BMP4-induced microRNAs in breast cancer cell lines

MASTER'S THESIS

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Rintasyöpä on yksi naisten yleisimmistä syöpätaudeista sekä Suomessa että muualla maailmassa. Useiden rinnan primaarikasvaimien ja rintasyöpäsolulinjojen tiedetään ilmentävän BMP4-kasvutekijää, joka saa aikaan solukasvun hidastumista, mutta joka toisaalta lisää solujen migraatiota ja invaasiota. Mikro-RNA:t (miRNA) ovat lyhyitä, noin 22 nukleotidin mittaisia yksijuosteisia RNA-molekyylejä, jotka vaikuttavat elimistössämme alkionkehityksestä aina aikuisten yksilöiden solujen säätelyyn, toimien lähinnä geenien ilmentymisen hienosäätelyssä. Epänormaalit tasot miRNA:iden ilmentymisessä saattavat osaltaan vaikuttaa sairauksien, kuten syövän syntyyn. Tässä pro gradu -työssä haluttiin tutkia, johtuvatko BMP4:n aiheuttamat muutokset solujen kasvussa ja migraatiossa siitä, että BMP4 muuttaa miRNA:iden ilmentymistasoa.

Tutkimusmenetelmät: BMP4:n aiheuttamia miRNA-ilmentymistasojen muutoksia tutkittiin qRT-PCR:n avulla seitsemässä rintasyöpäsolulinjassa ja yhdessä immortalisoidussa rintaepiteelisolulinjassa. Aluksi tarkasteltavia miRNA:ita oli kahdeksan, joista kaksi valittiin seuraavaan vaiheeseen yhdessä kahden solulinjan kanssa. Seuraavaksi tutkittiin näiden kahden miRNA:n vaikutusta rintasyöpäsolulinjojen kasvuun ja migraatioon käsittelemällä soluja sekä BMP4:llä että miRNA-estäjillä. Solukasvua tutkittaessa solumäärä laskettiin kahdessa aikapisteessä ja migraatiokokeessa käytettiin ns. scratch-määritystä, jossa tarkkaillaan solujen liikkumista tyhjälle alueelle. Migraatiokokeessa käytettiin ainoastaan MDA-MB-231 -solulinjaa.

Tutkimustulokset: qRT-PCR -tulosten perusteella valikoituivat kaksi solulinjaa (BT-474 ja MDA-MB-231) sekä kaksi miRNA:a (miR-16-5p ja miR-106b-5p). Näissä solulinjoissa BMP4 lisäsi molempien miRNA:iden ilmentymistä selvästi. Molemmat miRNA:t toimivat samankaltaisesti, lisäten rintasyöpäsolujen kasvua. Lisäksi molemmat miRNA:t vaikuttivat selvästi solujen migraatioon, mutta siten, että miRNA:iden toiminnan esto lisäsi migraatiota.

Johtopäätökset: BMP4:n aiheuttamat muutokset rintasyöpäsolujen kasvussa näyttävät johtuvan osittain kahden tutkitun miRNA:n (miR-16-5p ja miR-106b-5p) korkeasta ilmentymisestä..

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Abstract

Background and aims: Breast cancer is the leading cause of mortality in women worldwide. In cancer, deregulation of signaling pathways, such as of the growth factor BMP4, is a common phenomenon. BMP4 is known to have a dualistic role in breast cancer, as it simultaneously decreases cell growth, but also increases migration and invasion. miRNAs are small regulators of several important cellular processes, and they have also been linked to cancer. Here, I wanted to study if microRNAs (miRNAs) partly affect these phenotypes.

Methods: The expression level of eight miRNAs after BMP4 treatment was studied in seven breast cancer cell lines and in one immortalized breast epithelial cell line using qRT-PCR. In the functional studies cell growth and migration were studied in two cell lines using proliferation assay and scratch assay, respectively. However, cell migration was studied only in MDA-MB-231 cells. For the proliferation assays, cells were treated with BMP4 and anti-miR inhibitors and the cells were counted at two time points, four and six or seven days after seeding. In the scratch assay a scratch was introduced to a confluent monolayer of cells and the closure area was measured at two hours' intervals until the scratch closed up.

Results: Based on the qRT-PCR results two cell lines (BT-474 and MDA-MB-231) and two miRNAs (miR-16-5p and miR-106b-5p) were chosen for functional studies. In the functional studies it was seen that both of these miRNAs were upregulated after BMP4 treatment and the inhibition of these miRNAs increased cell growth. In addition, both miRNAs had a clear effect on cell migration. The inhibition of these miRNAs increased breast cancer cell migration further than the BMP4 treatment alone.

Conclusions: Both studied miRNAs, miR-16-5p and miR-106b-5p apparently cause part of the BMP4-induced reduction in breast cancer cell growth. On the other hand, it appears that high miR-16-5p and miR-106b-5p levels reduce the migration capability of MDA-MB-231 cells.

ABBREVIATIONS

AGO2	Argonaute-2
BCL2	B-cell lymphoma 2
BMP4	Bone morphogenetic protein 4
co-SMAD	Common mediator SMAD
DGCR8	DiGeorge critical region 8
eIF	Eukaryotic initiation factor
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
I-SMAD	Inhibitory SMAD
miRISC	miRNA-induced silencing complex
miRNA	microRNA
PABP	Poly(A)-binding protein
PACT	Protein kinase R-activating protein
PR	Progesterone receptor
R-SMAD	Regulatory SMAD
RLC	RISC (or miRISC) loading complex
siLUC	Control siRNA for luciferase gene
siRNA	small interfering RNA
SMURF	SMAD ubiquitin regulatory factor
TGF-β	Transforming growth factor β
TNRC6	Trinucleotide repeat-containing gene 6 protein
TRBP	Transactivation-response RNA-binding protein

CONTENTS

1. INTRODUCTION

All the normal functions of the cells in our body are under a strict control of regulatory signaling networks. If a cell escapes this control, growing unmanageable, it may lead to formation of a tumor or eventually with malignant events, into cancer (Weinberg 2014). The initial cause leading to cancer is accumulation of several mutations in a single cell (Hanahan and Weinberg 2011). The development of cancer through numerous mutations may take decades, allowing time for different combinations of gene alterations to occur (Macaluso et al. 2003). Thus instead of a single disease, cancer is a group of diseases characterized by uncontrolled cell growth and spreading to other sites of the body. Genomic alterations may arise for example from gain-of-function mutations of proto-oncogenes or loss-of-function mutations of tumor suppressor genes (Macaluso et al. 2003). Also genome structure or copy number changes, as well as epigenetic changes have critical roles in malignant tumor progression (Gray and Collins 2000). One of the most common tumor types is breast cancer, which is also the leading cause of cancer-related death in women worldwide (Siegel et al. 2013). It is estimated that in Western countries even 1 out of 8 women will develop this malignancy during their lifetime, making it a globally remarkable burden (Jemal et al. 2008).

The small non-protein coding RNAs - microRNAs (or miRNAs) - are known to be aberrantly expressed or mutated in cancer (Iorio and Croce 2012). These short (~22 nt) RNA molecules target messenger RNA (mRNA) and thus control gene expression at post-transcriptional level. miRNAs are involved for example in regulating cell growth, differentiation and apoptosis (Esquela-Kerscher and Slack 2006). Being capable of modulating even hundreds of target genes, it is not surprising that alteration of miRNA expression is implicated in tumorigenesis (Lujambio and Lowe 2012).

Bone morphogenetic proteins (BMPs) are signaling molecules that have been intensively studied in cancer. One member of this protein family, BMP4, is known to be essential in development, contributing to formation of several tissues, including the lungs, kidney, limbs and also mammary gland (Cho et al. 2006, Kallioniemi 2012). BMP4 has also been linked to breast cancer where it shows interesting effects to breast cancer phenotypes. At the same time, BMP4 decreases cell growth but also increases migration and invasion in breast cancer cells, thus acting as a dualistic regulator (Ketolainen et al. 2010. In addition, increased expression of

BMP4 was linked to decrease in growth and recurrence of primary breast tumors (Alarmo et al. 2013).

The mode of action of BMP4 has not yet been revealed, although some of the target genes of BMP4 have already been identified by Rodriguez-Martinez and colleagues (2011). Our research group has also studied the deregulated miRNAs induced by BMP4 treatment (Alarmo et al. 2015). In this thesis, I explore whether the selected miRNAs, which are upregulated by BMP4, contribute to BMP4-induced phenotypes. This information would help to understand the molecular background of breast cancer progression, especially of cancer cell growth and migration.

2. REVIEW OF THE LITERATURE

2.1 Breast cancer

According to the GLOBOCAN 2012 there were estimated 14.1 million new cancer cases in 2012, whereas the number in 2008 was 12.7 (Ferlay et al. 2013). The most common cancer in men was lung cancer and in women breast cancer. Breast cancer is thus one of the most common cancers with 1.7 million new cases diagnosed annually worldwide. Breast cancer is also diagnosed in men, but only a fraction the patients are male (Siegel et al. 2013). The worldwide incidence rates have been relatively stable since the end of the 1990's, but mortality rates have decreased because of earlier detection and improvements in treatment (Siegel et al. 2013). Even nine out of ten diagnosed with breast cancer are alive after five years' time (SEER 2014). However, due to the high prevalence, breast cancer remains to be the leading cause of cancer death among women in less developed countries and the second leading cause of cancer death in more developed regions (Jemal et al. 2011). The main part of breast cancer -related deaths is caused by metastatic disease, which means the spreading of the tumor from the original site to the other parts of the body (Christofori 2006). Typical for metastasis is epithelial-to-mesenchymal transition (EMT) where cancer cells acquire mesenchymal cell -like characteristics developing into more invasive phenotype (Christofori 2006). EMT is normally linked to progression of cancer as only about 20% of cancer patients with metastatic disease are alive after five years' period (Weigelt et al. 2005).

The human breast consists of fat and connective tissue, and is composed of approximately twenty lobules which are connected to the nipple via milk ducts (Ross and Wojciech 2006). Breast cancer most often originates from hyperproliferation of the epithelial tissue of these ducts and lobules, at first leading to in situ carcinoma and finally into metastatic cancer (Polyak 2007). Ductal and lobular carcinomas are two of the most common histopathological classes of breast cancer, comprising approximately 90% of all cases, while the remaining 10% are rare histological types (Weigelt et al. 2010). In addition to histopathological classification, several clinical factors as well as the hormone receptor status are used to describe breast cancer (Schnitt 2010). Important clinical features of the tumor, for example, are the size and the metastatic status of the original tumor (Shah et al. 2014). Furthermore, the expression of hormone receptors ER (estrogen receptor) and PR (progesterone receptor) and the human epidermal growth factor receptor 2 (HER2, also called as ERBB2, receptor tyrosine-protein kinase) are

used in classification (Koboldt et al. 2012). Based on these receptors, breast cancer has been divided clinically roughly in to three therapeutic groups: the ER or PR positive group, HER2 amplified group and in triple negative breast cancers (TNBC) that do not express ER, PR and HER2 (Koboldt et al. 2012). Sørlie and colleagues (2003) classified breast cancer intrinsically in five molecular subtypes based on the gene expression profiles of breast tumor samples. These subtypes are luminal A, luminal B, basal like, normal breast like and HER2-enriched. Both luminal A and B subtypes are ER-positive. Molecular level differences in breast cancer subtypes appear as distinct clinical outcomes and as different kind of responses to treatment. Usually, the basal like and HER2-enriched tumors have the worst, and luminal A-type tumors the best prognosis (Sørlie et al. 2003).

Breast cancer has multiple hormonal and non-hormonal risk factors. Most of the hormonal risk factors are related to the time of exposure to estrogen. For example late age at menopause, early age of menarche, a low number of pregnancies and a short term of breast feeding all prolong the exposure time of breast tissue to estrogen and thus increase the risk of cancer (Fasching et al. 2011). The non-hormonal risk factors include age, alcohol consumption, dense breast tissue and obesity (Shah et al. 2014). The risk is also increased with positive family history of the disease, which is the case for approximately 20-25% of the patients (Shah et al. 2014). Of these, only 5-10% show autosomal dominant inheritance.

Genetic susceptibility to breast cancer is highly increased with some mutations. The most prominent risk factors are mutations of *BRCA1* or *BRCA2*, both of which are important tumor suppressor genes (Roy et al. 2012). BRCA1 and BRCA2 proteins function in double-stranded DNA repair, and a mutation in one copy of a gene increases the risk of breast cancer by 50-80% (Roy et al. 2012). Also mutations in other DNA repair genes as DNA damage response kinases (*CHK2*) or ataxia-telangiectasia mutated (*AMT*) slightly increase the risk of breast cancer (Roy et al. 2012). Other breast cancer susceptibility genes are tumor suppressor gene *TP53*, phosphatase tensin homolog (*PTEN*) and serine–threonine protein kinase 11 (*SKT11*) (Economopoulou et al. 2015). Sporadic mutations - mostly amplifications - in genes such as *HER2*, *MYC* or *CYCLIN D* are frequently observed in breast cancer comprising approximately 90% of all breast cancer cases (Rizzolo et al. 2011, Shah et al. 2014). Other alterations include epigenetic changes such as DNA methylation and changes in microRNA (miRNA) expression (Rizzolo et al. 2011).

