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Expression and functional properties of selected miRNAs in human cancer tissue and cancer cell lines

A study of expression and function of miR-143, miR-145 and miR-126 in breast cancer and non-small cell lung cancer

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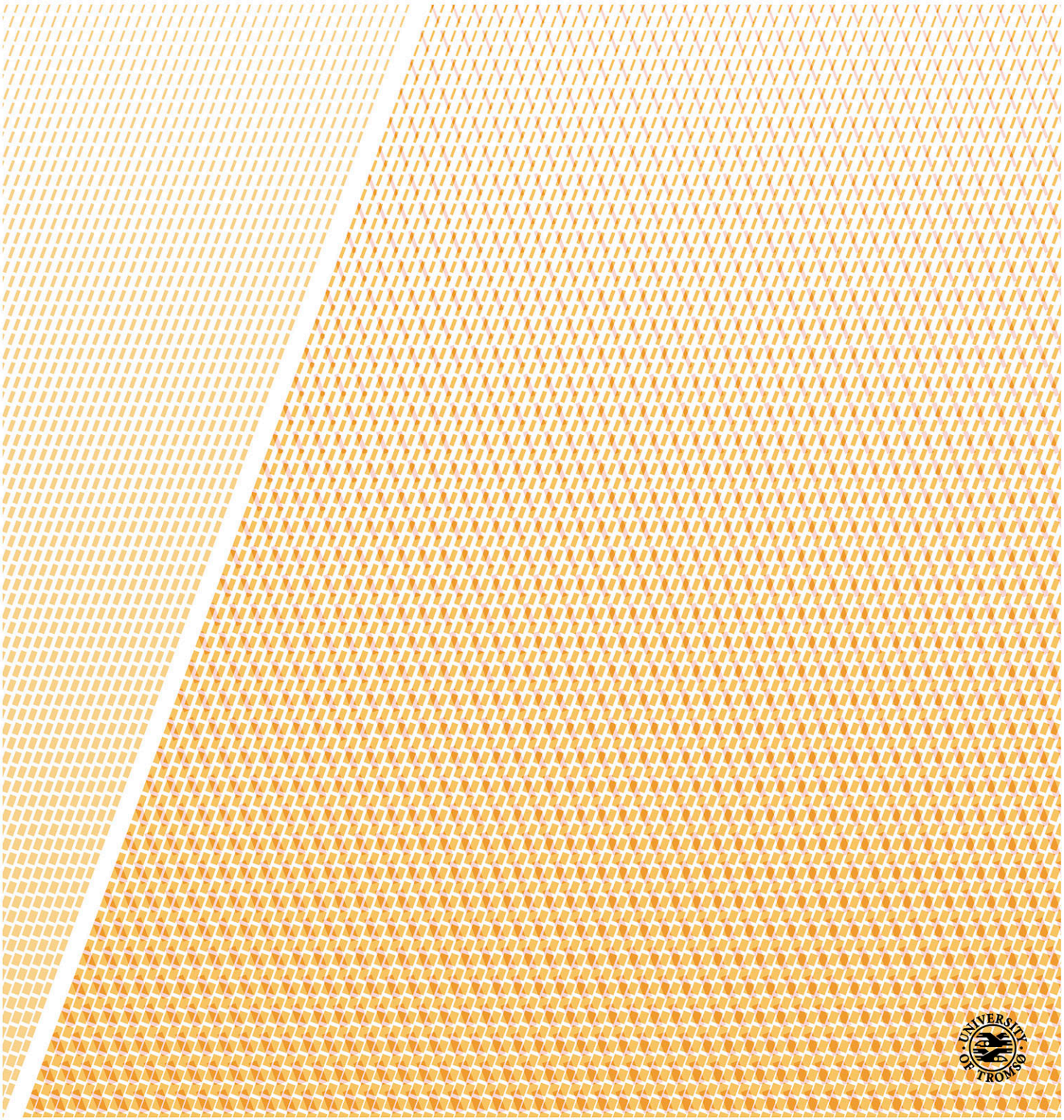
Charles Walquist Johannessen

A dissertation for the degree of Philosophiae Doctor – November 2018



Expression and functional properties of selected miRNAs in human cancer tissue and cancer cell lines

A study of expression and function of miR-143, miR-145 and miR-126 in breast cancer and non-small cell lung cancer

Doctoral Thesis

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Tromsø, 2018

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Summary

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) involved in the regulation of gene expression, and they are often seen dysregulated in cancer. For this reason, it is of great scientific interest to study these ncRNAs to better understand their distribution in human tissues and their mode of function. Based on a comprehensive miRNA microarray from 108 breast cancers in the Norwegian Woman and Cancer Study and 44 healthy controls, this thesis sought to investigate tissue expression and functional properties of the miRNA cluster miR-143/145 in breast and lung cancer tissues and cell lines, as well as miR-126 in breast cancer (BC) tissue and cell lines.

Our analysis found the miR-143/145 cluster to be downregulated in tumor samples from both breast and lung, as well as in their corresponding cancer cell lines. The transfection of miR-143 into a number of cancer cell lines from both breast and lung, promoted proliferation in some, whilst having opposite or no effect in others. In contrast, all cell lines suffered inhibition in both proliferation and migration when transfected with miR-145.

