: increased resistance to hypoxia-induced apoptosis and promotion of motility and invasiveness of breast cancer cells (Ye *et al.* 2009). The expression pattern of BMP6 in breast cancer does not differ much from normal cells, but inactivation of BMP6 by hypermethylation has been implicated in ER-negative breast cancer patients (Alarmo and Kallioniemi 2010; Zhang *et al.* 2007). Downregulation of BMP6 has been shown to enhance cell proliferation and chemoresistance, implicating that BMP6 inhibits growth and migration (Lian *et al.* 2013).

BMP4 expression is increased in a subgroup of breast cancer cell lines and primary tumors (Kallioniemi 2012). The expression of BMP4 has been shown to be elevated in 25% of breast cancer patients, and strong expression was related to low proliferation index and increased tumor recurrence frequency (Alarmo *et al.* 2013). BMP4, like BMP2, shows reduced cancer cell growth after treatment or overexpression (Ampuja *et al.* 2013; Ketolainen *et al.* 2010). The effect of BMP4 on migration and invasion is somewhat controversial, with one study showing reduced (Shon *et al.* 2009), and some others increased migration and invasion after BMP4 treatment (Guo *et al.* 2012; Ketolainen *et al.* 2010). Guo *et al.* (2012) demonstrated that overexpression of BMP4 promoted migration and invasion, and on the other hand, BMP4-expression knockdown resulted in inhibited migration and invasion activity.

BMP7 is widely expressed in breast cancer (Alarmo *et al.* 2007; Alarmo *et al.* 2006). Decreased BMP7 expression has been suggested to confer a specific bone metastatic potential to human breast cancer cells (Buijs *et al.* 2007). The BMP7 protein expression in primary tumors is associated with accelerated bone metastasis formation, and BMP7 expression is also a prognostic factor for early bone metastasis (Alarmo *et al.* 2008). BMP7 function has been studied in breast cancer cell lines, and in one study, the cell proliferation was stimulated by BMP7 in some cell lines, and inhibited in others (Alarmo *et al.* 2009). Furthermore, exogenous BMP7 increased migration and proliferation in MDA-MB-231 cells (Alarmo *et al.* 2009). Ying *et al.* (2015) demonstrated that BMP7 significantly inhibits the activation of EMT-related genes induced by TGF-b1, which resulted in a significant reduction in TGF-b1 triggered cell growth and metastasis.

## 2.4. Estrogen and BMP interactions

TGF-b and estrogen have extensive interactions with each other (Band and Laiho 2011), and since BMPs and TGF-b share common signaling pathways, the involvement of estrogen with BMPs is apparent. The co-SMAD of both BMP and TGF-β signaling pathways, SMAD4, was found to be a co-repressor of transcription of ERα (Wu *et al.* 2003).

### 2.4.1. Cross-talk between TGF-β and estrogen

TGF-b and estrogen have been indicated to be co-regulated in mammary glands in a study which found that phosphorylated SMADs and nuclear ERα were co-localized (Ewan *et al.* 2005). This suggested that ERα-mediated proliferation could be restricted by TGF-b (Ewan *et al.* 2005). ER activation has been reported to inhibit transcriptional activity of TGF-b reporter assays and TGF-b induced cell migration (Band and Laiho 2011). In a microarray of TGF-b treated MCF-7 breast cancer cells, TGF-b increased the expression of 956 genes more than twofold, and of these genes, estrogen treatment decreased the expression of 683 genes, which suggests ERα is a major modifier of TGF-b signaling cascade (Band and Laiho 2011). Ito *et al.* (2010) showed that ERα enhances the degradation of SMAD proteins and thus inhibits TGF-b induced transcription using an ubiquitin-proteasome pathway. The antiestrogen (tamoxifen and ICI 182.780) induced inhibition of breast cancer cell growth has been reported to be mediated by TGF-b (Band and Laiho 2011).

### 2.4.2. Cross-talk between BMPs and estrogen

Estrogen is known to modulate both BMP ligands and BMP induced responses, and evidence for a possible relationship between ER and BMP or BMPR signaling has been shown in recent studies (Alarmo and Kallioniemi 2010; Ye *et al.* 2009). Estrogen is apparently able to regulate BMP and BMPR expression, and it can repress the expression of some BMP receptors, e.g. BMPRIA, BMPRIB, ACVR2A, and ACVR2B, but has no effect on ACVR1 and BMPRII (Ye *et al.* 2009). The interaction between BMPs and estrogen also seems to work the other way round with BMPs repressing ER action.

Cross-talk between estrogen and BMPs has been studied in several different tissues and environments. Antiestrogens have been found to specifically upregulate BMP4 promoter activity in human osteoblastic cells, and the effect seemed to be bone cell specific and dependent on ERα presence (van den Wijngaard *et al.* 2000). The apoptotic effects of BMP7 on tissue remodeling have been found to be opposed by estrogen in an animal study (Monroe *et al.* 2000). The estrogen metabolite 16a-hydroxyestrone (16αOHE) has been implied to regulate BMPRII protein levels and inducing angiogenesis or vascular injury–related pathways in pulmonary arterial hypertension (Fessel *et al.* 2013). A BMP4-SMAD-ER molecular regulatory mechanism has been identified to regulate the prolactin promoter's transcriptional activity, inducing prolactin secretion in the pituitary gland (Giacomini *et al.* 2009). The interaction of ER and BMP4 may play a role in the prolactinoma formation in the pituitary gland (Giacomini *et al.* 2009).

A study by Helms *et al.* (2005) supported the view that the BMP/SMAD pathway may have a crucial role in ER-positive breast cancer advancement, and that BMP signaling is directly linked to ER expression, or might even be controlled by intact ER signaling. BMP2 has been shown to inhibit E2-induced proliferation of MCF-7 breast cancer cells by inducing the expression of cyclin kinase inhibitor p21 (Ghosh-Choudhury *et al.* 2000). The effect seems to be conveyed by positive cell cycle regulatory protein inhibition (Ghosh-Choudhury *et al.* 2000). A study by Yamamoto *et al.* (2002) was the first to connect the interactions between BMP-regulated SMADs to estrogen, by examining the connection between ERα, SMADs and BMP2. The expression of a novel ERα-36 type ER, a splice variant of the ERα, has been reported to be induced by BMP2, but not the expression of the more common ERα-66 (Wang *et al.* 2012).

BMP6 promoter was found to be activated by ERα in different cell types, but it had tissue-specific actions via antiestrogen stimulation of BMP6 activity in the osteoblast-like cells, and E2 stimulation in breast cancer and hepatoma cells (Ong *et al.* 2004). A study by Zhang *et al.* (2005) supported this

view, and further investigated the molecular mechanisms behind the activation of BMP6 transcription, noting that E2 induction of BMP6 expression happens by ERα binding to an ERE in the BMP6 promoter.

BMPs, by inhibiting MAPK pathways and estrogenic enzyme expression, have been reported to have inhibitory effects on the estrogen-induced mitosis of MCF-7 breast cancer cells (Takahashi *et al.* 2008). BMP2, BMP4, BMP6 and BMP7 were used alone or in combination with E2, and the cell proliferation –inducing effect of E2 was attenuated by all of them, BMP6 and BMP7 more effectively than BMP2 and BMP4 (Takahashi *et al.* 2008). However, the expression of BMP6 and BMP7 was also noted to be reduced by estrogen treatment, indicating that estrogen also alters BMP signaling (Takahashi *et al.* 2008). The fibroblast growth factor 8 (FGF8) has also been connected to BMP and estrogen cross-talk, and it has been indicated to enhance endogenous estrogenic actions and suppress BMP receptor signaling in ER-positive breast cancer cell lines, thus easing cell proliferation (Masuda *et al.* 2011). All in all, the interaction between BMPs and estrogen has been studied quite fractionally, and in most studies, only in MCF-7 breast cancer cell line. Thus, more information on the BMP/estrogen –cross-talk is needed to better understand its background.

# 3. Objectives

Estrogen has a significant role in breast carcinogenesis and progression, and is known to promote breast cancer cell growth. BMPs also take part in breast cancer pathogenesis, and affect e.g. breast cancer cell proliferation and migration. In contrast to estrogen, BMP4 has a breast cancer cell growth reducing effect. Estrogen and BMP signaling have been noted to be interconnected, but information on the cross-talk between them is still insufficient.

The specific aims of this study were:

1) to assess the effect of estradiol (E2) treatment to the expression levels of BMP4 and BMP7 in breast cancer cell lines
2) to examine how the combination of E2 and BMP4 treatments affects the proliferation of breast cancer cells.