2.2 Small non-coding RNAs

Only a small portion of human genome is translated into proteins while it is known that most part of the genome is transcribed into RNA (Lander et al. 2001, Birney et al. 2007). RNAs have traditionally considered as molecules which are the intermediate between DNA and proteins, but recent studies have revealed that also the non-coding RNAs have a remarkable role in gene regulation.

The importance of small non-coding RNAs in gene regulation was discovered in the 1990's in a study of larval development of a nematode *Caenorhabditis elegans* (Carthew and Sontheimer 2009). Today it is known that small RNAs are involved in many cellular processes such as cell growth, differentiation and proliferation (Wilson and Doudna 2013). In gene regulation level they regulate for example mRNA stability and heterochromatin formation, and also function in translational control (Kim et al 2009).

There are several distinct classes of small non-coding RNAs, which are defined as non-coding RNAs that are commonly ~30 nucleotides in length (Morris and Mattick 2014). The most studied small RNAs in animals include miRNAs, small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs), but also new classes have been recently identified (Carthew and Sontheimer 2009, Morris and Mattick 2014). The effect of small RNAs is generally inhibitory and the mechanism is commonly known as RNA interference (Carthew and Sontheimer 2009). In this thesis, I'll concentrate on miRNAs, small non-coding RNAs which participate in post-transcriptional gene regulation. To date more than 2500 mature human miRNAs have been identified, and it is estimated that they regulate more than half of all the human mRNAs (Kozomara and Griffiths-Jones 2014, miRBase release 21 2014).

2.2.1 The structure and biogenesis of miRNAs

miRNAs are evolutionarily conserved small RNAs, about 18-22 nucleotide in length. They act together with specific proteins to exert their function in mRNA binding and subsequently in translational repression. The role of a miRNA in the protein complex is to guide the complex to an mRNA by base-pairing with it (Kim et al. 2009, Berezikov 2011). The most typical consequence of miRNA binding is eventually exonucleolytic degradation of mRNA, but in case of higher complementarity also endonucleolytic cleavage of mRNA is possible (Kim et al. 2009). The effect of miRNA action may vary from a binary switch-like mode to fine tuning of gene expression (Bartel et al. 2009).

Similarly to protein-coding genes, miRNAs are transcribed from the genome. Large part, approximately 40% of the miRNA loci, is located in the introns, and about 10% in exons of non-coding genes (Kim et al. 2009). The remaining 50% of the miRNA genes are estimated to be located in introns of pre-mRNAs of protein-coding genes. Approximately one third of all miRNAs show significant sequence homology and are thus grouped into distinct miRNA families (Berezikov 2011). Such miRNAs are often derived from gene duplication. In addition, miRNAs that differ only in their termini, arise from minor differences in miRNA hairpin processing (Berezikov 2011). Many of the miRNAs are located as clusters and are transcribed as a polycistronic unit separately of their host genes (Ozsolak et al. 2008). Some miRNA genes, which reside in introns of protein coding genes, share the promoter with the host gene (Ozsolak et al. 2008, Ha and Kim 2009).

MicroRNAs are processed into mature forms through several phases. miRNAs are first transcribed by RNA polymerase II in nucleus into primary transcripts called Pri-miRNAs (Winter et al. 2009). Pri-miRNAs are two-stranded structures, with length of few hundred to several thousand nucleotides and they contain stem-loop structures as well as single-stranded 5'- and 3'-terminal overhangs (Figure 1) (Kim et al. 2009, Saini et al. 2007). Similarly to messenger RNAs, many primary miRNAs have a 5'cap structure and a poly(A) tail (Winter et al. 2009).

In humans, the 3' and 5' overhangs of the pri-miRNA are removed in the nucleus by an enzyme called Drosha - an RNAse that specifically acts on double-stranded RNA - leading to the formation of precursor-miRNA or pre-miRNA (Carthew and Sontheimer 2009) (Figure 1). Drosha is part of a microprocessor complex, which also includes DGCR8 (DiGeorge syndrome critical region 8) protein. DGCR8 recognizes the boundary between single- and double-stranded RNA and thus facilitates Drosha to the right cleavage site (Kim et al. 2009). Interestingly, all miRNAs do not require the Drosha-mediated step in case they form as a result from mRNA splicing (Winter et al. 2009). These precursor structures are called miRtrons, referring to the formation from introns. Pre-miRNAs are transported from the nucleus to the cytoplasm, where the next events in microRNA biogenesis take place. Export is mediated by a member of the nuclear transport receptor family, Exportin-5, which binds the pre-miRNA together with the GTP-bound form of the cofactor Ran (Kim et al. 2009). With GTP hydrolysis, pre-miRNA is released to the cytoplasm.

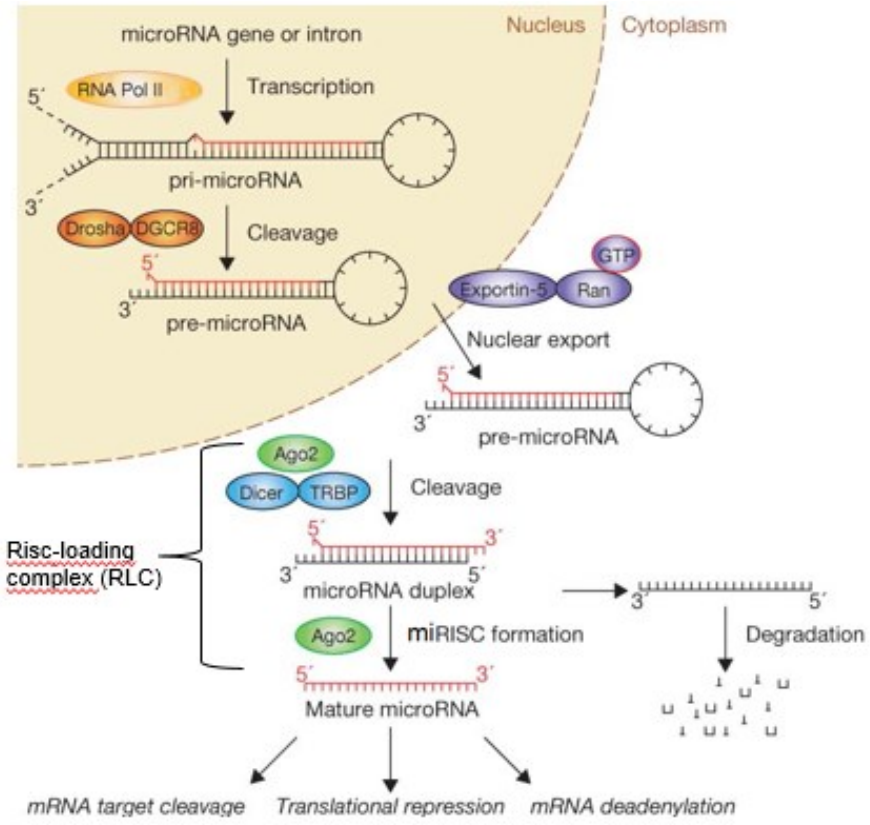

Figure 1. The canonical pathway of microRNA biogenesis. miRNAs are transcribed from the genome to pri-miRNAs. Pri-miRNAs are further cleaved to pre-miRNAs which are exported to the cytoplasm. Next miRNA ends are processed, the duplex is rewound and the single-stranded short RNA is ready to be loaded to miRISC-complex. The miRISC complex can subsequently act in several post-transcriptional events (Figure modified from Winter et al. 2009).

The further cleavage of the pre-miRNA is carried out by an RNase III enzyme, Dicer, which is part of a miRISC-loading complex (RLC, where RISC stands for RNA-induced silencing complex) (Carthew and Sontheimer 2009, Meister 2013). The RLC is formed before the attachment of the miRNA (Winter et al. 2009). In addition to Dicer, RLC consists of an Argonaute family protein 1-4 (AGO1-4) and either the double-stranded transactivation-response RNA-binding protein (TRBP) or protein kinase R-activating protein (PACT) (Figure 1), which partly refine the substrate specificity of Dicer (Winter et al. 2009, Ameres and Zamore 2013). There are multiple ways that lead to the formation or the miRNA duplex, depending on the length and degree of complementarity of the partially two-stranded pre-miRNA. However, almost all known miRNAs follow the canonical miRNA biogenesis pathway (Ha and Kim 2014). In the canonical pathway, for miRNAs without high complementarity, the terminal loop of the ~70 nt pre-miRNA is directly cut by Dicer, so that an approximately 22 nt long duplex

RNA product is formed (Winter et al. 2009). If, however, the pre-miRNA shows high degree of complementarity, an additional cleavage step by AGO2 is required before Dicer function (Winter et al. 2009). This step is assumed to aid in a subsequent dissociation of the two strands. Out of the four AGO-proteins found in humans, only AGO2 is capable of endonucleolytic cleavage of mRNA and it only cleaves RNA in case of high complementarity (Ha and Kim 2014).

In addition to pre-miRNA processing, RLC mediates the formation of the miRNA-induced silencing complex (miRISC) that basically means the transfer of miRNA from Dicer to AGO (Winter et al. 2009). The transfer of the duplex is aided by heat shock protein 90 (HSP90) (Meister 2013). Because Dicer and TRBP/PACT are not needed in miRISC, they dissociate after pre-miRNA cleavage, leaving only the AGO and the miRNA duplex. However, in functional miRISC, there are accessory proteins such as trinucleotide repeat-containing gene 6 protein A-C (TNRC6A-C) (Ameres and Zamore 2013). TNRC6 probably aids in recruiting PABP, a poly(A)-binding protein that has a function in inhibition of translation initiation of mRNAs (Meister 2013).

Only one miRNA strand is required for the silencing complex to work, so at first the two strands must be separated (Kim et al. 2009). There is evidence from siRNAs that the thermodynamic properties of the duplex define which one of the strands remains in the complex, favoring the strand which has thermodynamically less stable 5' end (Kim et al. 2009). In the miRISC-complex, miRNA forms the mRNA recognition part and guides the complex to silence targeted mRNAs. The functional miRISC then works in translation repression and mRNA degradation (Pratt and MacRae 2009).

2.2.2 A Short introduction to miRNA nomenclature

Before continuing to examine the function of miRNAs, I would like to underline a few things about nomenclature, which may be somewhat confusing. We can take hsa-miR-20a-5p as an example. The first letters, typically three or four refer to the organism (here Homo sapiens). In this thesis, all miRNAs used in the experimental part are has-miRs. The mature miRNAs in databases are written as 'miR', whereas 'mir' refers to precursor hairpin form or the genomic locus. The number usually tells about the time of discovery, and the bigger it is the more recently it is found (Kozomara and Griffiths-Jones 2014). The miRNAs that are paralogs but differ only by one or two nucleotides, are referred to as "a" or "b", as miR-20a-5p in the example. However, if the mature miRNA sequences are the same, but are derived from different

genomic loci or different precursors, the original gene or precursor gets an additional digit (Kozomara and Griffiths-Jones 2014). As an example, miR-16-5p can be derived from the stem-loop structure mir-16-1 in chromosome 13 or alternatively from mir-16-2 in the chromosome 3 (miRBase release 21).

In the light of increasing evidence that both miRNA strands can be functional, the whole name of the miRNA should be denoted. Previously, the assumed functional strand was simply named miR-20a and the passenger strand miR-20a*, with an asterisk. However, the new guidelines advise to describe which arm of the hairpin-precursor the miRNA is derived from. If it is excised from 3'-arm, the miRNA is named -3p and consequently -5p if excised from the 5'-arm. (Griffiths-Jones et al. 2008, Kozomara and Griffiths-Jones 2014).

2.2.3 miRNA target recognition

One mRNA may have several miRNA binding sites and it is thus estimated that even 30% - 60% of the human mRNA is under influence of microRNAs (Lewis et al. 2005, Friedman et al. 2009). Equally, one miRNA can regulate multiple mRNAs (Lewis et al. 2005). The key nucleotides in target mRNA recognition are nucleotides 2-7 close to the 5′ end of miRNA, called the "seed" region (Iorio and Croce 2012). The binding sites in target mRNAs usually lay at the 3' UTR-regions, although binding to the coding region and 5' UTR regions have been reported (Brennecke et al. 2005, Bartel 2009). The seed may pair with mRNA with perfect Watson-Crick base pairing, often leading to target cleavage, but the most common scene in mammalian systems is partial recognition, with mismatches and bulges (Carthew and Sontheimer 2009, Pratt and MacRae 2009).

2.2.4 The function of microRNAs

miRNAs are known to trigger the degradation of mRNAs and act in translational repression. However, the exact mechanism of translational repression, as well as the timing of these two processes, remains to be unresolved. Recent studies suggest that the primary action of miRISC complex is interestingly translational repression, which is often followed by mRNA degradation (Fabian and Sonenberg 2012, Hu and Coller 2012, Wilczynska and Bushell 2015). However, there is still debate, whether miRNA degradation instead, would be the principal method of miRNA-induced silencing (Ameres and Zamore 2013). These two events can also be uncoupled, meaning that the translational repression can occur in the absence of mRNA deadenylation or destabilization.