In BC tissue, expression of miR-143 and miR-145 was lower in malignant compared to benign breast tissue, and lower in the more aggressive tumors. Interestingly, miR-145 was mainly expressed in the myoepithelial cells of benign breast tissue, and at the subcellular level located to the nuclei. In lung cancer tissue, expression of the miR-143/145 cluster was found to correlate with expression of several sex steroid hormone receptors. Also, stromal expression of miR-143 was an independent positive prognostic factor in female patients, whereas stromal expression of miR-145 was associated with improved disease specific survival for male patients.

Both miR-126-3p and its passenger strand, miR-126-5p, was verified as downregulated in BCs as well as in all tested BC cell lines. The passenger strand had a strong proliferation promoting

effect in the most aggressive BC cell line, whilst having the opposite effect in the other cell lines. The introduction of miR-126-3p resulted in decreased proliferation and invasion in all BC cell lines. In BC tissue, expression of miR-126-5p was significantly higher in high grade tumors, and both miR-126 strands were downregulated in lymph node positive BCs when compared to tumors with no nodal involvement.

This thesis depicts interesting findings, and contributes to a better understanding of context-specific expression patterns, functions and prognostic impacts of the three selected miRNAs.

List of papers

Paper I

Charles Johannessen, Line Moi, Yury Kiselev, Mona Irene Pedersen, Stig Manfred Dalen, Tonje Braaten, Lill-Tove Busund.

Expression and function of the miR-143/145 cluster *in vitro* and *in vivo* in human breast cancer

PLoS One. 2017 Oct 26;12(10):e0186658. doi: 10.1371/journal.pone.0186658.

Paper II

Charles Johannessen, Yury Kiselev, Mona Irene Pedersen, Stig Manfred Dalen, Lill-Tove Rasmussen Busund, Line Moi.

Different functional roles and expression of miR-126-3p and miR-126-5p in breast cancer cell lines and tissues

Manuscript

Paper III

Kaja Skjefstad, Charles Johannessen, Thea Grindstad, Thomas Kilvaer, Erna-Elise Paulsen, Mona Pedersen, Tom Donnem, Sigve Andersen, Roy Bremnes, Elin Richardsen, Samer Al-Saad, Lill-Tove Busund.

A gender specific improved survival related to stromal miR-143 and miR-145 expression in non-small cell lung cancer

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List of abbreviations

3'UTR	3’untranslated region
ADC	Adenocarcinoma
BC	Breast cancer
BL	Basal-like
bp	Base pair
CAF	Cancer associated fibroblast
CK	Cytokeratin
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic acid
dsRBD	Double-stranded RNA binding domain
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EXP5	Exportin 5
HER2	Human epidermal growth factor receptor 2
IHC	Immunohistochemistry
ISH	*In situ* hybridization
MeSH	Medical subject heading search
miRNA	MicroRNA
ncRNA	Non-coding RNA
NOS	Not otherwise specified
NOWAC	Norwegian women and cancer study
NSCLC	Non-small cell lung cancer
NST	No special type
p63	Transformation-related protein 63
PR	Progesterone receptor
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
qPCR	Quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
rRNA	Ribosomal RNA
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
TMA	Tissue microarray
TN	Triple-negative
TNBC	Triple-negative breast cancer
TRBP	TAR RNA-binding protein
tRNA	Transfer RNA
TTF-1	Thyroid transcription factor-1
WTS	Whole-tissue section

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1. Background

1.1 Breast cancer

Worldwide, there are reported more than 1.6 million new cases of breast cancer (BC) every year, and the annual BC death rate is in excess of 500.000 [1]. This makes BC the most diagnosed cancer in the female population, where it contributes to 25% of all new cancer cases, and 15% of all cancer related deaths [1]. The five year survival rate of BC is 89% [2], but once metastases to distant organs occur, the five year survival rate drops below 25% [3].

Although metastatic BC rarely presents at the initial time of diagnosis, as many as 30% of patients diagnosed with early stage BC will later develop recurrent advanced disease or metastasis [4, 5]. The majority of BC metastases occur in the liver, bone, lung or central nervous system [6]. Although there has been some controversy regarding the effects of mammography [7], public screening programs are considered important for early detection and prevention of BC [8].