# 4. Materials and Methods

## 4.1. Gene expression analysis

### 4.1.1. Cell lines

The estrogen receptor (ER) positive human breast cancer cell lines BT-474, MCF-7, MDA-MB-361, T-47D and ZR-75-30, and the estrogen receptor negative cell line MDA-MB-231 were obtained from the American Type Tissue Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultured in their respective conditions as presented in Table 1, at 37°C and 5% $CO_2$. Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM), L-15 medium, RPMI medium, fetal bovine serum (FBS), and supplements L-glutamine, penicillin-streptomycin solution (P/S), sodium pyruvate, sodium bicarbonate, non-essential amino acid solution (NEAA) and HEPES buffer were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protaphane human insulin was obtained from Novo Nordisk (Bagsværd, Denmark).

**Table 1**: Cell line media and supplements. The glucose column indicates the glucose concentration already existing in the medium.

| Cell line | Medium | FBS | Supplements | Glucose (g/L) |
|---|---|---|---|---|
| BT-474 | DMEM | 10% | Sodium pyruvate, glucose, L-glutamine, P/S | 1 |
| MCF-7 | EMEM | 10% | Sodium pyruvate, NEAA, sodium bicarbonate, insulin, L-glutamine, P/S | 1 |
| MDA-MB-361 | L-15 | 10% | L-glutamine, P/S | 0.9 galactose |
| ZR-75-30 | RPMI | 10% | L-glutamine, P/S | 2 |
| T-47D | RPMI | 10% | Sodium pyruvate, HEPES buffer, sodium bicarbonate, glucose, insulin, L-glutamine, P/S | 2 |
| MDA-MB-231 | L-15 | 10% | L-glutamine, P/S | 0.9 galactose |

The supplements shown in Table 1 were added as instructed by ATCC.

### 4.1.2. Stripping

For the estrogen treatment, the cells were seeded to 24-well plates. The number of cells used for each cell line varied depending on their growth characteristics and is shown in Table 2. Cell numbers were counted with Z1 Coulter Particle Counter (Beckman Coulter, Fullerton, CA, USA). The cells were trypsinized, and 100 µl of cell solution added to 7.9 ml of the cell counter's electrolyte solution, Coulter ISOTON II diluent from Beckman Coulter. The cells were diluted to their respective

numbers, shown in Table 2, in their growth medium and allowed to attach overnight on the 24-well plate. An exception were MDA-MB-361 and MDA-MB-231 cell lines, which were normally cultured in $CO_2$-depleted conditions in L-15 medium, but in DMEM based medium on 24-well plates. The medium contained the same supplements as the usual growth medium.

**Table 2**: Cell numbers for different cell lines for seeding to 24-well plates.

| **Cell line** | **Cell number / 0.5 ml** |
|---|---|
| BT-474 | 70 000 |
| MCF-7 | 50 000 |
| MDA-MB-361 | 200 000 |
| T-47D | 50 000 |
| ZR-75-30 | 60 000 |
| MDA-MB-231 | 10 000 |

The day after seeding to 24-well plates, the cells were washed twice with PBS (Sigma-Aldrich, St. Louis, MO, USA), and the growth medium was changed to estrogen-free "stripping" medium, which consisted of phenol-red free DMEM, 10% charcoal-stripped FBS (both by Gibco by Life Technologies, Fischer Scientific, Waltham, MA, USA), and each cell lines' respective supplements as shown in Table 1. The phenol-red free DMEM did not contain glucose, so the same amount of glucose was added to each cell lines' estrogen depletion medium as there had been in their normal growth mediums.

### 4.1.3. Estrogen treatment

The cells were cultured in the estrogen-free medium for three days, after which estrogen treatment or vehicle treatment medium was applied. The estrogen treatment medium was prepared the same way as the estrogen-free medium earlier, but 17β-estradiol (later called E2) (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 100 nM. The E2 was dissolved to absolute ethanol, with stock concentration of 70.6 mM, and serially diluted into phenol-red free DMEM to achieve the desired concentration. The vehicle medium was prepared the same way, without E2. The treatment and vehicle media were added to the 24-well plate.

After 24h and 48h in the E2 or vehicle medium, the cells were lysed with the lysis buffer of the RNA Plus RNA extraction kit (Macherey-Nagel, Düren, Germany) and three parallel wells were pooled. The lysed cells were stored in -80°C freezer.

### 4.1.4. RNA extraction and cDNA synthesis

RNA was extracted from the lysed cell samples with the RNA Plus RNA isolation kit by Macherey-Nagel according to the instructions. First, genomic DNA was removed by centrifuging the sample through a binding column. Then, RNA binding conditions were adjusted with a binding solution, and the RNA was bound to a RNA binding column. The column was washed with washing solutions, and the bound RNA was eluted into RNAse-free water. The RNA samples were stored in -80°C freezer. The RNA concentrations were measured by spectrophotometry with Tecan Infinite® F200 PRO plate reader using a NanoQuant Plate (Tecan, Männedorf, Switzerland) to ensure successful RNA extraction.

For qRT-PCR analysis, the RNA samples were synthesized into cDNA with the Invitrogen SuperScript® III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA), using random hexamers as primers. For each sample, half of the instructed reaction component amounts were used. To start the cDNA synthesis, 50 ng/µl random hexamer primers (0.5 µl), 10 mM dNTP mix (0.5 µl) and the RNA sample (4 µl) were incubated at 65°C for 5 minutes, and placed on ice for at least 1 minute. The cDNA synthesis mix was prepared as shown in Table 3, and 5 µl was added to the samples, mixed, centrifuged briefly, and first incubated for 10 minutes at 25°C, and then 50 minutes at 50°C. The reaction was terminated by incubating the samples at 85°C for 5 minutes, and the samples were chilled on ice. 0.5 µl of RNAse H (2 U/µl) was added to samples, and the samples were incubated at 37°C for 20 minutes. The cDNA samples were stored in -20°C freezer.

**Table 3**: cDNA synthesis mix components in pipetting order

| **Component** | **½ reaction** |
|---|---|
| 10X RT buffer | 1 µl |
| 25 mM $MgCl_2$ | 2 µl |
| 0.1 M DTT | 1 µl |
| RNAseOUT™ (40 U/µl) | 0.5 µl |
| SuperScript® III RT (200 U/µl) | 0.5 µl |

### 4.1.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

The qRT-PCR analysis was conducted using the Roche LightCycler® 2.0 device, with software version 4.05 (Roche, Basel, Switzerland). SYBR Green I dye based assay was used for *GREB1*, *TFF1*, *BMP4* and *BMP7*. The *GREB1* primer sequence was obtained from Rae *et al.* (2005), and *TFF1* sequence from Bosma *et al.* (2002). *BMP4* and *BMP7* had been used earlier in the group's research. Primers were ordered from Sigma-Aldrich. For the housekeeping gene *HPRT*, TaqMan®-based

Universal ProbeLibrary Human HPRT Gene Assay (by Roche) was used. In the SYBR Green I based assay, LightCycler® FastStart DNA Master SYBR Green I kit by Roche was used. The gene specific primers are shown in Table 4. The cDNA samples to be used in qRT-PCR were diluted 1:10.

**Table 4**: Sequences of the primers used in qRT-PCR analysis

| **Gene** | **Forward primer (5'→3')** | **Reverse primer (5'→3')** |
|---|---|---|
| *GREB1* | CAAAGAATAACCTGTTGGCCCTGC | GACATGCCTGCGCTCTCATACTTA |
| *TFF1* | GAGGCCCAGACAGAGACGTG | CCCTGCAGAAGTGTCTAAAATTCA |
| *BMP4* | GGGACTTCGAGGCGACA | TTCTCCAGATGTTCTTCGTGGT |
| *BMP7* | GCTTCGACAATGAGACGTTC | TGGACCTCCGTGGCCTT |

At the beginning of the SYBR Green I assay, the components (shown in Table 5) were mixed to prepare a reaction mix. 18 µl of the reaction mix was pipetted to each LightCycler® capillary, and after that, 2 µl of the sample cDNA (1:10 dilution) was added.

**Table 5**: The components and their amounts in SYBR Green I qRT-PCR analysis

| **Components** | **1 x** |
|---|---|
| H2O | 11.6 µl |
| 10 µM F+R primer mix (5 µM each) | 2 µl |
| 10 x LightCycler® FastStart DNA Master SYBR Green I | 2 µl |
| 25 mM $MgCl_2$ | 2.4 µl |

The SYBR Green I assay started with a 10 minute denaturation step at 95°C. The amplification protocol is shown in Table 6. After amplification, the melting curve analysis for PCR product identification was carried out. Finally, the reaction was cooled to 40°C for 30 seconds.