It seems that in most cases, silencing occurs by inhibiting translation initiation (Wilczynska and Bushell 2015). The exact mechanism is not yet known, but miRISC is for example suggested to facilitate the dissociation of translation initiation factors, for example eIF4AI and eIF4AII (eukaryotic translation initiation factor 4AI and AII, respectively) from the mRNA (Fukao et al. 2014). miRNAs can also interfere with the initiation of translation by inhibiting the formation of the closed-loop mRNA (Petersen et al. 2006, Fabian and Sonenberg 2012). The closed-loop structure is formed between the poly(A) tail at the 3' end and the cap-structure at 5' end with help of accessory proteins. These accessory proteins are PABP, which associates with poly(A) tail, an eIF4E transcription factor which interacts with the cap structure and another translation initiation factor, eIF4G (Derry et al. 2006). The miRISC complex with bound TNRC6, is able to inhibit the circularization of mRNA, probably by competing with PABP binding (Wilson and Doudna 2013). In addition, miRISC may also inhibit the assembly of translation machinery by competing with cap-binding protein eIF4E, which is required for cap-dependent translation (Kiriakidou et al. 2007). Furthermore, eIF6 is suggested to be bound by AGO2 thus preventing the two ribosomal subunits to associate, leading to inhibition of translation initiation (Chendrimada et al. 2007). In addition to inhibiting the initiation of translation, there are also studies which suggest miRNA-induced repression in the elongation phases of protein synthesis. For example, it is proposed that the miRISC complex causes actively translating ribosomes to drop off from mRNA by binding to the nascent mRNA chain (Petersen et al. 2006).

The mechanism of mRNA degradation is quite well understood. A miRISC component TNRC6 may lead to the recruitment of either of the two deadenylase complexes CCR4-NOT or PAN2–PAN3 (Wilczynska and Bushell 2015). Together with miRISC, these proteins direct the mRNA for degradation by deadenylation (Eulalio et al. 2008, Wilczynska and Bushell 2015). In addition, this complex is able to recruit a decapping activator protein, DDX6, leading to the removal of the 5' cap structure and exonucleolytic decay of the mRNA (Rouya et al. 2014). The level of miRNA-induced mRNA degradation is believed to depend of the strength of interaction between miRNA and mRNA (Eulalio et al. 2008, Wilczynska and Bushell 2015). To summarize, recent findings propose that miRNAs usually act by inhibiting the initiation of translation or by guiding the mRNA for degradation.

2.2.5 Regulation of microRNA levels

MicroRNAs are regulated at multiple levels from transcription to their degradation (Winter et al. 2009). Because there are such many ways of regulation, only few, the most important are presented here. The first phases of regulation are at the level of miRNA transcription, which is controlled by transcription factors and epigenetic regulators associated with RNA polymerase II (Ha and Kim 2014). Similarly to protein coding genes, miRNAs have promoter sequences which are recognized by specific transcription factors. For example, Davis and colleagues (2010) showed that SMADs, the transducers of bone morphogenetic protein (BMP) signaling pathway may directly regulate miRNAs by binding to miRNA promoters. The promoter sequences of miRNAs were similar to SMAD-binding elements in protein coding genes that are regulated by BMP4 and transforming growth factor β (TGF-β) (Davis et al. 2010). Most of the promoter sequences of miRNAs, however, are still unknown (Ha and Kim 2014).

It has been shown, that miRNA genes are under the influence of epigenetic control such as DNA methylation and histone modifications (Lujambio and Esteller 2009). Methylation usually results in gene silencing, thus hypermethylation of miRNA promoters may lead to downregulation of some miRNA genes. Some of the miRNA genes are known to be methylated in a cancer specific manner (Wang et al. 2013). Histone modifications are mainly studied using histone deacetylase inhibitors (HDAC), and in such studies it has been shown that HDACs often increase miRNA levels (Wang et al. 2013). Thus miRNA expression levels can be modified by targeting the epigenetic machinery.

The microRNA processing phase is regulated most importantly by Drosha activity and specificity. Drosha and its partner DGCR8 can be modified post-transcriptionally to affect their stability, localization or affinity to miRNAs (Ha and Kim 2014). Furthermore, Drosha processing is regulated by SMAD transcription factors. For example the expression of miR-21 is regulated by TGF-β and BMPs by their downstream signaling molecule SMAD which binds to pri-miRNA facilitating its processing by Drosha (Davis et al. 2008). In addition, Drosha and DGCR8 are susceptible to autoregulation, where DGCR8 stabilizes Drosha and Drosha in turn destabilizes DGCR8 (Han et al. 2009).

Pre-miRNAs and Dicer-dependent cleavage, in turn, can be regulated by binding of the accessory proteins. Proteins such as TRBP and PACT are not essential for Dicer to work properly, but it seems that their presence or absence affect miRNA processing (Winter et al. 2009). Yet another regulation step occurs in additional AGO2-mediated cleavage of the pre-

miRNA duplex before Dicer processing (Winter et al. 2009). This step is thought to aid in miRNA duplex formation. The Drosha and Dicer -mediated cleavage may produce miRNAs that differ only by nucleotides in their termini (Berezikov 2011). These closely related small RNAs are called isomiRs. Usually one form of the isomiRs is clearly dominating and it is thus annotated as the mature miRNA sequence (Berezikov 2011).

The final regulation steps in miRNA pathway are editing of the double stranded miRNA and passenger strand degradation. microRNAs are susceptible to A-to-I editing, which means converting an adenosine residue to inosine in the double-stranded RNA (Berezikov 2011). This conversion alters the structural properties of the miRNA transcript and it may have effects on Dicer or Drosha recognition, target gene recognition or even miRNA degradation (Kume et al. 2014, Winter et al. 2009). However, these modifications are quite rare (Berezikov 2011). In addition, generally only one miRNA strand is retained and the other, the passenger strand is degraded. However, it is known that also the passenger strands may accumulate in cells, bind to AGO-proteins and regulate target mRNAs (Berezikov 2011). The strand selection provides one regulation step more because some tissues may favor the 5' -arm over the 3'-arm or vice versa (Berezikov 2011). This kind of arm-switching may be caused by alternative splicing by Drosha, as explained earlier in the text. All in all, miRNAs are regulated at multiple levels, making it possibly to fine-tune target gene expression.

2.2.6 microRNAs in cancer

Keeping in mind the fact that miRNAs coordinate many cellular processes and target a vast amount of mRNAs, it is not a surprise that aberrant miRNA expression contributes to diseases such as cancer. The first miRNAs that were studied to be involved in cancer were miR-16-1 and miR-15a in chronic lymphocytic leukemia (CLL) as the deletion of these miRNAs was shown to cause CLL (Croce 2009). This finding led to mapping of miRNA-genes and it was noticed that several miRNA-genes were located in chromosomal regions that commonly have many genetic alterations, such as amplifications or deletions (Croce 2009, Farazi et al. 2011). In addition, miRNAs located in common chromosomal breakpoints are found to be associated in development of malignancies (Farazi et al. 2011, Lujambio and Lowe 2012).

Abnormal expression levels of miRNAs, likewise in protein-coding genes, may lead to malignant transformations. If the normal function of a miRNA – for example - is to repress an oncogenic protein, the deletion of such miRNA would lead to the increase of the oncogenic protein, possibly leading to cancer. Conversely, amplification of a miRNA, which targets a

tumor suppressor mRNA, would lead to decrease of the tumor suppressor mRNA and again, to malignancies. Similarly to protein coding genes, also miRNAs can be called as oncogenes or tumor suppressor genes depending on the targets and context (Lujambio and Lowe 2012). However, due to high complexity and multiple targets Croce (2009) suggests that miRNAs should be described with those terms only if the tissue or the studied cell type is specified. The reason for this is the fact, that some miRNAs acts as a tumor suppressor in some carcinomas, but have oncogenic effect in others (Lujambio and Lowe 2012).

Dysregulated miRNA levels may also result from elevated levels of growth factors or transcription factors or decrease in core enzymes of the miRNA biogenesis pathway (Chang et al. 2008, Lujambio and Lowe 2012). In addition, epigenetic changes, as altered patterns in DNA methylation or histone deacetylation might lead to deregulated expression of miRNAs (Farazi et al. 2011). To make the function of these small RNAs even more complicated, miRNAs often target the same oncoproteins, which also regulate miRNAs, for example BCL2, RAS and MYC (Iorio and Croce 2012, Lujambio and Lowe 2012). This makes the feedback mechanisms and complicated regulatory circuits possible.

Due to abnormal expression compared to normal tissue, microRNAs could act as potential diagnostic or prognostic biomarkers for cancer (Iorio and Croce 2012, Shen et al. 2013). Good biomarkers are stable, accurate and easily accessible. These criteria are met by miRNAs, which are shown to be rather stable and, in addition to extraction from solid tumors, miRNAs can be extracted from body fluids, for example blood, urine or saliva (Iorio and Croce 2012, van Schooneveld et al. 2015). Several studies have shown that miRNA signatures reliable distinguish healthy controls from patients with cancer (Shen et al. 2013, van Schooneveld et al. 2015). Furthermore, miRNA profiles can identify the origin of poorly differentiated tumors and in some cases miRNA signatures can even discriminate between different tumor subtypes (Iorio and Croce 2012, van Schooneveld et al. 2015). In addition, miRNA signatures may help to determine therapeutic targets for distinct tumor types (Singh et al. 2013).

2.2.7 miRNAs in breast cancer

In recent years, miRNAs have also been implicated in breast cancer initiation, progression and metastasis (Singh et al. 2013, Pérez-Rivas et al. 2014, Shah and Chen 2014). Most studies either examine the effect of one particular miRNA on breast cancer cell lines or tumor samples or alternatively the studies try to reveal the expression profile of distinct breast tumors. Some of the most commonly studied miRNAs in breast cancer are miR-10, miR-21 and miR-17~92

cluster together with let7 and miR-200 families (Singh et al. 2013, Serpico et al. 2014). For example, upregulated miR-10b, miR-21 and miR-17~92 cluster are often associated with increased cell migration and invasion, whereas let7 and miR-200 family of miRNAs are often deleted of downregulated in breast cancer which thus contributes to tumorigenesis (Singh et al. 2013, Serpico et al. 2014).

2.3 Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) belong to the TGF-β family of growth factors that are essential regulators of growth and development (Miyazono et al. 2010). The name BMP stems from the fact that these proteins were originally found to induce the formation of cartilage and bone (Miyazono et al. 2010). Later it became clear that these molecules have multiple functions in morphogenesis of various tissues and organs (Hogan 1996). For example, BMP-null mice showed that BMP-signaling is required for normal development of the mesoderm, eye, kidney and heart (Hogan 1996).

There are more than 20 BMP ligands which are named either as BMPs or as growth and differentiation factors (GDF) (Sieber et al. 2009, Miyazono et al. 2010). All BMP molecules share a cysteine knot motif which is formed between six highly conserved cysteines to stabilize the monomer (Sieber et al. 2009). The functional signaling molecule, however, is a dimer. The dimerization occurs through a seventh cysteine (Nohe et al. 2004).

2.3.1 BMP receptors and mechanism of signaling

All TGF-β family proteins share a common signaling pathway that includes serine/threonine kinase receptors (Miyazono et al. 2010) (Figure 2). These receptors are transmembrane proteins consisting of three domains: the extracellular domain that interacts with the ligand, one transmembrane domain and an intracellular domain that acts as a kinase (Sieber et al. 2009). Serine/threonine kinase receptors act as a pair, consisting of both type I and type II receptors (Sieber et al. 2009). There are three of type I and three of type II receptors which bind BMP and all of which are highly homologous. Type I receptors include BMP receptor type IA (BMPRIA or ALK3) BMPR receptor type IB (BMPRIB or ALK6) and activin A receptor type I (ACVR1 or ALK2) and the type II receptors are BMP receptor type II (BMPR2), activin A receptor type IIA (ACVR2A) and activin A receptor type IIB (ACVR2B) (Nohe et al. 2004, Sieber et al. 2009). The diversity of the receptors, the affinity of a BMP molecule to the receptor

complex and the availability of the ligands as well as other interacting molecules all contribute to the versatility of BMP signaling (Sieber et al. 2009).

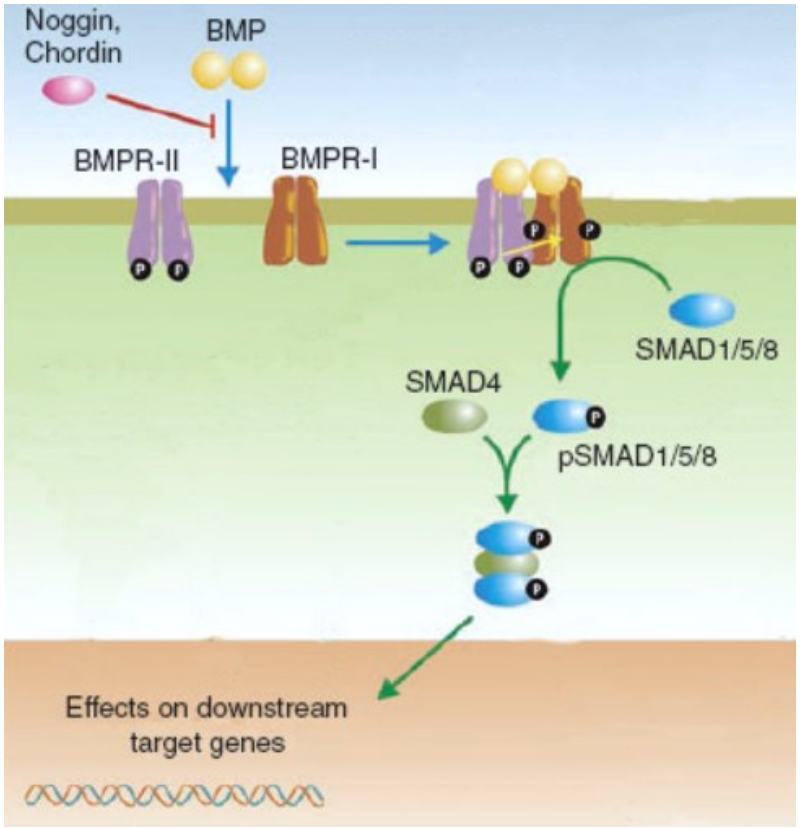

Figure 2. **The canonical pathway of BMP signaling.** BMPs are extracellular signaling molecules that exert their function through type I and II serine/threonine kinase receptors. BMP signaling requires the formation of heterotetrameric receptor complex and SMAD transcription factors. BMP signaling can be regulated by antagonists such as Noggin and Chordin (Modified from Anderson and Darshan 2008).