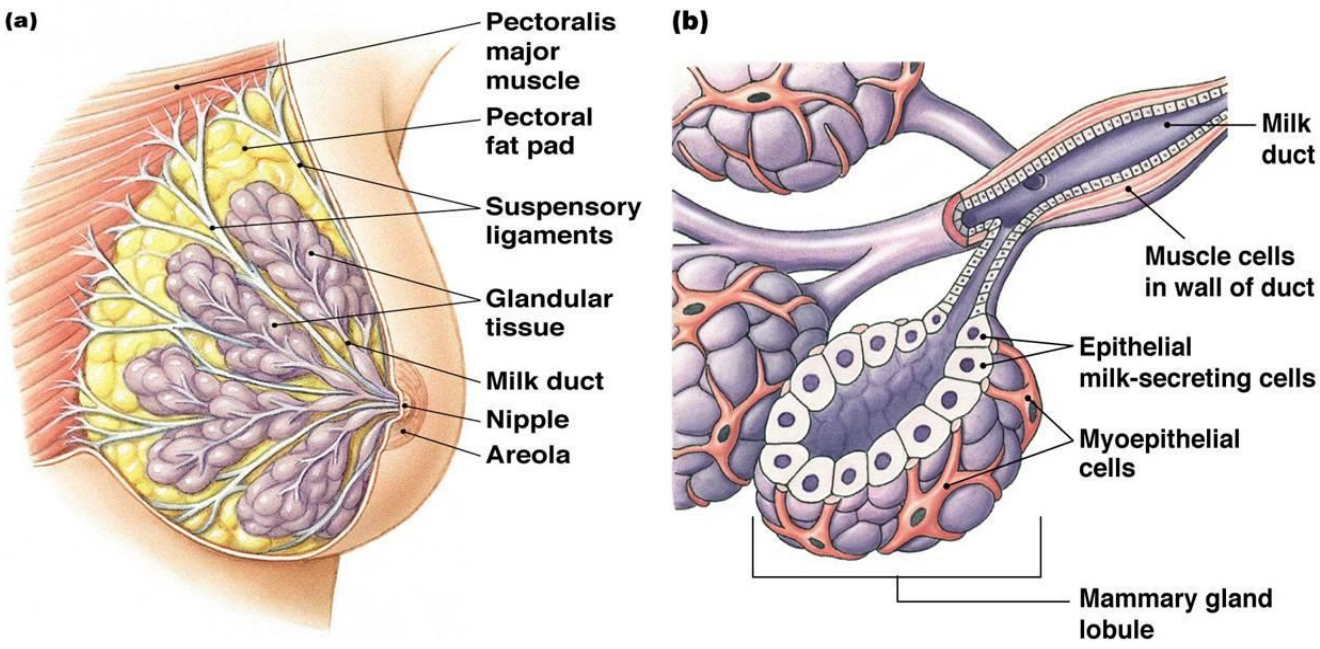

Figure 1: Anatomy and histology of the breast. Alvarado, 2016.

The mammary gland (figure 1) consists of several different cell types [9], and is a complex organ. The epithelial cells form the ductal network of the glands which are bilayered structures comprised of luminal cells (milk secreting epithelial cells) and basal cells [10]. The luminal cells form the ducts and the secretory alveoli, and makes up the inner layer of the glands' bilayered structure, whilst the basal cells form the outer layer which consists mainly of myoepithelial cells [10]. The function of the alveoli and the luminal cells is to secrete milk containing water and nutrients, whereas the contraction by the myoepithelial cells directs the milk through the ductal pathways [10, 11]. A relatively newly discovered cell type in this cellular landscape is the mammary stem cells which have been described as lineage-restricted cells with the capacity to differentiate into either luminal cells or myoepithelial cells [12, 13].

Precursor lesions of BC are annotated ductal carcinoma *in situ* (DCIS), and consist of clusters of neoplastic cells confined to the milk duct. It is estimated that up to 40% of DCIS cases progress into invasive carcinoma of no special type (NST), where cancer cells breach the basement membrane and infiltrate the surrounding tissue [14, 15]. Noteworthy, hereditary BC accounts for approximately 5-10% of all BC cases, and is primarily caused by mutations in one of the genes coding for the tumor suppressor proteins BRCA1 or BRCA2. Mutations in either of these genes are very potent cancer predictors, and women carrying these mutations have a 40-80% chance of developing BC before the age of 80 [16, 17].

1.1.1 Classification

BC is a highly heterogeneous disease composed of a diverse collection of molecular subtypes, which can be determined by gene expression patterns or immunohistochemistry (IHC) [18-21]. The identification of each tumor's molecular subtype is of great value, as each subtype has biological and clinical features [20] which are associated with prognosis and direct choice of treatment [22]. It is established that the different gene expression patterns observed in BC account for this heterogeneity [19]. The histopathological features of BC include characteristics such as histological morphology, receptor status, tumor size, tumor grade and nodal involvement. With the knowledge of both the molecular subtype and the histopathological status, the metastatic predisposition of the primary tumor may be determined [22, 23]. With the access to whole-genome profiling, one has been able to categorize BC into four general molecular subtypes. These subtypes are the luminal A, luminal B, human epidermal growth factor receptor 2 positive (HER2+) and the triple-negative (TN)/basal-like (BL) BCs [19, 24, 25]. Routinely, the classification of the tumors molecular subtype is basically based on the expression of relevant immunohistochemically stained receptors, and the expression of the proliferative marker Ki67. An illustration of immunohistochemical staining patterns for BCs with different receptor status is presented in figure 2.