**Table 6**: The protocol for the amplification step of qRT-PCR, differing values bolded.

| | | | **Amplification, 45 cycles** | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Pre-incubation** | | **Denaturation** | | **Annealing** | | **Extension** | |
| **Gene** | Time | Temperature | Time | Temperature | Time | Temperature | Time | Temperature |
| *GREB1* | 10 min | 95°C | 10 s | 95°C | 5 s | 60°C | 10 s | 72°C |
| *TFF1* | 10 min | 95°C | 10 s | 95°C | 5 s | 60°C | 10 s | 72°C |
| *BMP4* | 10 min | 95°C | 10 s | 95°C | 5 s | **55°C** | 10 s | 72°C |
| *BMP7* | 10 min | 95°C | 10 s | 95°C | 5 s | **55°C** | **13 s** | 72°C |

For *HPRT*, LightCycler® TaqMan® Master reaction mix by Roche was used with a 10 minute denaturation step at 95°C, 45 amplification cycles of 10 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C, and 1 second of elongation at 72°C, and finally 30 seconds of cooling at 40°C. The components of one reaction included 13.2 µl of $H_2O$, 0.4 µl of 20 µM F+R primer mix, 0.4 µl of 10 µM UPL probe, 4 µl of the TaqMan® master mix, and 2 µl of the sample cDNA (1:10 dilution).

### 4.1.6. Analysis of gene expression data

$C_t$ (threshold cycle) values for each gene in all samples and corresponding controls were acquired in the qRT-PCR analysis. The values were obtained with the LightCycler® software's Fit Points – method, in which the sample's exponential curve (as in Figure 5) is converted to linear. This exponential curve is attained by measuring the accumulating fluorescence during every cycle, which in turn is comparable to the amount of double-stranded DNA generated in the reaction. The beginning of the straight line is extended to cross with a horizontal crossing line, creating a crossing point (i.e., the threshold cycle, $C_t$). The background noise from the samples was eliminated by adjusting the noise band.

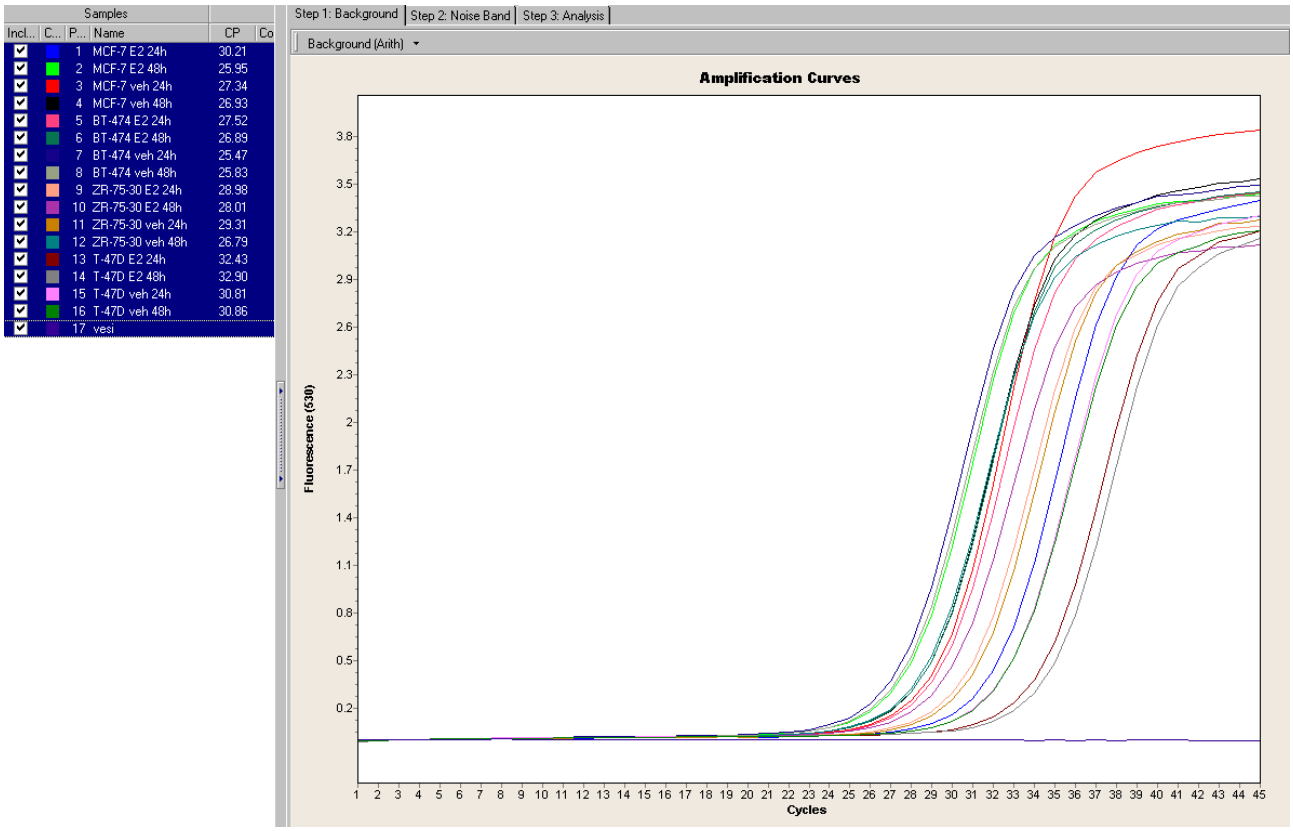



**Figure 5**: Screen capture from LightCycler® software depicting the amplification curves of E2 and vehicle treated MCF-7, BT-474, ZR-75-30 and T-47D cell lines at 24h and 48h, and their Ct values.

The fold change (FC) values were acquired by calculating the $\Delta C_t$'s and $\Delta\Delta C_t$ from the following equations (in which $C_t$ is the crossing point, GOI is the gene of interest, and normalizer is the housekeeping gene *HPRT*).

$$C_{t\ GOI}^{\ E2\ sample} - C_{t\ normalizer}^{\ E2\ sample} = \Delta C_{t\ E2\ sample}$$

$$C_{t\ GOI}^{\ vehicle} - C_{t\ normalizer}^{\ vehicle} = \Delta C_{t\ vehicle}$$

$$\Delta C_{t\ E2\ sample} - \Delta C_{t\ vehicle} = \Delta\Delta C_t$$

$$\text{Fold change} = 2^{-\Delta\Delta C_t}$$

## 4.2. Functional experiments with estrogen and BMP4

### 4.2.1. Cell culture

BT-474 and T-47D cell lines were chosen for the functional experiments. The cells were cultured in their usual culturing media as indicated in Table 1.

### 4.2.2. Estrogen and BMP4 treatment

For the estrogen and BMP4 treatment, E2 and recombinant human BMP4 protein (acquired from R&D Systems, Minneapolis, MN, USA) were used. E2 was used at 100 nM, as described earlier. Recombinant human BMP4 was used at a final concentration of 100 ng/ml, as determined earlier by our research group (Ketolainen *et al.* 2010). BMP4 was diluted in vehicle solution, which consisted of 4 mM HCl with 0.1% of bovine serum albumin (BSA). The vehicle for this experiment contained both the estrogen vehicle made earlier for the gene expression analysis experiment, and the BMP4 vehicle.

### 4.2.3. Cell proliferation assay

The cells were seeded to 24-well plates for the cell proliferation assay. In the first experiments with BT-474 and T-47D, the cells were seeded at 14000 cells per well with three parallel wells in each treatment. For BT-474, a following experiment was conducted with cell count of 40000 cells per well, with six parallel wells, so that statistical tests could be conducted. In following experiments with T-47D, 40000 cells per well were seeded on the 24-well plate with six parallel wells, and repeated twice. As earlier in the gene expression analysis experiments, the cells first needed to be depleted of estrogen. The estrogen-"stripping" was done the same way as earlier. The cells seeded to the 24-well plates the day before were washed with PBS, changed to estrogen-free medium, and cultured in this medium for three days. After this, the cells were trypsinized and counted with the Z1 Coulter Particle Counter as described earlier. The count was performed to ensure that all the wells contained same amount of cells.

The cells on the 24-well plate were changed into treatment media containing either 100 nM 17β-estradiol, 100 ng/ml BMP4, both, or vehicle. Fresh treatment media were changed every other day. The cells were counted using the Z1 Coulter Particle Counter on day six or seven after the first addition of the treatment media.

### 4.2.4. Statistical analyses for functional experiments

For experiments with six parallel samples, Mann-Whitney test was used to evaluate the statistical difference between the differently treated samples. Grubb's test was conducted to determine if one of the samples was a significant outlier.

# 5. Results

## 5.1. Effects of E2 treatment on BMP expression

The effect of E2 on breast cancer cell line's BMP expression was studied with gene expression analysis. The expression of the positive control genes *GREB1* and *TFF1*, and target genes *BMP4* and *BMP7* was assessed with qRT-PCR from six breast cancer cell lines: estrogen receptor (ER) positive BT-474, MCF-7, MDA-MB-361, T-47D and ZR-75-30, and ER negative MDA-MB-231. The positive control genes were genes known to be upregulated after estrogen treatment (Rae *et al.* 2005; Sun *et al.* 2005). In the ER negative MDA-MB-231 cell line the positive control genes were expressed on a very low level, and as expected, their expression did not change after E2 treatment. Similarly, *TFF1* expression levels were so low in the T-47D cell line that they could not be reliably measured. *GREB1* expression was upregulated in all ER positive cell lines (Figure 6). *TFF1* expression was increased in BT-474, MCF-7 and MDA-MB-361, but no significant change was seen in ZR-75-30 (Figure 6).