The downstream signaling of BMP pathway requires the presence of both types of receptors. The type II receptor has a constantly active kinase domain. When a BMP dimer binds, the type II receptor undergoes conformational changes that leads to the recruitment and phosphorylation of the type I receptor (Gordon and Blobe 2009). This, in turn, leads to the activation of the serine/threonine kinase activity of the type I receptor (Gordon and Blobe 2009, Sieber et al. 2009). Now that the type I receptor can act as a kinase, it is ready to phosphorylate other molecules.

The canonical BMP signaling route includes SMAD transcription factors. There are three types of SMADs, which are cytosolic receptor-regulated SMADs (R-SMADs), common

mediator SMAD (co-SMAD) and inhibitory SMADs (I-SMADS). R-SMADs include SMAD1, 5 and 9 (previously known as SMAD8), co-SMAD is called also as SMAD4 and I-SMADs include SMAD6 and SMAD7 (Sieber et al. 2009, Miyazono et al. 2010). R-SMADs have two conserved domains, MH1 and MH2. MH1 is responsible for DNA binding and MH2 has multiple functions in recognizing BMP receptors, other SMADs and mediating nuclear import (Sieber et al. 2009). MH2 is also the place for type I receptor phosphorylation. When unphosphorylated, these two domains interact rendering the molecule inactive. Upon R-SMAD phosphorylation by type I receptor, MH1 and MH2 domains are exposed and the affinity to SMAD4 increases and the nuclear import signal is revealed (Miyazono et al. 2010). When the complex between R-SMAD and SMAD4 forms, it can translocate into the nucleus, influencing gene expression (Miyazono et al. 2010). The affinity and selectivity to target DNA site depends on other DNA-binding factors and the core SMAD complex (Massagué et al. 2005).

BMP signaling can be regulated by both intra- and extracellular molecules. Intracellular regulator include I-SMADs, which are antagonists of R-SMAD and co-SMAD signaling (Miyazono et al. 2010). I-SMADs prevent the activation of R-SMADS by competing with association to type I receptor or they may form non-functional complexes with co-SMAD (Miyazono et al. 2010). In addition to I-SMADs, intracellular regulator molecules include for example SMAD ubiquitin regulatory factors (SMURFs) and protein phosphatases (Ali and Brazil 2014). SMURFs may target R-SMADs for degradation and they also act together with SMAD7 in inhibition of the SMAD pathway (Suzuki et al. 2002). Phosphorylation is regulated by Protein phosphatase-1 (PP1), which dephosphorylates BMP receptors (Ali and Brazil 2014). In addition, metal ion-dependent protein phosphatase 1A (PPM1A) may dephosphorylate, and thus inhibit R-SMADs. Extracellular regulators include BMP antagonists such as Noggin, Chordin and CAN (Cerberus and DAN) family of proteins (Walsh et al. 2010). These antagonists bind directly to BMPs, inhibiting their association with the receptors. The downstream effects of BMP signaling depend on concentration of both, BMPs and their inhibitors and antagonists, as well as plethora of other factors (Walsh et al. 2010).

2.3.2 BMPs in cancer

Members of TGF-β superfamily regulate targets genes, most of which are related to growth, differentiation, and apoptosis of several cell types (Kawabata et al. 1998). Alterations in TGF-β family proteins may thus result in various diseases, including cancer (Gordon and Blobe 2008, Ehata et al. 2013). The most prominent example of growth factor participation in cancer is

juvenile polyposis syndrome, a disease of the gastrointestinal tract (Howe et al. 2001). This illness is caused by germline mutation in BMP receptor 1A (*BMPR1A)* or *SMAD4* genes in about 50% of the cases (Schreibman et al. 2005). In addition to juvenile polyposis syndrome, genes of BMP signaling pathway are known to be mutated also in pancreatic and colon cancers (Massagué et al. 2000). *SMAD4*, for example, is mutated in half of pancreatic cancers, in one third of metastatic colon cancers as well as in a small subset of other carcinomas (Massagué et al. 2000). Although the aforementioned studies clearly demonstrate the connection between BMPs and cancer, only a few studies on sporadic mutations with BMP ligands have been reported. As an example, genetic variation in *BMP2*, *BMP4* and in some genes coding for BMP receptors, has been associated with higher risk of developing colon cancer (Slattery et al. 2012).

Aberrant BMP expression levels are often observed in many common malignancies including prostate, pancreatic and lung cancers as well as carcinomas of the breast (reviewed by Alarmo and Kallioniemi 2010, Singh and Morris 2010 and Ehata et al. 2013). BMP6 overexpression, for example, has been encountered in prostate cancer (Yuen et al. 2008), and overexpression of BMP2 in pancreatic and lung cancer (Kleeff et al. 1999, Langenfeld et al. 2003). In addition, Alarmo and colleagues (2007) showed that BMP4 and BMP7 are commonly expressed at high levels in breast cancer cells lines and primary tumors. These studies indicate the importance of BMPs in promotion of cancer, opening a field to study the downstream effects of BMPs.

The functional effects of BMPs have been mainly studied using cancer cell lines or animal experiments. Most of the studies show overexpression of BMPs, and demonstrate that BMPs influence the proliferation, migration, and metastasis of cancer cells (Alarmo and Kallioniemi 2010, Singh and Morris 2010, Ehata et al. 2013). The influence in proliferation is often seen as growth inhibitory effects. BMP2 and BMP4, for example, are shown to inhibit the proliferation of breast and prostate cancer cells (Brubaker et al. 2004, Ketolainen et al. 2010). However, the effects of BMPs vary depending on the type of tumor or cell line (Alarmo and Kallioniemi 2010, Singh and Morris 2010). This is demonstrated well in a study by Alarmo and colleagues (2007), which showed that out of the eight studied breast cell lines, BMP7 increased growth in two, decreased growth in four and had no effect in two. The effect of BMPs on cancer cell migration and invasion shows variability as well. BMP2, -4 and -7, for example are suggested to promote metastasis, EMT and invasiveness of most of the cancers (Singh and Morris 2010). However, in breast cancer BMP6 and BMP7 have been shown to have tumor suppressing

effects (Du et al. 2009). Taken together, the studies demonstrate that BMPs show tumor suppressing and tumor promoting effects in a context depending manner. Similar effect is seen in TGF-β, which acts as a tumor suppressor but at the same time promotes tumor metastasis (Ikushima and Miyazono 2010, Lebrun 2012). The tumor suppressive effects are seen in early carcinomas but the tumor promoting effects such as EMT, migration and invasion are observed in more aggressive tumors (Lebrun 2012).

2.3.3 BMP4 in breast cancer

Similarly to other BMPs, BMP4 is essential in development since it was shown that most BMP4-homozygous mice die soon after fertilization (Hogan 1996). Furthermore, BMP4 was shown to be important in hematopoiesis, and to act in a strictly regulated manner with other signaling molecules in formation of for example embryonic ectoderm, lungs, kidney, teeth and eye (Hogan 1996). It is interesting that BMP4 has also shown to be involved in mammary gland formation (Cho et al. 2006).

BMP4 protein expression has been associated with several types of cancer, including melanoma, ovarian and renal cell carcinomas as well as carcinoma of the breast (Kallioniemi 2012). In breast cancer, most of the studies report elevated levels of both BMP4 mRNA and protein, in cancer cell lines and in patient samples (Ketolainen et al. 2010, Guo et al. 2012, Alarmo et al. 2013). Increased levels of BMP4 usually inhibited cell growth, but nevertheless increased migration and invasion (Ketolainen et al. 2010, Guo et al. 2012). The growth inhibitory effect was indicated to be due to the G1 arrest and the migration was suggested to be partly caused by overexpression of matrix metalloproteinase-1 (MMP-1) and C-X-C chemokine receptor type 4 (CXCR4) (Ketolainen et al. 2010, Guo et al. 2012). The BMP4-induced decrease in proliferation and increase in migration was also seen in 3D culture system of breast cancer cells (Ampuja et al. 2013). Furthermore, similar dual role of BMP4 was observed in clinical breast cancer samples in which BMP4 expression associated with clinical parameters (Alarmo et al. 2013). This data is in good concordance with studies on breast cancer cells lines on BMP4-induced phenotypes.

The expression of BMP4-induced genes was studied in a time-dependent manner by Rodriguez-Martinez and colleagues (2011). This study showed that BMP4 first induces the transcription of genes that are involved in regulation of transcription and development, signal transduction and cell metabolism. The induction of genes controlling metabolic processes was prominent also at later phases, following the induction of genes involved in proliferation

(Rodriguez-Martinez et al. 2011). Among the genes with highest expression were *PTPRG* (protein tyrosine phosphatase, receptor type, G) and *DUSP2* (dual specificity phosphatase 2) which both are inked to carcinogenesis (Rodriguez-Martinez et al. 2011).

In addition to protein coding genes, BMP4 regulates the expression of microRNAs. In a study by Davis and colleagues (2008) BMP4 was shown to induce miR-21 production in human vascular smooth muscle cells, leading to a contractile phenotype (Davis et al. 2008). Furthermore, BMP4 repressed multiple members of the miR-302 family of miRNAs in pulmonary artery smooth muscle cells (Kang et al. 2012). Reciprocally, miR-302 was shown to inhibit BMPRII thus creating a feedback loop for BMP-miRNA signaling. There are yet no studies on BMP4-induced microRNAs in breast cancer, which makes it an interesting subject. In our research group, we have performed a miRNA microarray which studied BMP4-induced microRNAs in seven breast cancer cell lines and one immortalized breast epithelial cell line (Alarmo et al. 2015).

3. AIMS OF THE STUDY

The main goal of this work was to find out whether the selected microRNAs are behind the BMP4-induced phenotypes - decreased proliferation and increased migration. These phenotypes are caused by changes in expression of both protein coding genes and miRNA genes. To identify miRNA genes whose expression is altered in response to BMP4 treatment, our research group performed a microRNA screen. This screen revealed several deregulated miRNAs and eight with highest level of upregulation were selected for closer examination in this thesis. The knowledge on BMP4-induced miRNAs would increase our molecular level understanding of breast cancer with deregulated BMP4 expression.

The work was divided in two parts, validation of the miRNA microarray data and the functional studies.

1) In the *validation* part, the purpose was to quantitate the expression levels of the eight miRNAs using quantitative real time PCR (qRT-PCR). These results, were used to select ideal candidates for the functional characterization.
2) The purpose in the *Functional characterization* was to evaluate the functional contribution of the selected miRNAs on BMP4-induced phenotype - cell growth reduction and induction of migration. In this part of the study, proliferation and migration assays using anti-miRNA inhibitors together with BMP4 or vehicle were performed. These assays were expected to show what kind of role the selected miRNAs have on breast cancer cells phenotype.

4. MATERIALS AND METHODS

4.1 Cell lines and culture conditions

BT-474 and MD-MB-231 cell lines were obtained from ATCC and cultured according to standard conditions. Shortly, growth medium was changed three times per week and cells were subcultured when at 80 - 90% of confluence. The growth medium included either L-15 (MDA-MB-231 in T75 bottles) or DMEM (BT-474 and MDA-MB-231 in 24-well plates) as a basal medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, all purchased from Sigma-Aldrich (St. Louis, MO, USA). The BT-474 medium also included 1 mM sodium pyruvate and 4 mg/ml D-(+) glucose (both from Sigma-Aldrich). In addition, the following breast cancer cell lines from previously performed miroRNA microarray experiment were studied here: MCF-7, MDA-MB-231, MDA-MB-361, MDA-MB-436, HCC1954, BT-474, T-47D as well as an immortalized breast epithelial cell line, MCF-10A.

4.2 BMP4 treatment and microRNA inhibition

For the functional studies cells were seeded on 24-well plates (25,000 cells/well for MDA-MB-231 or 50,000 - 65,000 cells/well for BT-474). After 24 hours (day 1) growth medium was replaced with medium containing recombinant BMP4 (100 ng/ml) (R&D Systems, Minneapolis, MN, USA) or equivalent volume of vehicle (4 mM HCL in 0,1% BSA). Simultaneously with BMP4 or vehicle treatment, cells were transfected with anti-miR inhibitors (30 nM) (Ambion for LifeTechnologies, Carlsbad, CA, USA) or control siRNA for luciferase (siLuc) gene (30 nM) (Sigma-Aldrich). Because the studied cells do not code for the luciferase gene, no effect of siLuc transfection is expected to be observed.