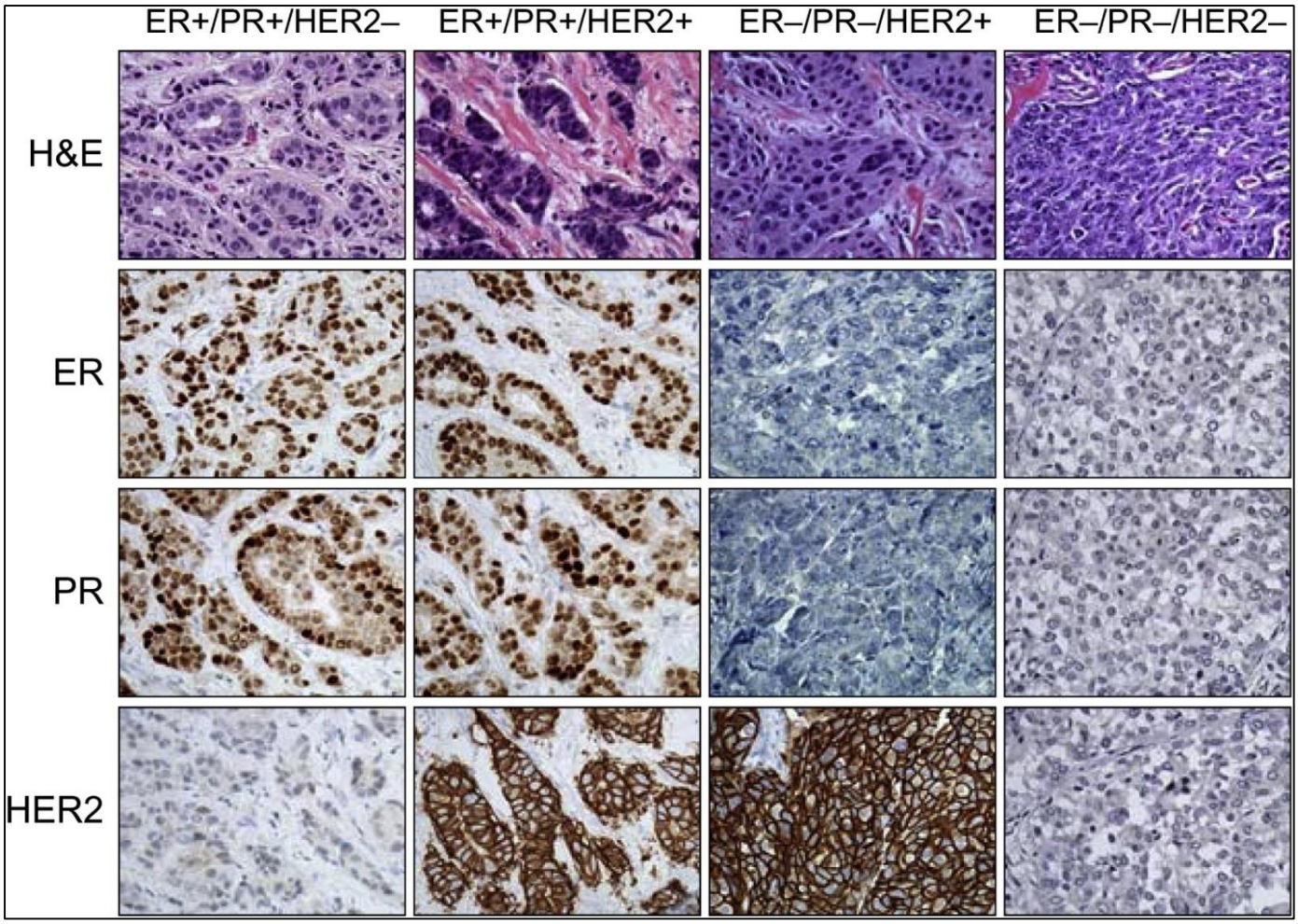

Figure 2: Representative immunohistochemical expression of ER, PR and HER2 in different subtypes of invasive BC. Rivenbark et al., 2013.

The luminal BCs are so named because their molecular profile has a similar gene expression to the normal luminal epithelium of the breast, and they are typically estrogen receptor positive (ER+) and progesterone receptor positive (PR+). The luminal A tumors display high expression of ER, low expression of HER2 and low expression of proliferation promoting genes [26, 27]. Luminal B tumors exhibit ER expression at lower levels compared to the luminal A subgroup, they can be either HER2+ or HER2-, and they typically have a higher expression of the proliferation-associated protein, Ki67 [27, 28]. The luminal BCs comprise the largest subgroup of BCs, where the luminal B cancers account for approximately 40% of all BCs [29], thus making it the largest individual subgroup. Due to the higher expression of ER and the lower

expression of proliferation markers in the luminal A tumors, this subgroup has a better prognosis than patients in the luminal B subgroup, with an overall survival rate 10 years after diagnosis of 70% vs 54%, respectively [22]. Notably, when further subgrouping the luminal B subgroup into luminal B/HER2+ and luminal B/HER2-, there is a significant difference in the overall survival rate at 10 years after diagnosis of 46% vs 54%, respectively [22]. Combined, however, the luminal BCs have a better prognosis compared to the non-luminal subgroups, which primarily is a result of their positive ER-status, hence allowing for targeted hormonal therapy [30].

The molecular subgroup HER2+ constitutes approximately 15-20% of all BC cases, where the *HER2* gene copy number can be as high as 50, resulting in a protein overexpression close to a 100-fold [31]. HER2 is a transmembrane receptor protein and is one of the four membrane receptor tyrosine kinases (RTKs) in the epidermal growth factor receptor family, first identified in rat neuroblastomas as a protein capable of transforming a mouse fibroblasts cell line into malignancy [32]. This family of receptors is important in communication between cell-cell and cell-stroma via signal transductors known as ligands. HER2 is a co-receptor for many ligands, and whilst the HER2 receptor has no known ligand of its own, the receptor forms heterodimers with other receptors of the HER family when activated by their ligand(s) [33]. Dysregulation of HER2, on both gene and protein level, is associated with a worse prognosis in both the lymph node-negative and lymph node-positive BC patients [34]. Adjuvant treatment of HER2+ BC with trastuzumab, a monoclonal antibody targeting the extracellular domain of the HER2 protein, has proven beneficial to patient outcome, with a 34% reduction in risk of death after two years [35]. Currently, adjuvant treatment with trastuzumab is the standard care of treatment for HER2+ tumors.