As for target genes *BMP4* and *BMP7*, the MDA-MB-231 cell line showed no measurable expression for *BMP7*, and insignificant fold changes for *BMP4* after estrogen treatment (data not shown). After

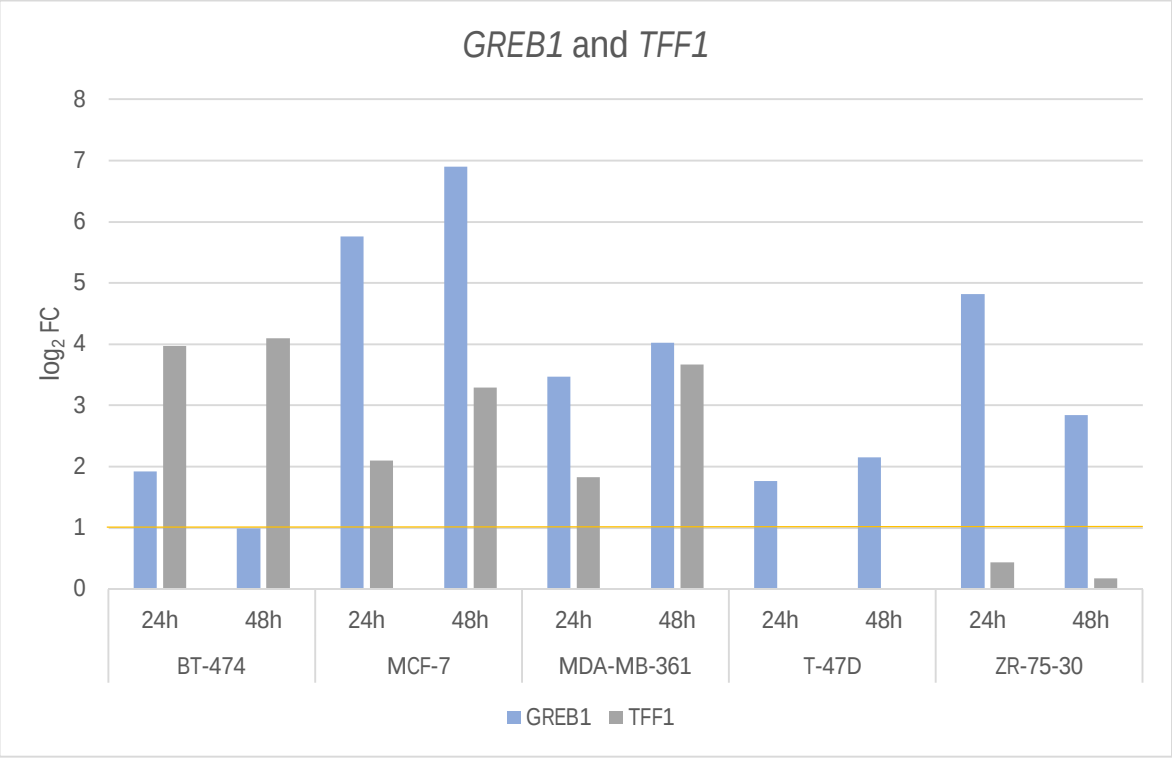



**Figure 6**: Fold change values of positive control genes GREB1 and TFF1 on a logarithmic scale. The orange line indicates a cut-off point for a fold change value considered as significant.

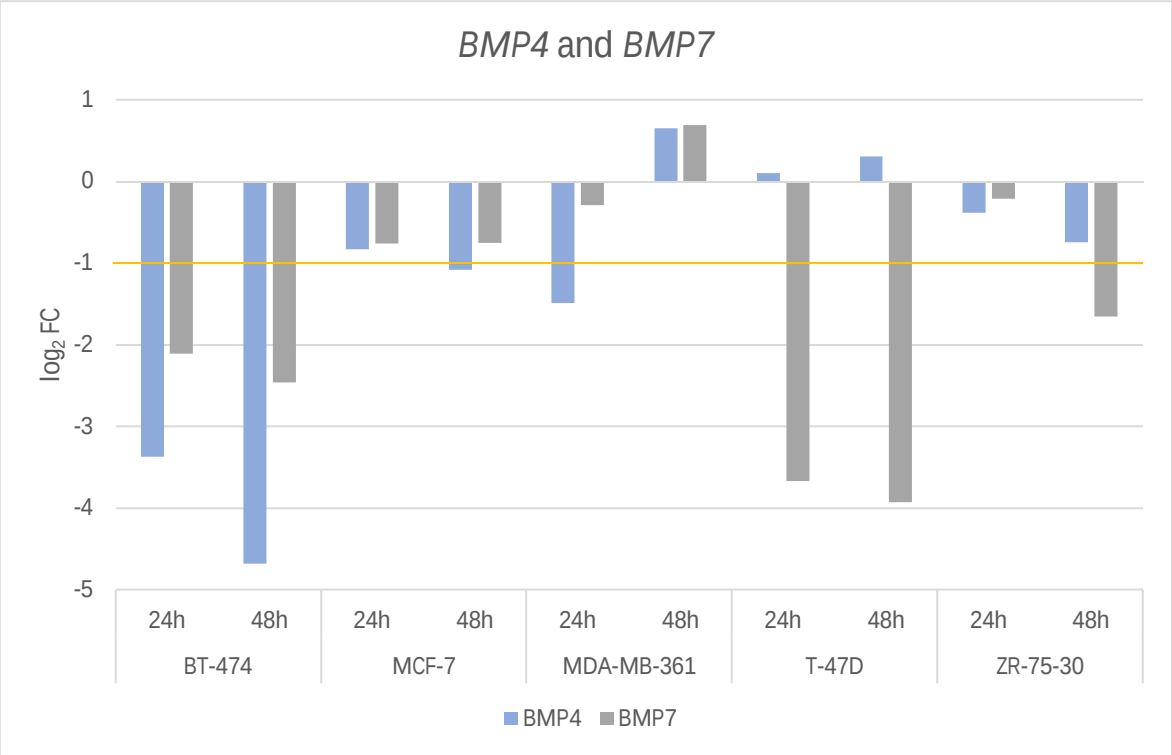



**Figure 7**: Fold changes for BMP4 and BMP7 on a logarithmic scale. The orange line indicates a cut-off point for a fold change value considered as significant.

estrogen treatment, *BMP4* expression was downregulated significantly in BT-474, MCF-7 (at 48 hours), and MDA-MB-361 (at 24 hours) cell lines (Figure 7). Other cell lines showed no significant change in *BMP4* expression after E2 treatment (Figure 7).

On the other hand, BT-474, T-47D, and ZR-75-30 showed downregulation of *BMP7* (Figure 7). In the case of ZR-75-30, this effect was only observed at 48 hours. MCF-7 showed small, but insignificant decrease in *BMP7* expression, whereas MDA-MB-361 had similar, insignificant change in expression than with *BMP4* (decrease at 24 hours, then increase at 48 hours). Biological replicates for the E2 treatment were assessed with similar results, and technical replicates for qRT-PCR were also performed.

## 5.2. Effects of E2 and BMP4 treatment on cell proliferation

In the functional experiments, the effect of estrogen and BMP4 treatment on breast cancer cell proliferation was assessed by observing cell growth. Light microscope pictures of the treated cells were taken to determine possible morphological changes caused by different treatments, and cells were counted to quantify the differences in cell number.