Transfection was done using the non-liposomal cationic amphiphilic transfection reagent Interferin, (Polyplus-Transfection, San Marcos, CA, USA) in six replicates for each treatment combination. Shortly, 100µl of transfection mix, consisting of anti-miR inhibitors or siLuc and 3µl of Interferin in serum-free medium, was vortexed for 10 s and incubated for 15 min in RT. During the incubation, 500µl of fresh growth medium containing BMP4 or vehicle was added to the cells. Subsequently 100µl of transfection was added in each well. If the cells were to be studied also at 6 or 7 days' time point, fresh growth medium was changed to cells at day 4. The transfection, however, was not repeated at day 4.

4.3 RNA extraction

The RNA was extracted using either Agilent Absolutely RNA miRNA kit (Agilent Technologies) or Nucleospin miRNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Briefly, for Agilent's kit, the cell lysate was first loaded into a prefiltration column and centrifuged to remove genomic DNA. Next, RNA binding conditions were adjusted with 100% ethanol and the mixture was transferred to an RNA-binding spin cup, washed and treated with DNAse. The following three wash steps were done to remove any contaminants. Finally, the RNA in the column was eluted with 50µl of 65°C RNase-free H_2O and centrifuged. To maximize the RNA yield, the final elution step was repeated. All centrifugation steps were performed at 14,500 rpm for 1 - 2 minutes.

In the Nucleospin miRNA kit the lysate was first filtrated using a NucleoSpin Filter to clear and homogenize the lysate. In the next step 70% ethanol was used to adjust the binding conditions of RNA. The sample was then loaded onto NucleoSpin RNA column and both the flow-through, containing small RNAs (<200 nt) and protein, and the column, in which large RNA was bound were treated separately until the final wash steps. The column with bound large RNA was treated with desalting buffer following a DNA digestion step. The flow-through instead was treated with protein precipitation buffer and centrifuged to pellet the protein. The supernatant was then purified with NucleoSpin Protein Removal Columns and treated with binding buffer to optimize binding conditions for small RNA. Next the sample with small RNAs was loaded to the column containing the large RNA to obtain total RNA. This was followed by three wash steps and elution to 30 µl of RNase free H_2O. All centrifugation steps were performed at 11,000 - 14,000 g for 30 s - 2 min. The concentration of RNA was measured by spectrophotometry with Tecan infinite F200 PRO plate reader using a NanoQuant Plate (Tecan, Männedorf, Switzerland).

In addition to RNA samples extracted for this study, RNA extracted for previously performed miRNA microarray screen was used in the two-step real time (RT) PCR.

4.4 Two-Step RT-PCR for quantitation of miRNA expression levels

4.4.1 Reverse Transcription

Two-step RT-PCR includes the reverse transcription and the quantitative real-time PCR (qRT-PCR) steps. In the first part of two-step RT-PCR, miRNAs are selectively transcribed into cDNA using Taq-Man® MicroRNA Reverse transcription Kit and miRNA-specific looped

primers (both from Applied Biosystems of Life Technologies). The looped primers (Figure 3) detect only mature miRNAs and they can discriminate miRNAs with closely related sequences.

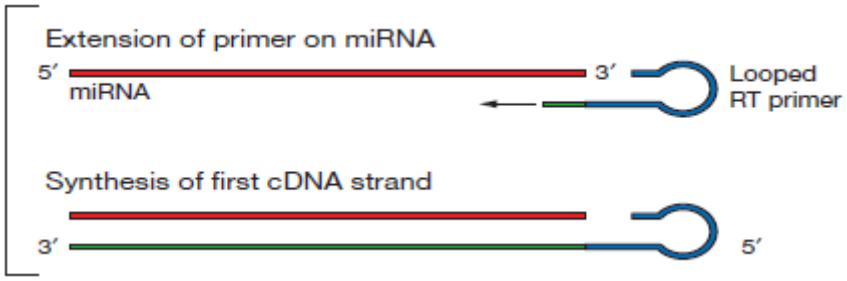

Figure 3. Reverse transcription with miRNA-specific looped primers. Looped primers detect only the short, mature miRNAs (Taqman small RNA assays protocol).

The reverse transcription reaction was performed according to manufacturer's instructions. Briefly, 10 ng of total RNA was combined in a 15 µl reaction with 1 mM dNTPs, 50 U MultiScribe Reverse Transcriptase, 1X Reverse Transcription buffer, 3.8 U RNase inhibitor and 3µl hairpin-looped miRNA-specific RT primers. The reaction was performed on a PTC-200 thermal cycler (Bio-Rad). The cDNA synthesis was carried out with following steps: 16°C for 30 min (priming), 42°C for 30 min (transcription) and 85°C for 5 min (enzyme inactivation).

4.4.2 Quantitative real-time (qRT) PCR

qRT-PCR of eight miRNAs (miR-16-5p, miR-17-5p, miR-20a-5p, miR-22-3p, miR-26a-5p, miR-29b-3p, miR-106b-5p and miR-141-3p) and two housekeeping genes (small nucleolar RNAs RNU44 and RNU48) was performed on a LightCycler 2.0 equipment (Roche) using miRNA-specific TaqMan® assays (Applied Biosystems of Life Technologies) which include miRNA-specific primers and fluorescent probes. The reaction mix contained 1X TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems of Life Technologies), 1X human miRNA-specific TaqMan® assays and 4 µl undiluted cDNA in a final volume of 20 µl. The reaction was run with the parameters seen in Table 1, using 45 or 50 cycles.

Ct values (Threshold cycle values) are obtained from the sample amplification plot of a qRT-PCR reaction (Figure 4). On the x-axis is the cycle number and on the y-axis is the fluorescence which is proportional to the amount of PCR product that is generated in each cycle. The plot is divided in two phases. In exponential phase the amount of PCR product is doubled in each cycle and in the plateau phase the reaction starts to slow down because of limited amount

of reagents (Bio-Rad Laboratories 2006). The Ct value is the intersection of the threshold line and the amplification plot. The threshold line indicates the cycle number at which the signal has accumulated enough to be separated from the background noise (Bio-Rad Laboratories 2006). Ct-value is inversely proportional to the amount of gene of interest.

Table 1. Program of the qRT-PCR reaction for amplification and detection of miRNAs.

Phase	**Temperature**	**Time**
Denaturation	95°C	10min
Amplification (45-50 cycles)	95°C	10sec
	60°C	60sec
	72°C (measurement)	1s
Cooling	40°C	30sec

The quantification of miRNAs was determined relative to a housekeeping gene that is expected to be transcribed at a relatively constant level. The relative miRNA expression was calculated using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (BMP4-treated) − ΔCt (vehicle-treated). ΔCt was calculated by subtracting the Ct-value of the housekeeping gene from the specific miRNA.

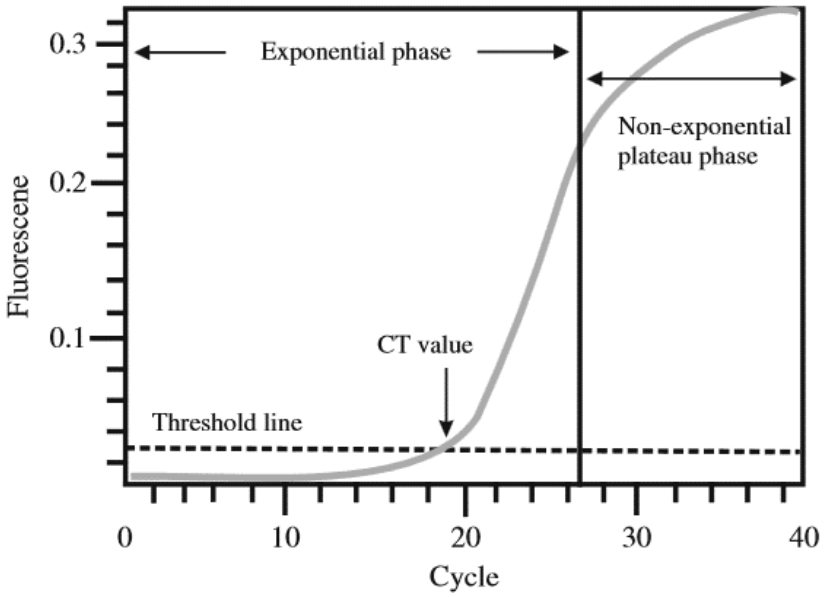

Figure 4. Ct value is obtained from the qRT-PCR amplification plot. It is the cycle number at the intersection of the threshold line and the amplification plot and it is inversely proportional to the amount of target nucleic acid (Bio-Rad Laboratories 2006).

4.5 miR-16-5p and miR-106b-5p functional studies

4.5.1 Cell growth assay

The number of BMP4-treated BT-474 and MDA-MB-231 cells was calculated to study the effect of miRNA inhibition on cell growth. Cells were counted 4 and 6 or 7 days after transfection from six replicate wells using Coulter Z1 Counter (Beckman Coulter, San Diego, CA). The counter uses Coulter Principle that is based on impedance measurement. Cells are suspended in low concentration electrolyte solution and the solution is passed through a small aperture in the wall of the measuring tube. There is an electrode inside and outside of the measurement tube. As an electric field is applied, the impedance of the electrolyte changes as particles flow through the aperture. The number of cells is obtained directly from the amount of changes in impedance. All assays were performed twice.

4.5.2 Migration assay

Cell migration was studied using a scratch assay. In this assay a scratch is applied to a confluent monolayer of cells on a 24-well plate using a pipette tip. The well is rinsed with PBS or cell culture medium to remove the detached cells and replaced with fresh medium. Next, the wells are imaged at chosen time points until scratches close up, and either the scratch width or migration area is used as a measure of cell migration.

Migration was studied only in MDA-MB-231 cells. After 24 hours of seeding, cells were treated with BMP4 or vehicle and simultaneously transfected with anti-miR inhibitors or siLuc as described in section *4.2 BMP4 treatment and microRNA inhibition*. When at confluence, at 4 - 7 days, a scratch was introduced to the cell monolayer with a 1 ml pipette tip. Fresh medium containing BMP4 or vehicle was added to cells at day 4 and after the scratch was applied.

Scratch assays were imaged every 2 hours for 30 hours using the Cell-IQ v.2 time-lapse imaging system (CM technologies, Tampere, Finland) equipped with a 0.13 NA/4x air objective (Nikon). During imaging cells were maintained at 37°C temperature and in 5% CO_2. Alternatively scratch was imaged with Olympus IX71 inverted microscope 0h, 6h and 24h after drawing the scratch. All treatment combinations were done in six replicate wells and two to eight images were taken from each scratch depending of the quality of the scratch. More images were taken from scratches with variable width or cells attached in the middle to obtain enough representative images. Regardless of the imaging method, all images were analyzed with Cell-IQ Imagen imaging analysis software, version MA2.2.0.1 (CM technologies) to identify the

area the cells had migrated. Migration area is a measure that describes the migration even if the scratch width is not the same. After the analysis with Cell-IQ software all images were visually checked for possible artifacts in image quality or analysis. The time point for analysis and comparison was chosen to be such, where the first scratches started to close up. All migration assays were performed twice.

4.6 Statistical analyses

Possible outliers were removed from cell proliferation measurements using GraphPad QuickCalcs online Outlier calculator that is based on Grubbs test ($p<0.05$). The statistical significance for proliferation and migration assays was evaluated in GraphPad Prism using Mann-Whitney test and student's t-test, respectively.

5. RESULTS

5.1 qRT-PCR studies on differentially expressed miRNAs

To study how BMP4 influences microRNA expression in breast cancer cells a miRNA microarray experiment was performed in our research group (Alarmo et al. 2015). For this microarray, seven breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-361, MDA-MB-436, HCC1954, BT-474 and T-47D) and one immortalized breast epithelial cell line (MCF-10A) were treated with BMP4 and vehicle, and subsequently the miRNA expression was studied. The microarray identified altogether 20 - 128 miRNAs per cell line, which were differentially expressed after the 4h BMP4 treatment in at least 2 cell lines. Of these, eight miRNAs with increased expression following BMP4 treatment (fold change, FC ≥1 in log2 scale, between BMP4 and vehicle-treated samples), were selected for qRT-PCR validation. These miRNAs were miR-16-5p, miR-17-5p, miR-20a-5p, miR-22-3p, miR26a-5p, miR-29b-3p, miR-106b-5p and miR-141-3p. In qRT-PCR, the same RNA samples were used as in the miRNA microarray.

The purpose of the validation was to study the BMP4-induced miRNA expression with an assay based on a different method. The output from the microarray was in some cases similar to the qRT-PC, however this did not apply to all studied cell lines (Figure S1 in Supplements). The cell line with the highest association to both methods was BT-474. BT-474 showed the highest level of upregulation of the eight selected miRNAs after the BMP4-treatment: six out of eight miRNAs (miR-16-5p, miR-17-5p, miR-20a-5p, miR-22-3p, miR-106b-5p and miR-141-3p) were upregulated both on the array and by the qRT-PCR. Out of the remaining two miRNAs, miR-26a-5p showed high expression on array, but not in qRT-PCR, whereas the expression of miR-29b-3p was low using both methods.