The TN/BL subgroup is highly heterogeneous, is immunohistochemically defined as BCs not overexpressing HER2, and is both ER and PR negative. It is considered an especially aggressive subtype, in particular affecting the younger female population, and targeted therapeutic options are limited to clinical trials [36]. The terms TN and BL are often used as synonyms, and although most TN cancers are of a BL phenotype, and most tumors expressing basal-markers are TN, this is not always the case [37, 38]. In fact, studies have revealed that 70-90% of TN cancer are of a BL subtype, and 50-80% of molecular BL tumors are TN [38-40]. However, pooling the TN and the BL cancers into one subgroup is sensible, as they exhibit great similarities in that they affect younger patients, are more likely to present as interval cancers, and they are significantly more aggressive than tumors in other subclasses [41, 42].

The TNBCs account for 10-20% of all BCs, depending on the methods and thresholds used to evaluate ER, PR and HER2 expression [41]. There are histological subtypes which are typically TN, but the TN cancers as a group lack distinctive histological characteristics [43]. With their expression of epidermal growth factor receptor (EGFR) together with cytokeratins 5, 14 and 17, their genomic characteristics are similar to the normal myoepithelial (basal) cells of the breast [44], and interestingly, the gene expression patterns of myoepithelial mammary cells are similar to those found in squamous cell carcinoma of the lung [45].

As a result of the tumors' negative receptor status, the TNBC patients benefit from neither endocrine therapy, nor trastuzumab (HER2 antagonist). The current backbone in TNBC management is treatment with cytotoxic chemotherapy, where TNBCs have a higher pathologic complete response rate when compared to hormone receptor-positive BC treated with neoadjuvant chemotherapy [46]. Still, however, patients harboring the TN subtype have a worse outcome after chemotherapy compared to patients with BC of other subtypes [44, 47].

The highly heterogeneous nature of TNBC argues for personalized treatment strategies targeting molecular tumor-specific sites. This is further substantiated by the fact that the response rate of TNBC patients treated with chemotherapy is 40% or less [43]. Indeed, there are currently several ongoing clinical trials investigating the potential for targeted therapy for TNBC. These targets include, but are not limited to, the androgen receptor (phase II), the AKT-pathway (phase II) and poly (ADP-ribose) polymerase PARP (phase III) [36]. The future of treating TNBCs may be brighter, as the treatment regime shifts towards more personalized, molecular targeted therapies.

1.2 Non-small cell lung cancer

Worldwide, lung cancer is the leading cause of cancer related death, and annually 1.8 million new lung cancer cases are reported [48]. Additionally, it is also one of the cancers with the highest death rate, with just short of 1.6 million people dying each year [48]. It is estimated that approximately 80-90% of all lung cancer cases in high-income countries are directly related to smoking [49], and although lung cancer incidence in men is slowly declining due to fewer smokers, the incidence in women continues to rise, also in Norway [50] (figure 3).