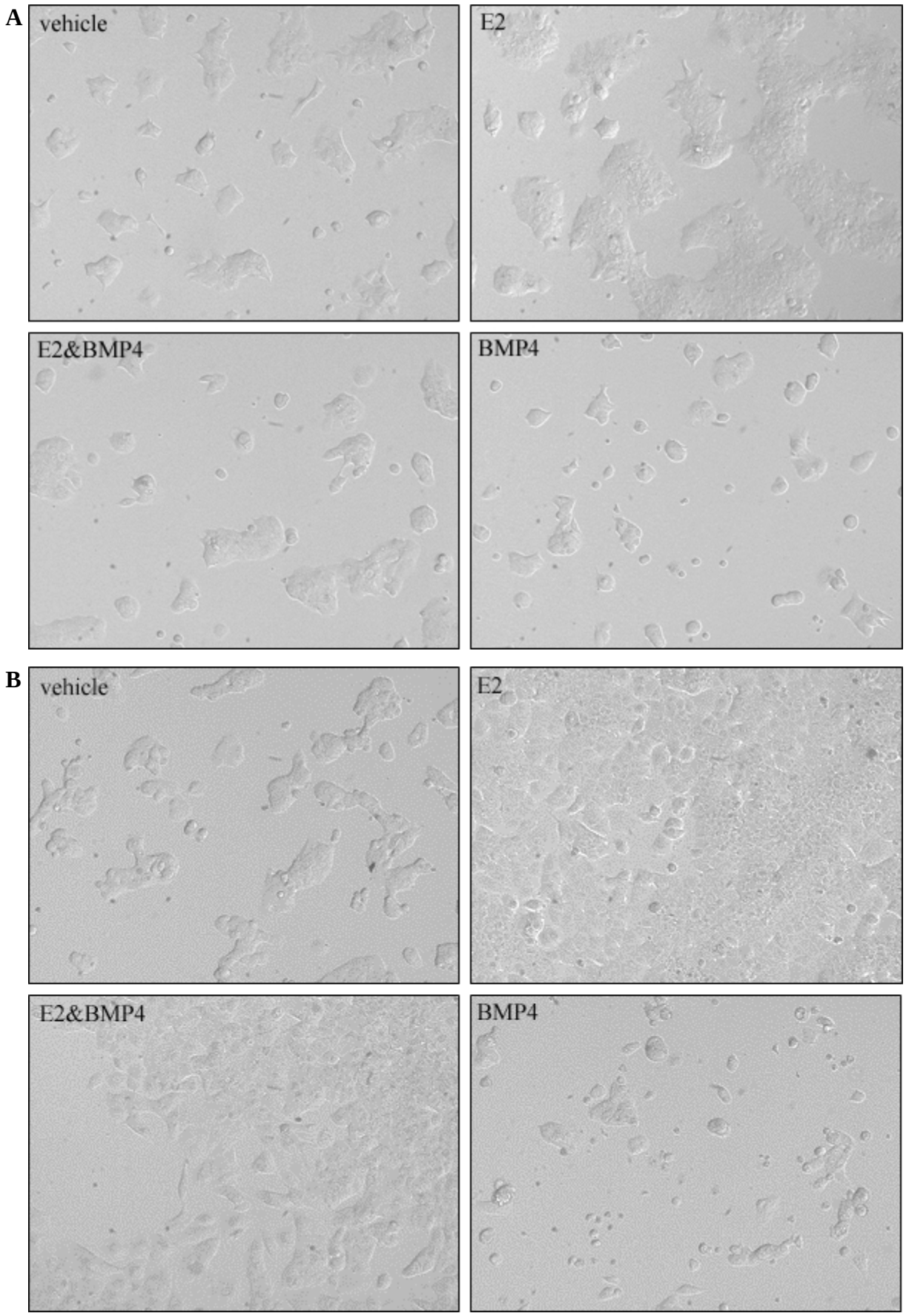



**Figure 8**: Light microscope pictures (10X magnification) of vehicle, estrogen (E2), E2 and BMP4, and BMP4 treated (A) BT-474 cells at day 7 and (B) T-47D cells at day 5.

The BT-474 cell morphology, seen in Figure 8A, was notably different in estrogen treated cells compared to BMP4 treated cells, as they grew in raft-like formations as usual for this cell line, with seemingly more cells. The vehicle treated cells made raft-like formations, but with seemingly less cells, as did the cells treated with both estrogen and BMP4. The BMP4 treated cells on the other hand, formed smaller, more round formations (Figure 8A). The cells seemed to spread out more in the presence of estrogen, and when there was only BMP4, the cells remain in their place.

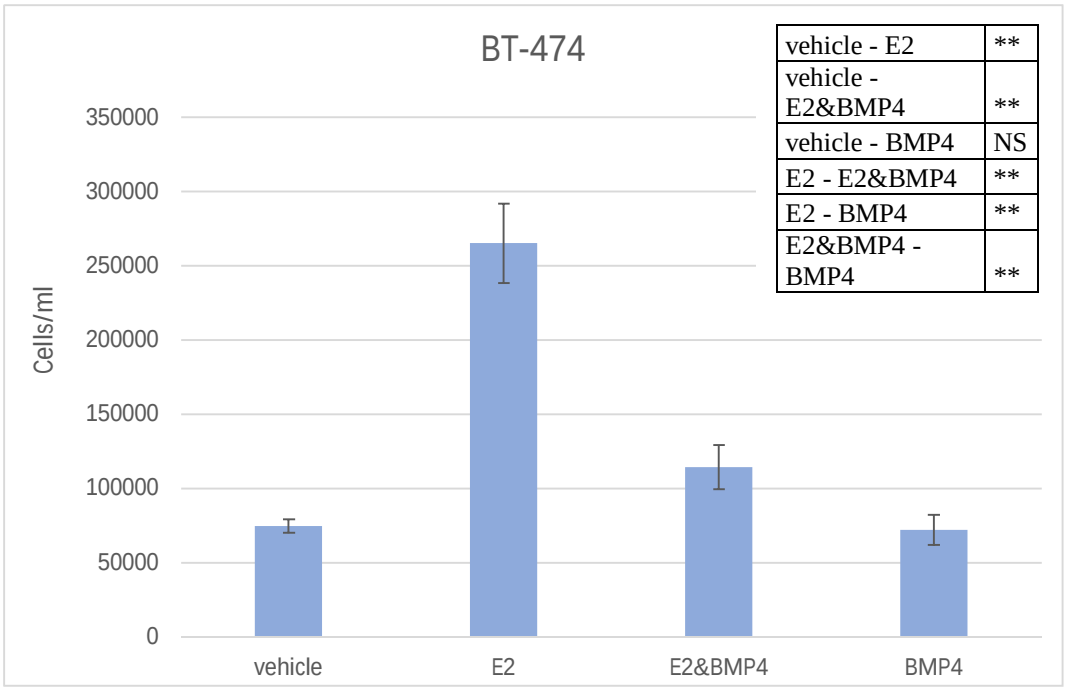
The morphology of T-47D cells, seen in Figure 8B, changed drastically when estrogen was present. Both estrogen and estrogen and BMP4 treated cells spread out and formed a confluent layer of cells at the bottom of the well, whereas the cells in estrogen-free environment grew in patches. The BMP4 treatment seemed to affect the cell growth quite negatively. The E2 treated cells were most like T-47D cells usually, when grown in the normal phenol red containing culture medium with FBS.

The effect of different treatments on cell growth was assessed by counting the cells. The cells were counted on day 0 (after 72h of estrogen depletion) to determine the actual amount of cells in the wells, because the estrogen depletion affected cell growth negatively. The cells were also counted on day 3 but no differences in growth were detected at that point (data not shown).

The count of BT-474 cells on day 7, seen in Figure 9A, showed that the vehicle control and BMP4 treated cells were the least proliferative, with around 70 000 cells/ml, when originally the cells were seeded at 80 000 cells/ml. Estrogen treatment had a significant proliferative effect on the cells. With the combination of E2 and BMP4 treatment, the cell count was significantly higher than with the vehicle or BMP4 treated cells, but significantly less than with only E2 treated cells. The BT-474 cell proliferation was decreased 57% when treated with both E2 and BMP4 as compared to only E2 treated cells. The differences in cell numbers are statistically significant between all except vehicle and BMP4 treatments.

The T-47D cell count, seen in Figure 9B, showed slightly different results comparing to BT-474 (Figure 9A) with vehicle cells. The estrogen depletion didn’t seem to affect T-47D as much, so the difference in cell counts between vehicle and BMP4 treated cells was significant. BMP4 thus showed a growth decreasing effect in T-47D cells. The estrogen treatment significantly increased proliferation, whereas combined E2 and BMP4 treatment decreased it by 65% compared to the E2 treatment. There was no significant change between E2 and BMP4 combination treatment, and BMP4 only treated cell counts, but between other treatments significant differences could be detected.