After the BMP4 treatment, MDA-361 showed high expression (FC ≥1) of all miRNAs on microarray, but only miR-22-3p and miR-29-b-3p were upregulated by qRT-PCR. Similar effect was seen in HCC1954 cell line, in which all studied miRNAs, apart from miR-26a-5p, were upregulated on array, whereas the only upregulated miRNAs in qRT-PCR were miR-20a-5p and miR-106b-5p. Part of the miRNAs, on the contrary, showed increased expression on qRT-PCR, but not on microarray. For example MCF7 and MDA-436 expressed only miR-29b-3p on array (FC ≥1), while six and five miRNAs showed increased expression by qRT-PCR, respectively. MicroRNA expression in T-47D was affected the least after BMP4 treatment,

because none of the miRNAs were upregulated with neither of the study methods. MDA-MB-231 was another cell line with no increase in miRNA expression on microarray; nevertheless six out of eight miRNAs showed elevated expression using qRT-PCR. MCF-10A showed increased expression of three miRNAs on microarray, and five in qRT-PCR. Of these, miR-29b-3p and miR-141-3p were upregulated according to both methods.

Out of the eight cell lines, BT-474 and MDA-231 were selected for functional studies. BT-474 was chosen for the highest miRNA expression levels after BMP4 treatment. In addition, it is known that BMP4 induces a clear growth-reduction phenotype in this cell line (Ampuja et al. 2013). MDA-MB-231 was chosen for two reasons. First, this cell line has an interesting feature - high migration capability that is induced by BMP4 treatment - and secondly, according to qRT-PCR, six out of eight miRNAs were upregulated in this cell line.

Two miRNAs were selected for functional studies based on the frequency and level of upregulation (FC ≥ 1 in log2 scale). miR-16-5p and miR-106b-5p were both highly induced after BMP4 treatment in 5/8 of the cell lines when studied by qRT-PCR. In addition, miR-16-5p was upregulated in 3/8 of the cell lines on microarray and miR-106b-5p in 4/8 of the cell lines (Figure 5).

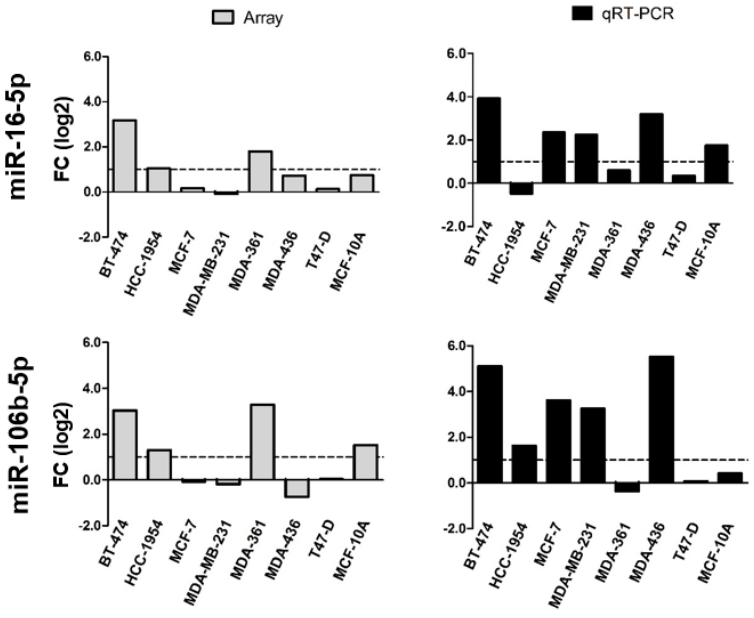

Figure 5. qRT-PCR validation of microarray data. Cells were treated with BMP4 or vehicle for 4h. The expression levels of miRNAs were studied in the same samples by miRNA microarray and qRT-PCR. The expression levels are shown as fold change (FC) in log2 scale, between BMP4 and vehicle-treated samples. The dashed line indicates the cut-off value of 1.

The expression levels of miR-16-5p and miR-106b-5p were also higher compared to other miRNAs. An additional criterion for selection was the Ct-value, so that the miRNAs with low overall expression level (Ct-value cutoff 35) were omitted. Thus out of the eight cell lines and eight miRNAs two cell lines (BT-474 and MDA-MB-231) and two miRNAs (miR-16-5p and miR-106b-5p) were selected for functional studies.

5.2 Functional studies of miRNAs

To study the contribution of microRNAs in BMP4-induced phenotypes (cell growth and migration), the cells were treated with anti-miR-inhibitors or control siRNA (siLuc) together with BMP4 or vehicle.

5.2.1 Anti-miR inhibitors effectively inhibit miRNA expression

The efficacy of miRNA silencing with anti-miR inhibitors was assessed using qRT-PCR after each proliferation and migration assays, at 4 days and 6/7 days' time point. For miR-16-5p, silencing resulted each time in over 90% inhibition and at for miR-106-5p in at least 69% inhibition at 4 days' time point. These silencing efficacies were considered adequate to see the possible functional effects of miRNA inhibition. At day 6/7, the strength of miRNA inhibition varied considerably, but successful silencing until day 4 was thought to exert the effects on phenotypes also at day 6/7.

5.2.2 Inhibition of miR-106b-5p leads to increased breast cancer cell growth and migration in BMP4-treated cells

BMP4 treatment decreased proliferation by 52% in BT-474 cells and by 43% in MDA-MB-231 cells at 7 and 6 days' time point, respectively, compared to vehicle treatment ($p<0.005$) (Figure 6). For MDA-MB-231 the effect of BMP4 was seen already at day 4, although no effect for BT-474 was seen in this time point. The observations of growth reduction were in good concordance with earlier studies (Ketolainen et al. 2010, Ampuja et al. 2013).

The BMP4-induced decrease in growth was partially reversed by anti-miR-106b-5p inhibitor at day 6 or 7. This can be seen as a slightly higher cell number at day 6/7 in cells that were treated with both BMP4 and anti-miR-inhibitors. Cell growth decreased only by 34% in BT-474, and by 39% in MDA-MB-231 cells ($p < 0.005$), compared to cells treated with vehicle and siLuc control. In BT-474 cells the effect of miRNA inhibition was clearer. miRNA inhibition alone - that is without BMP4 - had a remarkable effect on cell growth in BT-474 cells

at day 7, the increase in growth being 54% ($p < 0.005$). In MDA-MB-231 cells transfected with miRNA inhibitors, there was a slight increase in growth at day 4 but that was not detected anymore at day 6.

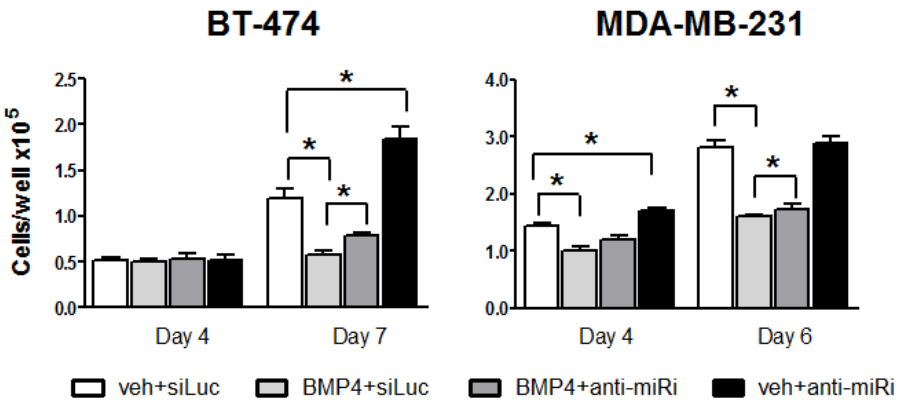

Figure 6. The effect of miR-106b-5p inhibition on cell proliferation. Cells were treated with BMP4 or vehicle in combination with miRNA inhibitors or siLuc control. The experiments were repeated twice with consistent results. The graph shows data from a representative experiment with error bars indicating the standard deviation of 5-6 replicates. An asterisk (*) represents p-value < 0.005.

Migration was studied only in MDA-MB-231 cells by a scratch assay using Cell-IQ Imagen automated imaging system and image analysis was performed with Cell-IQ Analyzer. Representative images of Cell-IQ image analysis from four time points (0h, 6h, 12h and 24h after the indicated treatment combinations) of the miR-106-5p migration assay are presented in Figure 7. The analysis software recognized well the borders of the scratches, marked with magenta color. The effects of different treatment combinations were seen already at 12 h time point.

To quantitate the data, the migration area was determined. The average of all migration areas from six wells of each treatment combination is shown in Figure 8. BMP4 treatment increased migration by 53% in MDA-MB-231 cells compared to vehicle treatment ($p < 0.0001$) as expected (Figure 8) (Ketolainen et al. 2010). In BMP4-treated cells, inhibition of miR-106b-5p increased migration even more, altogether 80% compared to vehicle treatment at 18 h time point ($p < 0.0001$). There was no statistically significant difference between vehicle-treated samples, whether or not the miRNA inhibitor was present.

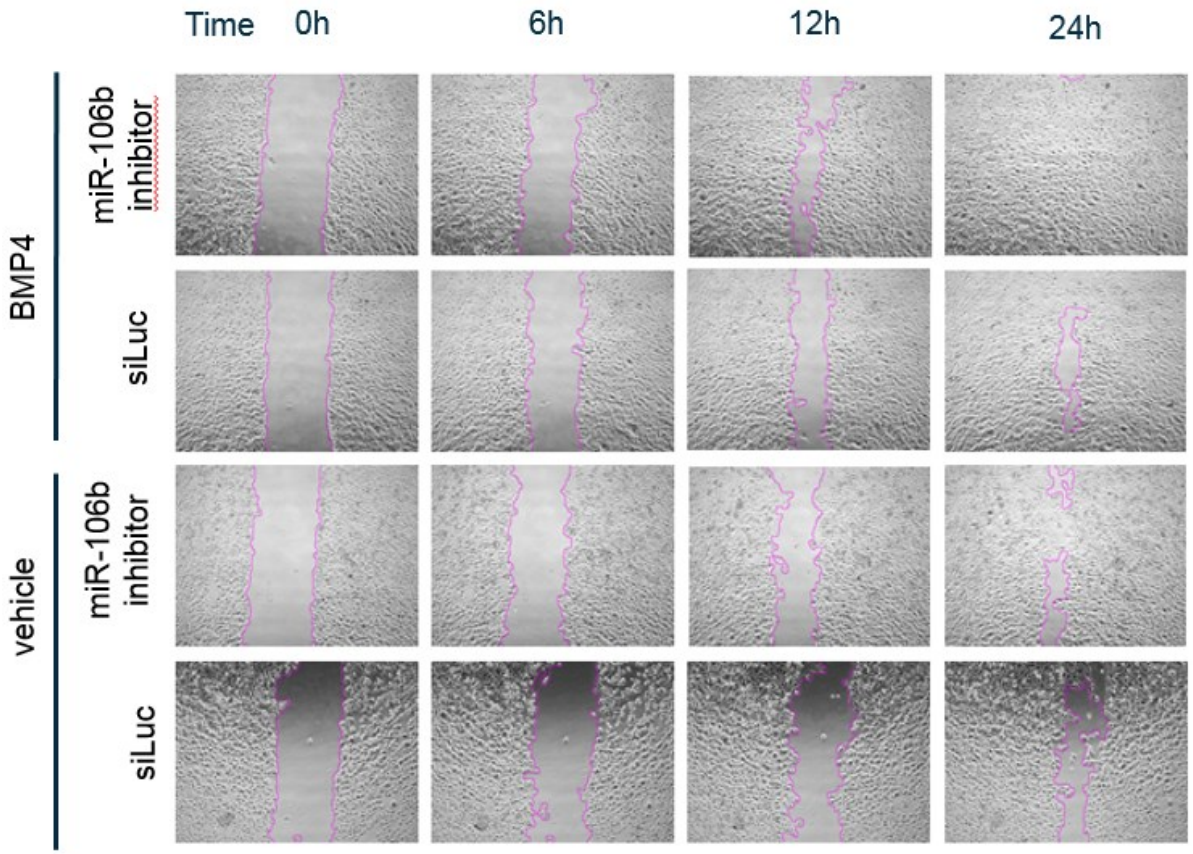

Figure 7. Scratch assay to study migration in MDA-MB-231 cells. Representative images of cell migration in a monolayer scratch assay. MDA-MB-231 cells treated with BMP4 or vehicle in combination with anti-miR-106b-5p or siLuc control at 0, 6, 12 and 24 h.

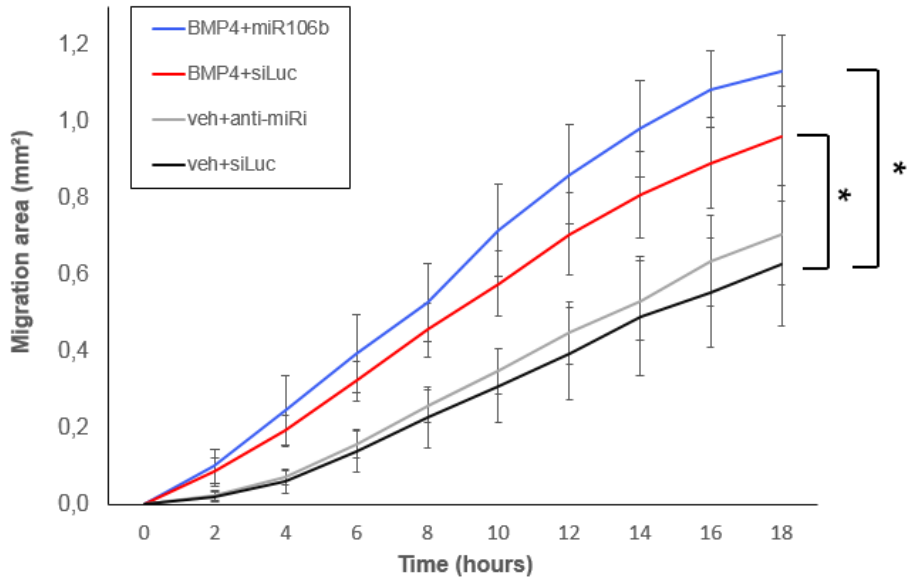

Figure 8. The effect of miR-106b-5p on migration of MDA-MB-231 cells. Cells were treated with BMP4 or vehicle in combination with anti-miR-106b inhibitor or siLuc control and imaged at two hours' intervals for 30 hours. The graph illustrates the migration from the beginning of

imaging to the state where scratches started to close up (i.e. at 18 h). An asterisk (*) represents p-value < 0.005.