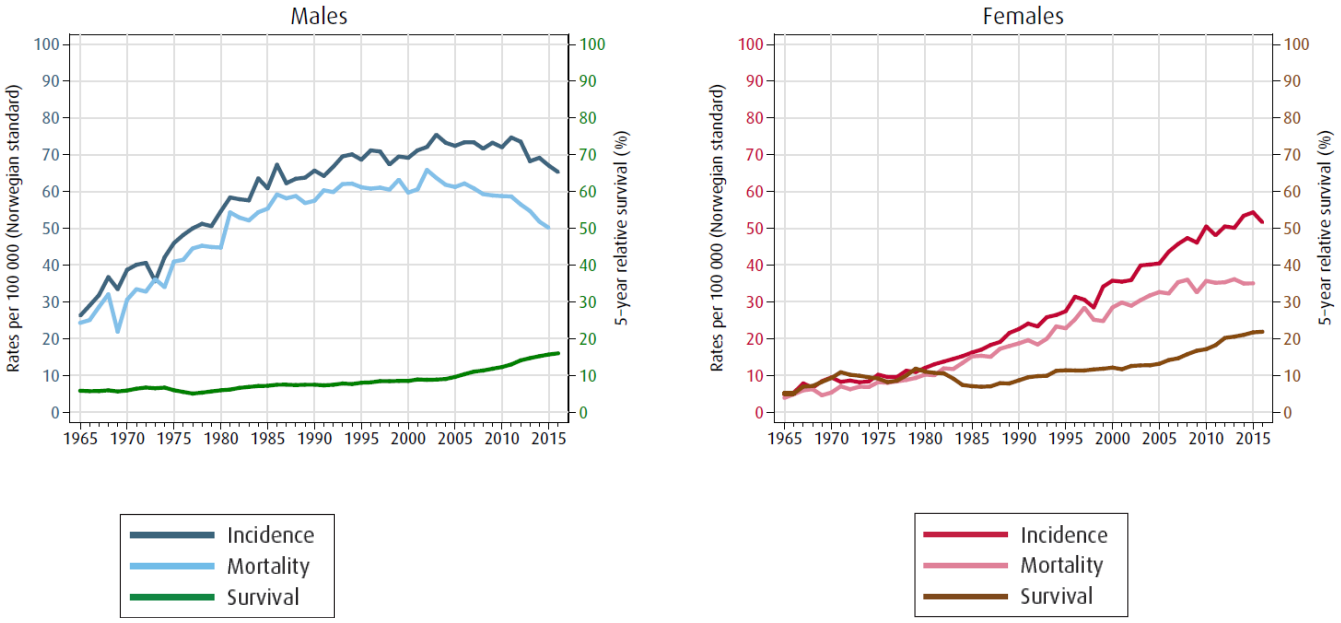

Figure 3: Incidence and mortality rates in lung cancer patients from 1965-2015.
(Adapted from www.kreftregisteret.no; Cancer in Norway 2016)

Lung cancer is a disease that presents great challenges, as 70% of lung cancer patients have advanced stage at the time of diagnosis [58]. Metastases to distant organs are responsible for 70% of all lung cancer deaths, regardless of histological subtype, and the most frequent sites for metastases include bone, brain, adrenal glands and liver [59]. An obvious way to decrease lung cancer mortality (in addition to reducing smoking in the population) is through achieving early diagnosis, and hence treatment, by screening programs. However, studies involving chest radiographs and sputum cytology for early detection did not significantly reduce lung

cancer mortality [60]. On the other hand, patients in high-risk groups participating in The American National Lung Screening Trial had significantly lower mortality, but there are still unanswered questions in regards to over-diagnosis and over-treatment [60, 61].

1.2.1 Classification

Histologically and clinically, lung cancer is typically classified into two major groups: Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC accounts for approximately 15% of all diagnosed lung cancers and is a very aggressive type of cancer, generally observed in smokers [51].

NSCLC is the largest group and accounts for approximately 85% of all lung cancer cases, and is further classified into subgroups [52]. The classification of NSCLCs was recently revised by the 2015 WHO Classification of Lung Tumors, and consist of squamous cell carcinoma (SCC), adenocarcinoma (ADC) and NSCLC not otherwise specified (NOS) [53]. ADC is the most common subtype observed in never-smokers [54], it typically forms glandular structures, and the production of mucus is sometimes observed. SCCs are typically more aggressive than ADCs, and can be keratinizing, non-keratinizing or display a low differentiated basaloid morphology, somewhat similar to that of TNBCs [45, 55].

Previous classification of NSCLC was based on light microscopy of hematoxylin and eosin stained tissue samples, and focused mainly on morphology. However, new IHC analyses have been applied to classify subgroups more accurately. These analyses include the thyroid transcription factor-1 (TTF-1), which is expressed by pneumocytes and is primarily associated with ADCs [53]. Similarly, the transformation-related protein 63 (p63), its isoform p40, and cytokeratin 5/6 (CK 5/6) are predominantly expressed in the bronchial epithelium and are

typically associated with SCCs [53]. On rare occasions (3% of cases), if no distinct IHC staining pattern is evident to indicate either ADC or SCC, then the NOS subtype is applied, and merely as a diagnosis of exclusion [53].

The histological and molecular distinction between ADC and SCC is crucial when it comes to therapeutic decisions and to predicting the clinical course, especially for patients with advanced-stage disease [56]. In Norway, the recent availability of targeted treatments has resulted in the screening of all non-SCCs for mutations in both the EGFR and the anaplastic lymphoma kinase (ALK) gene at the time of diagnosis. In addition, the list of potential future candidates for targeted treatment of NSCLC is still growing, and include KRAS, BRAF, HER2, RET, ROS1, MET, PIK3CA, NTRK [57].

1.3 MicroRNA

In the early era of DNA research, 3% of genes were considered to be protein-coding while the other 97% was considered little more than 'junk' DNA [58]. However, decades later, our understanding of the human transcriptome has changed fundamentally. Not too long ago, the Encyclopedia of DNA Elements (ENCODE) project reported that as much as 75% of the human genome is transcribed into RNA, which is a big statement to the fact that non-coding RNAs (ncRNAs) comprise the majority of the human transcriptome [59, 60]. The first ncRNAs were discovered in the 1950s, and they include the ribosomal RNA (rRNA) and the transfer RNA (tRNA). Since then, a vast variety of various ncRNAs have been described, where microRNAs (miRNAs) are the best described class of short ncRNAs [61, 62].

The first miRNA, named lin-4, was initially described in the transparent nematode *Caenorhabditis elegans*, and was reported as a small RNA with antisense complementarity to the mRNA transcript of the gene *lin-14* [63]. Today, more than 2500 human miRNAs later, we understand that the regulation and dysregulation of miRNAs are involved in virtually all types of cellular processes, both benign and malignant, as miRNAs' involvement in a vast number of different intracellular processes has been revealed [64-69].

The mature miRNA is typically 22 nucleotides in length and is normally processed by the two RNase III proteins Drosha and Dicer [70]. In RNA silencing, the miRNA sequence function as a guide by base pairing with the complementary 3'untranslated region (3'UTR) of its target mRNA, and when united with the AGO proteins, this ultimately results in either translational repression, mRNA deadenylation or mRNA degradation [70].