**A**




**B**

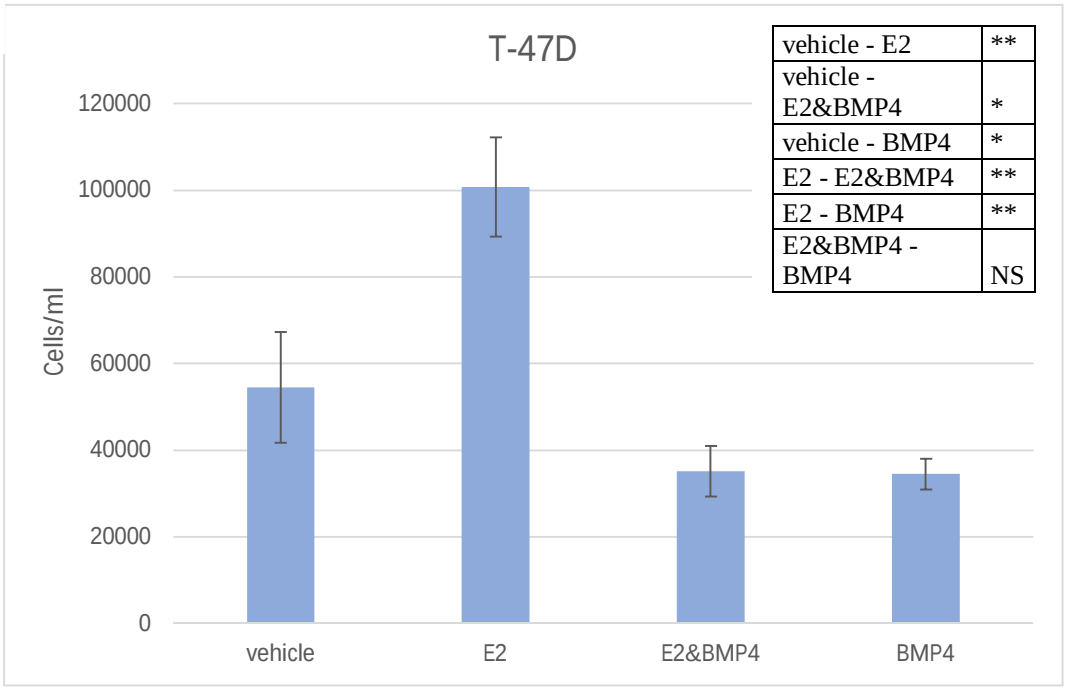



**Figure 9**: Results of (A) BT-474 and (B) T-47D cell count on day 7 after E2, E2 & BMP4, BMP4 and vehicle treatment. The box in the upper right corner shows statistical difference between samples, *=p<0.05, **=p<0.01.

# 6. Discussion

This study aimed to evaluate the possible cross talk between estrogen and BMP signaling. First, the possible effect of estrogen treatment on *BMP4* and *BMP7* expression was studied, and known estrogen responsive genes, *GREB1* and *TFF1*, were used as positive controls. More specifically, the expression levels of *BMP4*, *BMP7*, *GREB1* and *TFF1* were screened in five ER-positive breast cancer cell lines and one ER-negative control breast cancer cell line 24 and 48 h after estrogen treatment. Additionally, the effect of estrogen and BMP4 treatment on the growth of two breast cancer cell lines was studied.

The cells used in the experiments were grown in estrogen-free conditions ("stripped") before estrogen treatment, which notably affected cell growth. The phenol red and normal FBS -containing medium provides the ER-positive cell lines a growth-inducing environment. Phenol red has weak estrogenic properties, binding to ERs with a 0.001% affinity compared to estradiol (Berthois *et al.* 1986). It also stimulates cell-proliferation like estrogen, in dose-dependent manner, and it is usually present in high concentrations in cell culture media (Berthois *et al.* 1986). Charcoal-stripping of FBS is unspecific and removes not only steroid hormones, but also other vital components of the serum such as folic acid, vitamins (e.g. $B_{12}$), glucose and phosphorus are extracted (Cao *et al.* 2009). Untreated FBS contains high levels of estradiol conjugates, and the charcoal stripping procedure does not necessarily remove all of the existing conjugates, which some cells can use to convert to estradiol (Cao *et al.* 2009). Specific removal of estradiol from FBS would be the optimal situation, but as it happens, apparently no such method exists at the moment. Serum-free cell culture is an option, but also then the cell growth would be stunted due to the absence of essential growth factors and nutrients.

## 6.1. Estrogen inhibits the expression of BMP4 and BMP7 in a cell line dependent manner

In the gene expression experiments, positive control genes *GREB1* and *TFF1* were used to assess the functionality of the E2 treatment. *GREB1* and *TFF1* expression are known to be elevated in breast cancer cells after E2 treatment (Bosma *et al.* 2002; Ghosh *et al.* 2000; Mohammed *et al.* 2013; Rae *et al.* 2005; Sun *et al.* 2005). The results of the positive control gene expression studies mostly confirmed this. *GREB1* expression was elevated after E2 treatment in all ER-positive cell lines, and *TFF1* expression was notably upregulated in all but ZR-75-30 cell line, and the *TFF1* expression was so low in the T-47D cell line that it could not be reliably measured. However, Rae *et al.* (2005) showed *TFF1* gene expression upregulation by E2 treatment in the T-47D cell line in their study.

Their primers for *TFF1* were obtained from the study by Bosma *et al.* (2002) as in this study, and other conditions were quite similar. Also, as expected, the ER-negative MDA-MB-231 cell line demonstrated very low expression of both *GREB1* and *TFF1*, and did not show any change after E2 treatment.

*BMP4* expression after E2 treatment has not been studied before, at least not in the studies found. The *BMP4* expression was notably downregulated after E2 treatment in three of the five ER-positive cell lines: BT-474 MCF-7 and MDA-MB-361. BT-474 was the sole cell line in which gene expression was downregulated in both time points, and only decreased more with time. In MCF-7, *BMP4* showed insignificant decrease at 24h and barely significant decrease at 48h. In MDA-MB-361, the *BMP4* expression was at first downregulated notably, but then reverted back at 48h.

The expression of *BMP7* has been found to be downregulated after E2 treatment in the MCF-7 cell line (Takahashi *et al.* 2008), but no other cell lines have been evaluated. In this study, the decrease in *BMP7* expression in MCF-7 cells was apparent, but did not reach the set cut-point level. However, *BMP7* expression was significantly reduced in BT-474, T-47D and ZR-75-30 cell lines. In both BT-474 and T-47D the expression was downregulated at both time points, and in ZR-75-30 only at 48h time point.

Taken together, it seems that E2 has different effects on the BMP expression of different cell lines. The only cell line in which the expression of both *BMP4* and *BMP7* was reduced notably, was BT-474. Different cell lines are derived from different individuals, which is why their genetic backgrounds can be very variable (Heiser *et al.* 2009). Several different factors are needed to regulate transcription, and the expression of these factors varies depending on the cell line. Moreover, other factors than just E2 are needed to regulate BMP expression, and perhaps some of these factors were not present in those cell lines in which E2 didn't have a notable effect. More studies should be done on the molecular mechanisms of E2 regulation of BMP expression to find out why it acts in such a variable manner in breast cancer cells.

## 6.2. BMP4 treatment attenuates the effects of estrogen on breast cancer cell growth

The cell proliferation experiment was conducted with two cell lines, with three days of estrogen "stripping", and counting the cells after six or seven days after treatment with E2, BMP4, or both. BT-474 and T-47D cell lines were chosen for the cell proliferation experiment for two reasons: i) BT-474 showed significant response to E2 treatment with BMP4 gene expression decreasing significantly

and ii) BMP4 treatment had earlier been shown to decrease cell growth significantly in T-47D, BT-474 was not used in the study (Ampuja *et al.* 2013; Ketolainen *et al.* 2010).

The effect of estrogen on BMP signaling and action has been studied more in the cell proliferation perspective. Takahashi *et al.* (2008) demonstrated that BMP6 and BMP7 has a growth-inhibiting effect on the MCF-7 cell proliferation induced by estrogen. BMP4 has not been studied in this respect, but it has been shown to inhibit breast cancer cell growth in several breast cancer cell lines (Ampuja *et al.* 2013; Ketolainen *et al.* 2010).

As expected, E2 significantly, and distinctly increased cell proliferation in both BT-474 and T-47D cell lines. When treated with both estrogen and BMP4, both cell lines showed significant reduction in cell proliferation, as in previous study using BMP6 and BMP7 (Takahashi *et al.* 2008). There was no significant difference in proliferation between vehicle and BMP4 in BT-474 cells, but in T-47D cells BMP4 treatment significantly decreased cell proliferation. This could be due to the cell line's different reactions to the "stripping" medium. T-47D might better tolerate the harsher environment, and thus, the addition of BMP4 reduces growth. In T-47D the treatment with both E2 and BMP4 reduces growth to the same level as treatment with only BMP4. In BR-474, however, the E2 and BMP4 treated cells proliferate more than only BMP4 treated cells, possibly due to the cell line's poor tolerance of the "stripping" medium. The effects of E2 and BMP4 could not be seen so clearly yet on day three after treatment, which indicates that the growth reducing effects are likely to happen via gene expression changes, and not the rapid response pathways.

Interestingly, the microscope images taken of T-47D cells on day 5 show that cells treated with both E2 and BMP4 have spread out, and seem like there are more cells than in the BMP4 treated well, when in fact, there are actually almost the same number of cells. This might be due to the migration and invasion inducing effect of BMP4, and it seems that the presence of estrogen is required for the spreading to happen. The effect is not seen with BT-474 cells. However, Ketolainen *et al.* (2010) show that migration actually decreases in T-47D cell line after BMP4 treatment. The study was not conducted in estrogen-free environment, so it might be that the estrogen "stripping" also affects this phenomenon.

Estrogen addition affected the morphology of both BT-474 and T-47D cells, and the cells looked more like usual (when grown in normal conditions). Otherwise, the cells grew quite poorly in the vehicle treated wells, and BMP4 treatment seemed to only intensify the suffering.