5.2.3 Inhibition of miR-16-5p leads to increased breast cancer cell growth and migration in MDA-MB-231 cells

Again, treatment with BMP4 showed growth inhibition in both cell lines (Figure 9). The amount of cells decreased by 52% in BT-474 (Day 7) and by 18% in MDA-MB-231 (Day 6) ($p < 0.005$) compared to vehicle treatment.

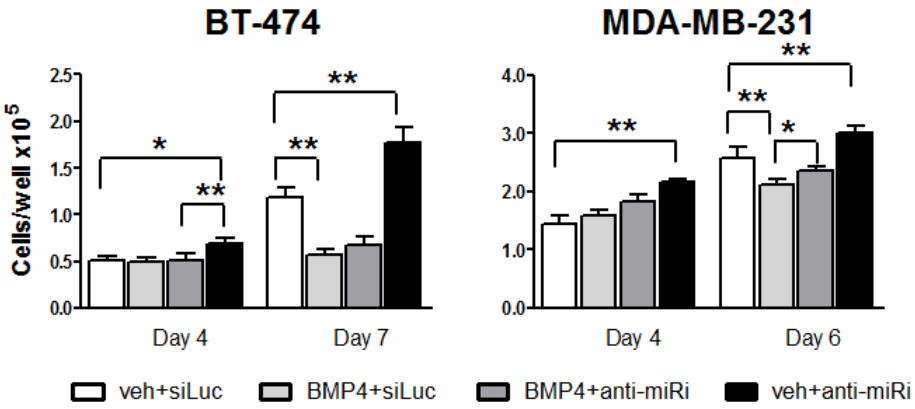

Figure 9. The effect of miR-16-5p on cell proliferation. Cells were treated with BMP4 or vehicle in combination with miRNA inhibitors or siLuc control. The experiments were repeated twice with consistent results. Images are representative examples with error bars indicating the standard deviation of six replicates. A double asterisk (**) represents p-value <0.005, and an asterisk (*) p-value <0.05.

Inhibition of miR-16-5p led to slight growth induction in BMP4-treated cells compared to solely BMP4-treated cells. However, the increase in growth was statistically significant only in MDA-MB-231 ($p < 0.05$) showing a partial growth reversal of 12%. The effect of miR-16-5p inhibitor alone was 49% increase in growth ($p < 0.005$) in BT-474, but only 12% in MDA-MB-231 ($p < 0.005$). In both cell lines, increase in cell growth was seen already at day 4.

The average of all migration areas from six wells of each treatment combination is shown in Figure 10. BMP4 treatment increased migration by 38% compared to vehicle-treated MDA-MB-231 cells as expected ($p < 0.0001$). Inhibition of miR-16-5p increased migration 24% points further, by 62% ($p < 0.0001$). The difference in migrated area between the two vehicle-treated samples was 20%, showing faster migration for cells with miRNA inhibitor treatment ($p < 0.0001$). The time point in which the analysis was done was chosen to be 14 hours, because in that time point scratches started to close up.

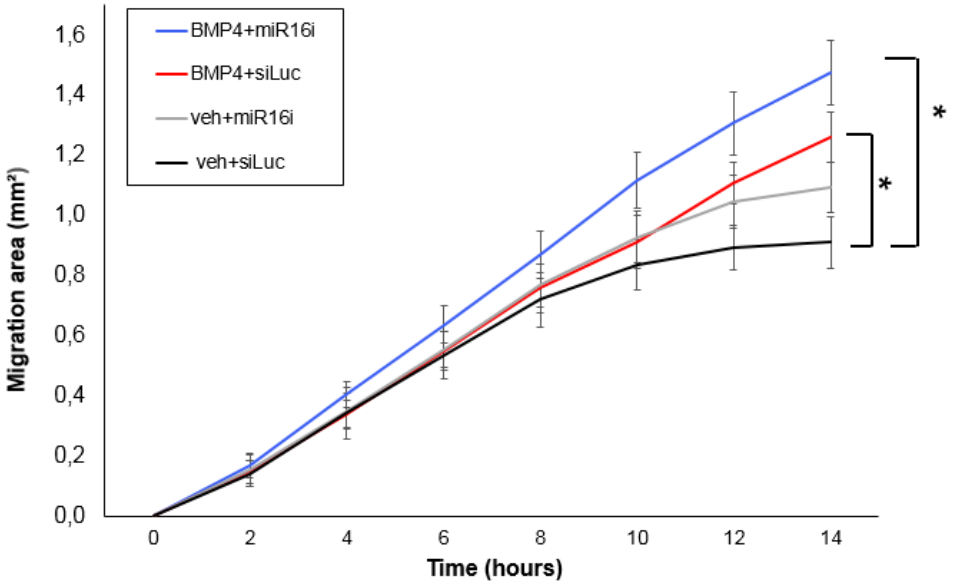

Figure 10. The effect of miR-16-5p on migration of MDA-MB-231 cells. Cells were treated with BMP4 or vehicle in combination with anti-miR inhibitor or siLuc control. The migration area was calculated every two hours for 30 hours after the introduction of the scratch, but the analysis was done at 14 h time point when the scratches started to close up. The figure is a representative example of two similar assays with error bars indicating standard deviation. An asterisk (*) represents p-value < 0.0005.

6. DISCUSSION

Deregulation of the short, single-stranded microRNAs is linked to various carcinomas (Lujambio and Lowe 2012). Because miRNAs target multiple mRNAs, altered miRNA levels can crucially affect the phenotype of cancer cells (Farazi et al. 2011, Lujambio and Lowe 2012). BMP4, a growth factor is known to act as a dualistic regulator of cancer cell behavior, causing decrease in proliferation but at the same time increase in migration (Ketolainen et al. 2010, Alarmo et al. 2013). This background created an interesting question: how do miRNAs contribute to these BMP4-induced phenotypes?

A common way to study miRNA expression in cells and tissues is a microarray screen. Therefore, our research group has performed a miRNA microarray to study the effect of BMP4 on miRNA expression in breast cancer cell lines (Alarmo et al. 2). The first part of this thesis was to study the differentially expressed miRNAs using a different method, qRT-PCR. This was done to achieve better understanding of BMP4-induced miRNA expression than the microarray alone. The other part was the *in vitro* studies where the effect of miRNAs on cell growth and migration were evaluated. Cell growth was studied by counting the cells at two time points, whereas cell migration was determined using a scratch assay, in which the migrated area of the cells was calculated at two hour intervals until the scratch closed up. The purpose of performing these experiments was to elucidate the effect of miRNAs on breast cancer cell growth and migration and to examine if BMP4 exerts its dual phenotype partly through miRNAs.

6.1 Studies of microRNA levels with qRT-PCR

Some of the miRNAs showed good correspondence between microarray and qRT-PCR. For example BT-474 and T-47D cell lines showed very similar results in both methods. In BT-474, most of the miRNAs - that is six out of eight - were overexpressed after BMP4 treatment according to both methods whereas no upregulation was seen in T-47D.

In general, the results from microarray and qRT-PCR varied although the same RNA samples were used in both methods. For example, MDA-MB-231 and MCF-7 cells showed contrary expression on microarray and qRT-PCR. On microarray, no effect of BMP4 treatment on miRNA expression was seen, whereas using qRT-PCR miRNA expression was elevated in both of these cell lines. Similar observations of discrepancy between microarray and qRT-PCR

have been made in other studies as well, thus comparing the results between two different types of methods inevitably faces challenges (Git et al. 2010, Meyer et al. 2012, Wang et al. 2014a). However, although the interplatform variation has reported to be large, the intraplatform reproducibility is considered generally good (Meyer et al. 2012). In this thesis, qRT-PCR for each miRNA and cell line was performed only once, thus the variation between qRT-PCR runs could not be estimated. In addition, a possible cause for the discrepancy is that there were no replicates. Also, for example Rivas and colleagues (2012) used 100 ng of RNA as starting material for the TaqMan two-step RT-PCR, whereas only 10 ng was used in this study. The protocol instructed to use 1-10 ng of RNA, but it seems that using amounts this small lead to high Ct values and thus cause possible variation in the results.

Out of these two methods, qRT-PCR is considered as more reliable to study miRNA expression, due to its higher specificity and sensitivity (Chen et al. 2009, Wang et al. 2014a). There are two main reasons for the often divergent results between these methods. Firstly, the basic concept of these methods is different because microarray is based on probe hybridization and qRT-PCR on PCR amplification. Secondly, the abundance of miRNAs in cells is low, which may lead to increased variation (Chen et al. 2009). It has also been reported that the choice and amount of reference genes may have effect on the results (Hellemans et al. 2007, Davoren et al. 2008). At least two reference genes are advised to be used in qRT-PCR data normalization. The recommendation of reference genes, however varies. For example Hellemans and colleagues (2007) stated that miR-16 together with *let-7* could be used as a stable reference gene in breast cancer studies. In this thesis, however, miR-16-5p levels varied considerably after BMP4 treatment, which made this miRNA a study object, not a reference gene.

6.2 Functional studies of miR-16-5p and miR-106b-5p

In the second part of this thesis I wanted to determine the functional effects of the two studied BMP4-induced miRNAs. In line with a study by Ketolainen and colleagues (2010), BMP4 treatment decreased cell growth significantly in both of the studied cell lines: BT-474 and MDA-MB-231. In addition, it is known that MDA-MB-231 is a cell line with high migration capability, and that BMP4 increases migration of these cells (Ketolainen et al. 2010). The main interest in this thesis was to examine if the two miRNAs would contribute to the BMP4-induced phenotypes. This was studied by inhibiting the miRNAs with anti-miR inhibitors and by

observing the effect of miRNA inhibition on cell growth and migration for up to seven days. At first, I'll discuss the contribution of miR-106-5p on cell growth and migration, then of miR-16-5p.

6.2.1 The effect of miR-106b-5p on breast cancer cell growth and migration

The inhibition of miR-106b-5p inhibition slightly increased the growth of BT-474 and MDA-MB-231 cells, indicating the role of this miRNA as growth repressor. An exception was MDA-MB-231 cells at 6 days' time point, where no such effect was seen. The cells in MDA-MB-231 proliferation assay reached high density at day 6, leading possibly to detachment of cells, which may have affected the results. In future studies, perhaps smaller cell number at seeding should be considered to get more reliable results of cell growth.

Likewise the results obtained in this thesis, similar effects of miR-106b-5p on cell growth were reported by Gong and colleagues (2013). They studied the effect of TGF-β -induced miR-106b-5p in both invasive (MDA-MB-231) and non-invasive (MCF-7) breast cancer cell lines and in an immortalized breast epithelial cells (MCF-10A) as well as in patient samples. In their study, miR-106b-5p was suggested to suppress the growth of low-invasive cancer cells, to which BT-474 belongs. On the contrary, they also showed that in the high-invasive cells, miR-106b-5p first decreases cell growth, but later acts as growth inducer. The growth-inducer effect was seen already at day 4 by Gong and colleagues (2013) but in this thesis no such effect was seen in high-invasive MDA-MB-231 cells neither at day 4 or day 6/7. The difference can be probably explained by the fact that they studied TGF-β which is different from BMP4, although they belong to the same protein family. These two molecules for example use different SMADs in their downstream signaling pathway and thus may have different consequences on target cells (Guo et al. 2012). A study by Zheng and colleagues (2014) revealed that upregulation of miR-106b in tissue and plasma samples of breast cancer patients correlated with increased tumor size. This finding thus indicates an opposite role for miR-106b in cell growth that was reported in this thesis and by Gong and colleagues (2013).