1.3.1 MiRNA biogenesis

The genes encoding miRNAs are typically located within intergenic regions, but approximately 30% of miRNA genes are positioned within introns or exons of protein-coding genes [71]. It is not unusual that miRNA loci are positioned in relative close proximity to each other, making a polycistronic transcription unit, often referred to as a miRNA cluster [72]. The miRNA cluster is generally subject to a common promoter region, and is hence cotranscribed. However, subsequent differentiated processing and regulation at the posttranscriptional level is common [72, 73]. The miRNA genes that reside in the introns or exons of protein-coding genes often share promoter with their host gene, but it has been shown that different miRNA genes can have several start sites for transcription, which is also the case for intragenic miRNA, whose promoter site can be distinct from the promoters of their host genes [74].

The vast majority of miRNAs are transcribed by RNA polymerase II (RNA pol II). This is apparent when considering the length of the primary miRNA (pri-miRNA) transcript, which is more than 1 kb longer than a typical RNA pol III transcript [75]. In addition, the pri-miRNA transcript contains sequences of uridine residues, which terminate transcription by RNA pol III [76]. Transcriptional regulation of miRNAs is typically directed by various factors, like the transcription factors p53, MYC, ZEB1 and ZEB2, as well as epigenetic factors, like DNA methylation and histone modification [73, 77, 78].

Transcribed pri-miRNA is typically >1 kb long and forms a specific hairpin-shaped stem-loop secondary structure where the mature miRNA sequence is embedded. Typically, a pri-miRNA consists of the mature miRNA-containing stem (33-35 bp), the terminal loop, and a single-stranded RNA segment at both the 5’ and 3’ side [73]. The nuclear maturation process of pri-miRNA is in essence initiated by the nuclear RNase III protein Drosha in collaboration with the DiGeorge syndrome chromosomal region 8 (DGCR8) protein. The RNA binding protein

DGCR8 identifies and binds pri-miRNA and the central part of the Drosha protein. Together, these proteins form the Microprocessor complex, which process the 5' and the 3' strand of the pri-miRNA into an approximately 65 bp hairpin-shaped RNA structure called precursor miRNA (pre-miRNA) [79, 80]. The pre-miRNA is subsequently exported into the cytoplasm, where maturation can be completed. The export of pre-miRNA is aided by the protein exportin 5 (EXP5), whose interaction with the GTP-binding nuclear protein RAN·GTP forms a transporter complex [81]. Upon reaching the cytoplasm through the nuclear pore, the GTP is hydrolyzed and the pre-miRNA/EXP5/RAN·GTP-complex is disassembled, resulting in the release of pre-miRNA into the cell's cytosol [82, 83].