All in all, combined E2 and BMP4 treatment of BT-474 and T-47D cells shows reduced growth compared to only E2 treated cells. Thus, these data indicate that BMP4 is able to reverse, at least partly, the proliferation inducing effects of estrogen. This is an important finding that may have clinical applicability for the treatment of breast cancer patients with ER positive tumors.

## 6.3. Future aspects

In this study, estrogen and BMPs demonstrate their cross-talk. It seems that in some breast cancer types, the interaction between them is more pronounced, as can be seen in the gene expression results. More research should still be done in regard of the proliferation reducing effect of BMPs on E2 induced cell growth, and assess the effects in other cell lines. The effects of BMPs and E2 on ER-negative cell lines should also be further studied to ensure that the effect is ER-dependent. Detailed studies highlighting the functional cross-talk of the estrogen and BMP signaling pathways should be done to better understand the factors that have an effect in it. Also the reasons behind why estrogen decreases BMP expression in some cell lines and not in others could bring light to the factors playing a role in the interactions between estrogen and BMPs.

The interaction between estrogen and BMPs has only been studied in very few breast cancer cell lines, although a lot of them are used in research nowadays. MCF-7 cell line is the most used one, but other cell lines should also be considered when studying E2 and BMP cross-talk, since breast cancer is inherently a very variable disease. Thus, breast cancer studies should be done with more cell lines than only one or two, and preferably with cell lines showing different ER/PR status, ethnic background, and other characteristics. This might help in the development of personalized medicines and treatments for breast cancer patients with different tumor types, responding to different treatments Furthermore, the molecular background of the interactions between E2 and BMPs in specific cell lines should be studied in more detail to assess the central factors influencing the different effects in different cell lines.

# 7. Conclusion

The objective of this study was to assess the relationship between estrogen and bone morphogenetic proteins in several different breast cancer cell lines. Both the effect of estrogen treatment on *BMP4* and *BMP7* expression, and the effect of estrogen and BMP4 on breast cancer cell proliferation were studied in this respect. The results show that the effects of estrogen on *BMP4* and *BMP7* expressions vary in different cell lines and different time points, with their expression being down-regulated in some cell lines or not notably altered in others. The proliferation study with E2 and BMP4 treatment showed consistent results that E2 and BMP4 treatment together reduce the proliferation induced by E2. The findings can be used to further study the E2 and BMP interactions, and may possibly later in the future have a clinical significance in the treatment of breast cancer patients with ER positive tumors.

# 8. References


Alarmo E and Kallioniemi A. 2010. Bone morphogenetic proteins in breast cancer: dual role in tumourigenesis? Endocrine-related cancer 17:R123-39.

Alarmo E, Korhonen T, Kuukasjärvi T, Huhtala H, Holli K, Kallioniemi A. 2008. Bone morphogenetic protein 7 expression associates with bone metastasis in breast carcinomas. Annals of Oncology 19:308-314.

Alarmo E, Kuukasjarvi T, Karhu R, Kallioniemi A. 2007. A comprehensive expression survey of bone morphogenetic proteins in breast cancer highlights the importance of BMP4 and BMP7. Breast cancer research and treatment 103:239-246.

Alarmo E, Huhtala H, Korhonen T, Pylkkänen L, Holli K, Kuukasjarvi T, Parkkila S, Kallioniemi A. 2013. Bone morphogenetic protein 4 expression in multiple normal and tumor tissues reveals its importance beyond development. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc 26:10-21.

Alarmo E, Pärssinen J, Ketolainen JM, Savinainen K, Karhu R, Kallioniemi A. 2009. BMP7 influences proliferation, migration, and invasion of breast cancer cells. Cancer letters 275:35-43.

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

Ampuja M, Jokimäki R, Juuti-Uusitalo K, Rodriguez-Martinez A, Alarmo E, Kallioniemi A. 2013. BMP4 inhibits the proliferation of breast cancer cells and induces an MMP-dependent migratory phenotype in MDA-MB-231 cells in 3D environment. BMC Cancer 13:1-13.

Band AM and Laiho M. 2011. Crosstalk of TGF-β and Estrogen Receptor Signaling in Breast Cancer. Journal of mammary gland biology and neoplasia 16:109-115.

Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. 1986. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proceedings of the National Academy of Sciences of the United States of America 83:2496-2500.

Bosma AJ, Weigelt B, Lambrechts AC, Verhagen OJHM, Pruntel R, Hart AAM, Rodenhuis S, van 't Veer LJ. 2002. Detection of Circulating Breast Tumor Cells by Differential Expression of Marker Genes. Clinical Cancer Research 8:1871-1877.

Boulware MI and Mermelstein PG. 2005. The influence of estradiol on nervous system function. Drug news & perspectives 18:631-637.

Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A. 2011. Bone Morphogenetic Proteins: A critical review. Cellular signalling 23:609-620.

Brazil DP, Church RH, Surae S, Godson C, Martin F. 2015. BMP signalling: agony and antagony in the family. Trends in cell biology 25:249-264.

Buijs JT, Henriquez NV, van Overveld PGM, van der Horst G, Que I, Schwaninger R, Rentsch C, ten Dijke P, Cleton-Jansen A, Driouch K, Lidereau R, Bachelier R, Vukicevic S, Clézardin P, Papapoulos SE, Cecchini MG, Löwik CWGM, van der Pluijm G. 2007. Bone Morphogenetic Protein 7 in the Development and Treatment of Bone Metastases from Breast Cancer. Cancer research 67:8742-8751.

Cao Z, West C, Norton-Wenzel CS, Rej R, Davis FB, Davis PJ, Rej R. 2009. Effects of resin or charcoal treatment on fetal bovine serum and bovine calf serum. Endocrine research 34:101-108.

Carreira AC, Alves GG, Zambuzzi WF, Sogayar MC, Granjeiro JM. 2014. Bone Morphogenetic Proteins: Structure, biological function and therapeutic applications. Archives of Biochemistry and Biophysics 561:64-73.

Chlebowski RT. 2014. Current concepts in breast cancer chemoprevention. Polskie archiwum medycyny wewnetrznej 124:191-199.

Clemons M and Goss P. 2001. Estrogen and the Risk of Breast Cancer. N Engl J Med 344:276-285.

Davies SR, Watkins G, Douglas-Jones A, Mansel RE, Jiang WG. 2008. Bone morphogenetic proteins 1 to 7 in human breast cancer, expression pattern and clinical/prognostic relevance. Journal of experimental therapeutics & oncology 7:327-338.

Diel P. 2002. Tissue-specific estrogenic response and molecular mechanisms. Toxicology letters 127:217-224.

Ehata S, Yokoyama Y, Takahashi K, Miyazono K. 2013. Bi-directional roles of bone morphogenetic proteins in cancer: Another molecular Jekyll and Hyde? Pathology international 63:287-296.

ESHRE Capri Workshop Group. 2004. Hormones and breast cancer. Human reproduction update 10:281-293.

Ewan KBR, Oketch-Rabah H, Ravani SA, Shyamala G, Moses HL, Barcellos-Hoff M. 2005. Proliferation of Estrogen Receptor-α-Positive Mammary Epithelial Cells Is Restrained by Transforming Growth Factor-β1 in Adult Mice. The American Journal of Pathology 167:409-417.

Feigelson HS and Henderson BE. 1996. Estrogens and breast cancer. Carcinogenesis 17:2279-2284.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. 2015. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. International Journal of Cancer 136:E359-E386.

Fessel JP, Chen X, Frump A, Gladson S, Blackwell T, Kang C, Johnson J, Loyd JE, Hemnes A, Austin E, West J. 2013. Interaction between bone morphogenetic protein receptor type 2 and estrogenic compounds in pulmonary arterial hypertension. Pulmonary Circulation 3:564-577.

Finnish Cancer Registry. 2016. Cancer Statistics, www.cancerregistry.fi.

Ghosh MG, Thompson DA, Weigel RJ. 2000. PDZK1 and GREB1 Are Estrogen-regulated Genes Expressed in Hormone-responsive Breast Cancer1, 2. Cancer research 60:6367-6375.

Ghosh-Choudhury N, Ghosh-Choudhury G, Celeste A, Ghosh PM, Moyer M, Abboud SL, Kreisberg J. 2000. Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1497:186-196.

Giacomini D, Páez-Pereda M, Stalla J, Stalla G, Arzt E. 2009. Molecular Interaction of BMP-4, TGF-β, and Estrogens in Lactotrophs: Impact on the PRL Promoter. Molecular Endocrinology 23:1102-1114.

Guo D, Huang J, Gong J. 2012. Bone morphogenetic protein 4 (BMP4) is required for migration and invasion of breast cancer. Molecular and cellular biochemistry 363:179-190.