The effect of miRNAs on cell migration was studied by scratch assay in MDA-MB-231 cells. Cells that were treated with BMP4 migrated more than vehicle-treated cells, consistently with previous result (Ketolainen et al. 2010). In this thesis I showed that the BMP4-induced migration of MDA-MB-231 breast cancer cells was further increased after anti-miR-106b-5p treatment. Because low levels of miR-106b-5p cause increased migration, it means that this miRNA may have tumor-suppressor effects. However, anti-miR-106b-5p alone did not

significantly alter proliferation. These results differed from the majority of other studies which showed that overexpression - not repression - of miR-106b-5p caused increased migration (Smith et al. 2012, Gong et al. 2013, Ni et al. 2014, Zheng et al. 2014). Smith and colleagues (2012), for example, presented that the miR-106-25 cluster is able to induce EMT in cancer cells and that particularly miR-106b predicted shortened relapse of breast cancer. In addition, Zheng and colleagues (2014) showed that high miR-106b levels were associated with shortened disease-free survival of breast cancer patients. Earlier recurrence of cancer may indicate that cells have achieved increased capacity to migrate and invade other tissues. Furthermore, Gong and colleagues (2013) showed that miR-106b is expressed at elevated levels in higher stage tumors. Thus the aforementioned studies suggested miR-106b to be an oncogene. On the contrary to these studies and in concordance with this thesis, it has also been reported that miR-106b-5p was downregulated during bone metastasis of breast cancer, and that it instead inhibits migration and invasion (Ni et al. 2014).

miR-106b-5p belongs to the miR-106b-25 cluster, which is highly conserved in vertebrates and contains also two other miRNAs: miR-93 and miR-25 (Gong et al. 2013). The cluster is located on chromosome 7q22 on intron of *Mcm7* gene and can be transcribed together with *Mcm7* (minichromosome maintenance complex component 7) primary RNA transcript (Ni et al. 2014). The miR-106b-25 cluster of miRNAs is reported to be upregulated and oncogenic in several cancers, including hepatocellular carcinoma, prostate cancer and glioma (Li et al. 2009, Hudson et al. 2013, Liu et al. 2014). In studies regarding breast cancer, however, the results seem to be more variable. Some part of the differences between the results in this thesis and other studies may be explained by different study set-up. To give an example, Smith and colleagues (2012) studied miR-106b in MCF-7 cells (which have low migration capability) as well as in human breast cancer tissue microarray whereas BT-474 and MDA-MB-231 cell lines were used in this thesis. Furthermore, also the study set-up of Zheng and colleagues (2014) was different as they studied miR-106b in tissue and plasma samples of early stage breast cancer patients. The effects of miRNAs thus seem to be context-dependent. In addition, neither Smith and colleagues (2012) nor Zheng and colleagues (2014) specified whether the miR-106b they studied was derived from the -5p or -3p arm of the precursor.

6.2.2 The effect of miR-16-5p on breast cancer cell growth and migration

Similarly to miR-106b-5p, miR-16-5p is suggested to act as a tumor suppressor, because its upregulation or forced overexpression often leads to inhibition of tumorigenesis (Calin et al.

2002, Rivas et al. 2012, Janaki Ramaiah et al. 2014, Mobarra et al. 2015). In fact, Janaki Ramaiah and colleagues (2014) studied miR-16 in MDA-MB-231 cells similarly to this work, stating that high levels of miR-16 lead to inhibition of cell proliferation. This was in concordance with my results, which showed that the inhibition of miR-16-5p increased cell growth. The miR-16 -attributable decrease in cell growth was shown to be caused by G1 cell cycle arrest and caspase-3 dependent apoptosis (Janaki Ramaiah et al. 2014). Interestingly, G1 arrest was shown to be the reason also in BMP4-induced inhibition of cell growth (Ketolainen et al. 2010). This may indicate the contribution of miR-16-5p in BMP4-induced G1 arrest. In a study by Rivas and colleagues (2012) mir-16 was also suggested as a tumor suppressor, because overexpression of miR-16 decreased *in vivo* and *in vitro* breast tumor growth that was caused by a hormone progestin.

miR-16-5p belongs to the miR-16 gene family together with miR-15a/b, miR-195, miR-424 and miR-497 (Caporali and Emanueli 2011). The stem-loop of mir-16 forms two miRNAs; miR-16-5p and miR-16-1-3p (miRBase release 21). These miRNAs are located on chromosome 13q14, in a region that is frequently deleted in B-cell chronic lymphocytic leukemia (Calin et al. 2002). The same region has found to be deleted also in prostate cancer, and loss of miR-16 has indicated to promote cell survival, proliferation and invasiveness of prostate cancer cells (Bonci et al. 2008). Although there were numerous studies of the effect of miR-16 on breast cancer cell growth, no studies of breast cancer cell migration were found. miR-16 was shown to inhibit the growth of prostate cancer cell lines and metastatic prostate tumors also in a study by Takeshita and colleagues (2010). Furthermore, in non-small cell lung cancer cells downregulation of miR-16 promoted migration and invasion (Ke et al. 2013). Also in glioma cells miR-16 was shown to act as a tumor-suppressor, as miR-16 mimics inhibited EMT (Wang et al. 2014b). These studies made with cells obtained from other cancer types, are in agreement with the results obtained in this thesis with breast cancer cell lines.

6.3 Further aspects

Additional information on the effect of these miRNAs on cell growth and migration could be obtained by performing more assays. For example, miRNA mimics, which should increase the miRNA levels and thus have the opposite effect as do the inhibitors, could be used to confirm the effects of the studied miRNAs. Also, a very interesting study set-up would be to study the effects of miR-16-5p and miR-106p-5p in the same experiment to see the combined effects of

these miRNAs. Keeping in mind the fact that miRNAs are often transcribed as a cluster, it could be possible that other miRNAs - for example miRNAs miR-93 and miR-25 together with miR-106b-5p - would contribute to the BMP4-induced phenotype much stronger than miR-106b-5p alone. The miRNA microarray preceding this thesis, however, did not recognize overexpression of other miRNAs in the clusters of miR-16 and miR-106b. One has to note, though, that neither did the microarray recognize the upregulation of miR-16-5p and miR-106b-5p in MDA-MB-231 cells on array, although it was seen by qRT-PCR.

The studies could be continued by searching for the target genes of the two microRNAs. In fact, some target genes of miR-16-5p and miR-106b-5p have already been pointed out. Best known targets for miR-16 are mRNAs of the genes *BCL2*, *CCND1* (encoding cyclin D1) and *WNT3A*, all of which contribute to tumorigenesis by promoting cell survival, proliferation and invasion (Bonci et al. 2008). In addition, miR-16, was shown to directly target RPS6KB1 (Ribosomal protein S6 kinase beta-1) mRNA by 3'-UTR binding, leading to further regulation of fundamental cellular processes such as transcription, translation and cell growth (Janaki Ramaiah et al. 2014). Moreover, miR-16 was shown to target Twist and EZH2 (Enhancer of zeste homolog 2), key proteins in EMT and thus repressing metastatic ability of these cells (Janaki Ramaiah et al. 2014). The other studied miRNA, miR-106b is known to regulate p21 (also called as CDKN1A, an important cell cycle inhibitor) expression in gastric cancer, leading to impairment of TGF-β pathway (Petrocca et al. 2008). There may also be a feedback loop between BMP pathway and miR-106b, as Smad7, a BMP inhibitor, is a target of miR-106b-25 cluster (Smith et al. 2012). In addition to the aforementioned target genes, there for sure are other targets of miR-16 and miR-106b that contribute to tumorigenesis, because the outcome of an individual miRNA is most likely mediated via multiple targets.

Although the results of the migration studies were statistically significant, the differences between BMP4-treated and vehicle control cells were smaller than reported by for example Ketolainen and colleagues (2010). It seems that migration chambers, instead of scratch assay, would highlight the differences better. Scratch assay, on the other hand, allows the controlling of migration at regular intervals. For further assays, these two methods should be compared to ensure that the results are similar. The critical step in a scratch assay, however, is to create scratches of approximately similar size, therefore this method needs more optimization.

In studies done with miR-16 or miR-106b, it should be acknowledged that only a small part of studies have reported if the miRNA at issue was derived from -5p or the other strand, -

3p. There opposing results between studies may also rise from the fact that miRNAs are either studied in different context, such as in tissue samples, or being regulated by other molecules than BMPs. The vast amount of miRNA targets makes the studies on miRNAs even more complicated. Taken together, increased expression of miRNAs is observed in a wide range of tumors comparing to normal tissue which leads to overexpression or repression of target genes. Thus, overexpression of miR-16-5p and miR-106b-5p, induced by BMP4, may depict a central event in the tumorigenic process

An intriguing question is what kind of role these miRNAs have in the big picture - that is in the BMP4-induced phenotype in breast cancer. Previously, we have studied the target genes of BMP4 where the purpose was to clarify the mechanisms through which BMP4 decreases cell growth and increases migration (Rodriguez-Martinez et al. 2011). That study was continued by the miRNA microarray study, where instead BMP4-induced microRNAs were studied. Also, in addition to protein coding genes and microRNAs, there are also other molecules such as non-coding RNAs that most probably have at least a minor importance. The pieces of information move us towards the elucidation of the role of BMP4 in breast cancer, hopefully revealing a major part of the complicated network of molecular changes leading to breast cancer.

7. CONCLUSIONS

The purpose of this thesis was to study the role of miR-16-5p and miR-106b-5p in BMP4-induced breast cancer cell phenotypes. I showed that miR-16-5p and miR-106b-5p cause statistically significant effects on breast cancer cell proliferation and migration between BMP4 and vehicle-treated samples.

Determining the role of miRNAs in breast cancer is important for discovering the mechanism of miRNA-mediated cancer development and progression. This kind of evidence may reveal new aspects for treatment and diagnosis of breast cancer. Based on the results of proliferation and migration assays in this thesis, miR-106b-5p seems to suppress the malignant properties of MDA-MB-231 cells by inhibiting cancer cell growth and migration. In addition, miR-16-5p has similar growth-reducing and migration-promoting effect in BMP4- and vehicle-treated cells. Thus the two studied miRNAs may partly cause the growth-reducing effects of BMP4, but these miRNAs are not responsible for the increased migration caused by BMP4.

Based on the results of this thesis, the effects of microRNAs on cell proliferation and migration are nonetheless indisputable. However the connection between BMP4 and microRNAs remains still unknown and further studies are needed to ensure that the changes in phenotypes are indeed a cause of BMP4-induced miRNAs.

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9. SUPPLEMENTARY DATA

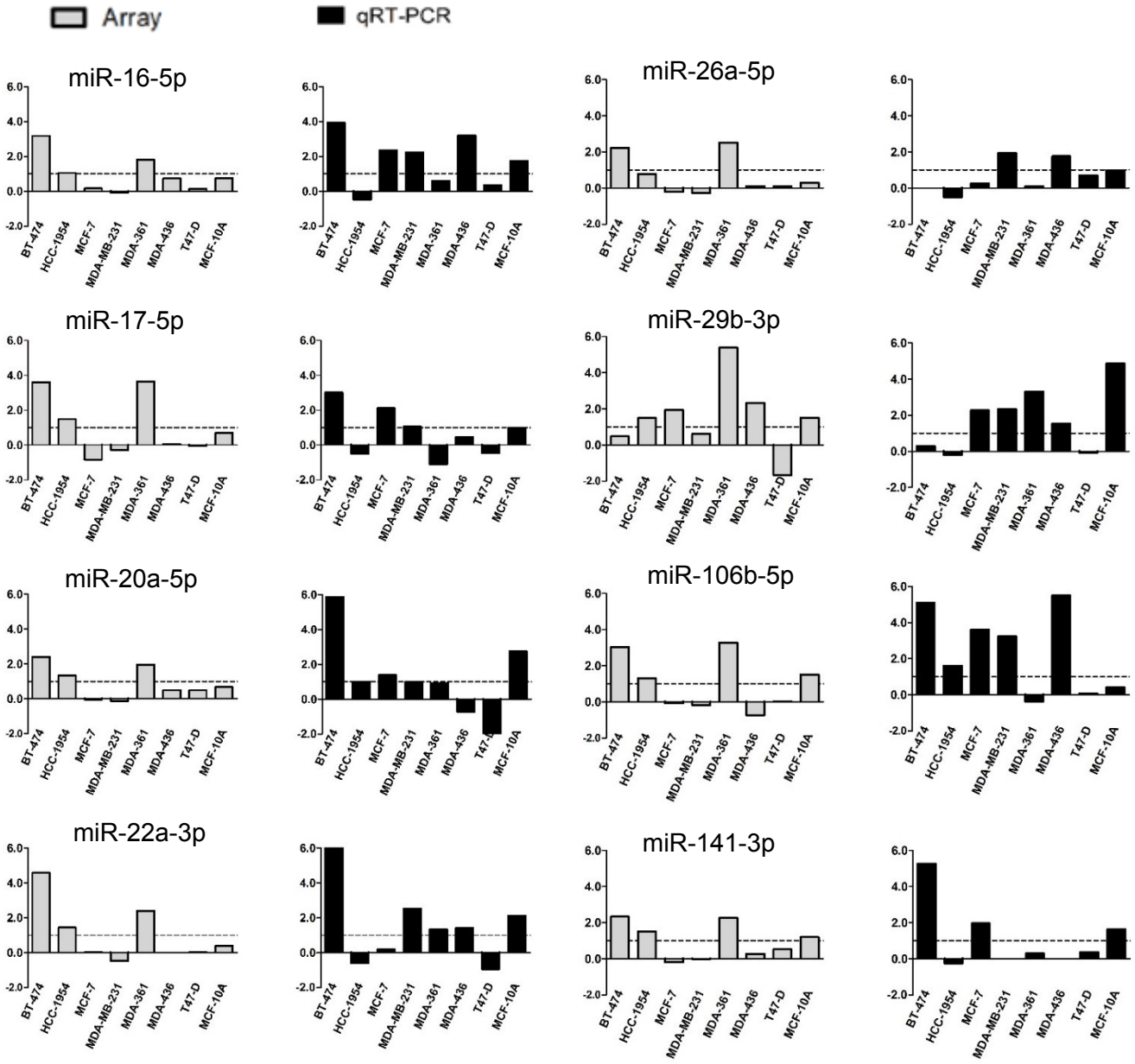

Figure S1. Comparison of miRNA microarray and qRT-PCR results. Bars depict the relative expression of each miRNA - compared to a housekeeping gene RNU48 (in log2 scale). The dashed line depicts the cut-off value of 1.