Heiser LM, Wang NJ, Talcott CL, Laderoute KR, Knapp M, Guan Y, Hu Z, Ziyad S, Weber BL, Laquerre S, Jackson JR, Wooster RF, Kuo WL, Gray JW, Spellman PT. 2009. Integrated analysis of breast cancer cell lines reveals unique signaling pathways. Genome biology 10:1-17.

Helms MW, Packeisen J, August C, Schittek B, Boecker W, Brandt BH, Buerger H. 2005. First evidence supporting a potential role for the BMP/SMAD pathway in the progression of oestrogen receptor-positive breast cancer. The Journal of pathology 206:366-376.

Hewitt SC, Winuthayanon W, Korach KS. 2016. What's new in estrogen receptor action in the female reproductive tract. Journal of Molecular Endocrinology 56:R55-R71.

Huang B, Warner M, Gustafsson J. 2015. Estrogen receptors in breast carcinogenesis and endocrine therapy. Molecular and cellular endocrinology 418, Part 3:240-244.

Hynes NE and Watson CJ. 2010. Mammary Gland Growth Factors: Roles in Normal Development and in Cancer. Cold Spring Harbor Perspectives in Biology 2:a003186.

Ito I, Hanyu A, Wayama M, Goto N, Katsuno Y, Kawasaki S, Nakajima Y, Kajiro M, Komatsu Y, Fujimura A, Hirota R, Murayama A, Kimura K, Imamura T, Yanagisawa J. 2010. Estrogen inhibits transforming growth factor beta signaling by promoting Smad2/3 degradation. The Journal of biological chemistry 285:14747-14755.

Kallioniemi A. 2012. Bone morphogenetic protein 4—a fascinating regulator of cancer cell behavior. Cancer Genetics 205:267-277.

Katsuno Y, Hanyu A, Kanda H, Ishikawa Y, Akiyama F, Iwase T, Ogata E, Ehata S, Miyazono K, Imamura T. 2008. Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway. Oncogene 27:6322-6333.

Ketolainen JM, Alarmo EL, Tuominen VJ, Kallioniemi A. 2010. Parallel inhibition of cell growth and induction of cell migration and invasion in breast cancer cells by bone morphogenetic protein 4. Breast cancer research and treatment 124:377-386.

Korde LA, Zujewski JA, Kamin L, Giordano S, Domchek S, Anderson WF, Bartlett JMS, Gelmon K, Nahleh Z, Bergh J, Cutuli B, Pruneri G, McCaskill-Stevens W, Gralow J, Hortobagyi G,

Cardoso F. 2009. Multidisciplinary Meeting on Male Breast Cancer: Summary and Research Recommendations. Journal of Clinical Oncology 28:2114-2122.

Lian WJ, Liu G, Liu YJ, Zhao ZW, Yi T, Zhou HY. 2013. Downregulation of BMP6 enhances cell proliferation and chemoresistance via activation of the ERK signaling pathway in breast cancer. Oncology reports 30:193-200.

Massagué J. 2008. TGFβ in Cancer. Cell 134:215-230.

Massagué J and Gomis RR. 2006. The logic of TGFβ signaling. FEBS letters 580:2811-2820.

Masuda H, Otsuka F, Matsumoto Y, Takano M, Miyoshi T, Inagaki K, Shien T, Taira N, Makino H, Doihara H. 2011. Functional interaction of fibroblast growth factor-8, bone morphogenetic protein and estrogen receptor in breast cancer cell proliferation. Molecular and cellular endocrinology 343:7-17.

Mohammed H, D'Santos C, Serandour A, Ali H , Brown G, Atkins A, Rueda O, Holmes K, Theodorou V, Robinson JL, Zwart W, Saadi A, Ross-Innes C, Chin S, Menon S, Stingl J, Palmieri C, Caldas C, Carroll J. 2013. Endogenous Purification Reveals GREB1 as a Key Estrogen Receptor Regulatory Factor. Cell Reports 3:342-349.

Monroe DG, Jin DF, Sanders MM. 2000. Estrogen Opposes the Apoptotic Effects of Bone Morphogenetic Protein 7 on Tissue Remodeling. Molecular and cellular biology 20:4626-4634.

Nelson LR and Bulun SE. 2001. Estrogen production and action. Journal of the American Academy of Dermatology 45:S116-S124.

Ong DB, Colley SM, Norman MR, Kitazawa S, Tobias JH. 2004. Transcriptional Regulation of a BMP-6 Promoter by Estrogen Receptor α. Journal of Bone and Mineral Research 19:447-454.

Polyak K. 2007. Breast cancer: origins and evolution. The Journal of clinical investigation 117:3155-3163.

Polyak K. 2001. On the birth of breast cancer. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 1552:1-13.

Rae JM, Johnson MD, Scheys JO, Cordero KE, Larios JM, Lippman ME. 2005. GREB1 is a critical regulator of hormone dependent breast cancer growth. Breast cancer research and treatment 92:141-149.

Santen R, Cavalieri E, Rogan E, Russo J, Guttenplan J, Ingle J, Yue W. 2009. Estrogen Mediation of Breast Tumor Formation Involves Estrogen Receptor-Dependent, as Well as Independent, Genotoxic Effects. Annals of the New York Academy of Sciences 1155:132-140.

Shah R, Rosso K, Nathanson SD. 2014. Pathogenesis, prevention, diagnosis and treatment of breast cancer. World Journal of Clinical Oncology 5:283-298.

Shon S, Kim A, Kim JY, Kim KI, Yang Y, Lim J. 2009. Bone morphogenetic protein-4 induced by NDRG2 expression inhibits MMP-9 activity in breast cancer cells. Biochemical and biophysical research communications 385:198-203.

Simpson E and Santen RJ. 2015. Celebrating 75 years of oestradiol. Journal of Molecular Endocrinology 55:T1-T20.

Springhouse. 2002. Lippincott Professional Guides. Philadelphia, US: Wolters Kluwer Health.

Sun J, Spencer VA, Li L, Yu Chen H, Yu J, Davie JR. 2005. Estrogen regulation of trefoil factor 1 expression by estrogen receptor α and Sp proteins. Experimental cell research 302:96-107.

Takahashi M, Otsuka F, Miyoshi T, Otani H, Goto J, Yamashita M, Ogura T, Makino H, Doihara H. 2008. Bone morphogenetic protein 6 (BMP6) and BMP7 inhibit estrogen-induced proliferation of breast cancer cells by suppressing p38 mitogen-activated protein kinase activation. Journal of Endocrinology 199:445-455.

Thawani JP, Wang AC, Than KD, Lin C, La Marca F, Park P. 2010. Bone morphogenetic proteins and cancer: review of the literature. Neurosurgery 66:233-246.

Tsuchiya Y, Nakajima M, Yokoi T. 2005. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. Cancer letters 227:115-124.

van den Wijngaard A, Mulder WR, Dijkema R, Boersma CJC, Mosselman S, van Zoelen, E. J. J., Olijve W. 2000. Antiestrogens Specifically Up-Regulate Bone Morphogenetic Protein-4 Promoter Activity in Human Osteoblastic Cells. Molecular Endocrinology 14:623-633.

Wang D, Huang P, Zhu B, Sun L, Huang Q, Wang J. 2012. Induction of estrogen receptor alpha-36 expression by bone morphogenetic protein 2 in breast cancer cell lines. Molecular medicine reports 6:591-596.

Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S, Idowu O, Li M, Shen C, Hu A, Haydon RC, Kang R, Mok J, Lee MJ, Luu HL, Shi LL. 2014. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes & Diseases 1:87-105.

Wu L, Wu Y, Gathings B, Wan M, Li X, Grizzle W, Liu Z, Lu C, Mao Z, Cao X. 2003. Smad4 as a Transcription Corepressor for Estrogen Receptor a. Journal of Biological Chemistry 278:15192-15200.

Yamamoto T, Saatcioglu F, Matsuda T. 2002. Cross-Talk between Bone Morphogenic Proteins and Estrogen Receptor Signaling. Endocrinology 143:2635-2642.

Ye L, Bokobza SM, Jiang WG. 2009. Bone morphogenetic proteins in development and progression of breast cancer and therapeutic potential (review). International journal of molecular medicine 24:591-597.

Ying X, Sun Y, He P. 2015. Bone Morphogenetic Protein-7 Inhibits EMT-Associated Genes in Breast Cancer. Cellular Physiology and Biochemistry 37:1271-1278.

Zhang M, Yan JD, Zhang L, Wang Q, Lu SJ, Zhang J, Zhu TH. 2005. Activation of bone morphogenetic protein-6 gene transcription in MCF-7 cells by estrogen. Chinese Medical Journal 118:1629-1636.

Zhang M, Wang Q, Yuan W, Yang S, Wang X, Yan J, Du J, Yin J, Gao S, Sun B, Zhu T. 2007. Epigenetic regulation of bone morphogenetic protein-6 gene expression in breast cancer cells. The Journal of steroid biochemistry and molecular biology 105:91-97.