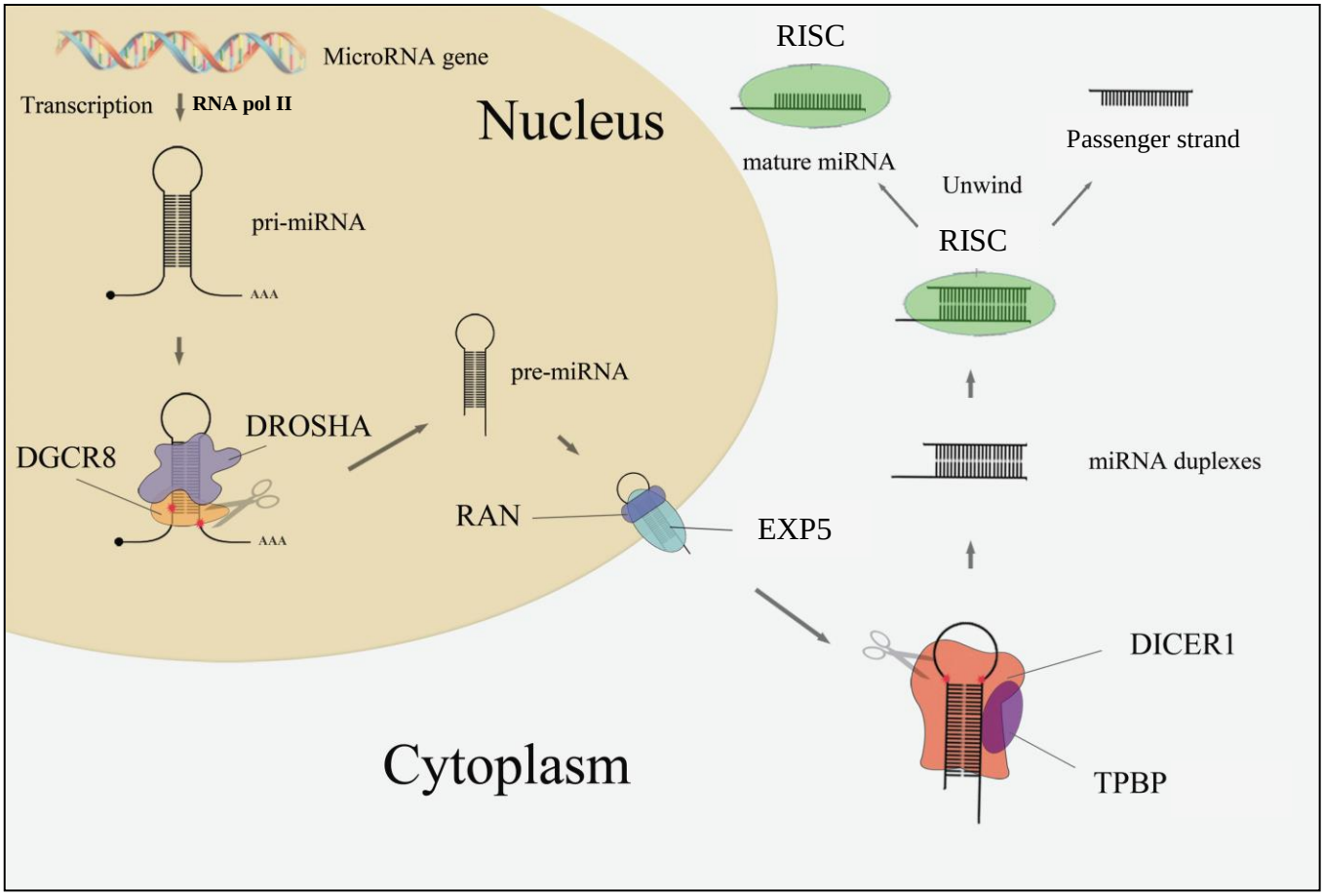

Figure 4: miRNA pathway. An illustration of the miRNA pathway from transcribed pri-miRNA to translational repression. Modified from He et al., 2016.

Once the pre-miRNA is released into the cytosol, the RNase III endonuclease Dicer carries out the next step in miRNA maturation. Dicer recognizes the 5' phosphate and the 3' overhang close to the terminal loop, and cleaves the double stranded pre-miRNA at this site, producing a roughly 22 bp long RNA duplex often referred to as a miRNA:miRNA* complex [73, 76, 84]. There are many different factors and pathways regulating the activity of Dicer, the most recognized being by the TAR RNA-binding protein (TRBP). TRBP contains three double-stranded RNA binding domains (dsRBDs) and is involved in the modulation of several pre-miRNAs, as well as in regulating the length of mature miRNAs [85, 86].

Next, the miRNA:miRNA* complex is loaded into the RNA induced silencing complex (RISC) to finalize the miRNA maturation. The catalytic component of the human RISC complex is the Ago2 protein, who is essential in both binding miRNA and in releasing Dicer [87]. Ago2 is a protein with endonuclease activity, and it cleaves the 3' arm of the miRNA before it is processed by Dicer, a course that is believed to aid in determining the mature lead miRNA strand [87, 88]. Selection of the miRNA lead strand is based on the relative thermodynamic stability of the two ends of the small RNA duplex, and the lead strand is generally the one with the more relatively unstable terminus at the 5′ side [73, 89]. In addition, the Ago2 protein selects the lead strand based on the first nucleotide sequence, and the lead strand presenting a U at nucleotide position 1 is typically selected [90].

The Arg2, using the lead miRNA as a guide, then directs the RISC complex to downregulate target genes. Based on the complementarity between the 3'UTR of the target mRNA and the RISC incorporated miRNA, gene expression is post-transcriptionally repressed by either translational repression, mRNA degradation or mRNA deadenylation [70, 91]. An illustration of the canonical miRNA pathway is presented in figure 4 [92].

1.3.2 The role of miRNAs in cancer

Ever since the first publication describing the deletion of the *miR-15* and *miR-16* loci in the majority of samples from patients with B-cell chronic lymphocytic leukemia [93], a large number of papers describing dysregulation of numerous different miRNAs in a vast diversity of cancers has been published [94]. In general, the mechanisms involved in miRNA dysregulation are chromosomal abnormalities, alterations in transcriptional control, epigenetic changes and defects in the miRNA biogenesis [95].

The deletion of the *miR-15* and *miR-16* loci was the first reported miRNA dysregulation due to a chromosomal abnormality (deletion). Similarly, the 5q33 region, harboring the *miR-143/145* gene cluster, is commonly deleted in lung cancer, resulting in reduced expression of both these miRNAs [96]. In contrast, duplication of the *miR-17-92* gene cluster has been reported in both B-cell lymphomas and lung cancer, resulting in overexpression of these miRNAs [97, 98]. It has become evident that both deletions and duplications (amplifications) of specific genomic regions, as well as chromosomal translocations, contribute to abnormal miRNA expression.

The expression of pri-miRNAs is subject to dysregulation similar to protein-coding genes. During tumorigenesis, alterations in tumor suppressors or oncogenic factors functioning as transcriptional activators or suppressors, will affect the expression of pri-miRNAs. One of these alterations involves the oncogenic miR-17-92 cluster, which is upregulated in several different cancers [99]. MYC stimulates expression of the miR-17-92 cluster, which in turn promotes tumorigenesis by regulating the post transcriptional expression of E2F1, THBS1, CTGF and other transcripts important for cell cycle progression and angiogenesis [94, 100]. Conversely, the miR-200 family is regularly reported as downregulated in human tumors. The miR-200 family is involved in targeting important transcription factors involved in suppression of epithelial genes in order to facilitate the epithelial-mesenchymal transition, which is key to

invasion and metastasis [101]. There are many transcription factors associated with cancer that regulate expression of miRNAs, making transcriptional dysregulation a crucial mechanism in altered miRNA expression in cancer.

Modifications of histones and DNA control the chromatin structure of the chromosome, and these epigenetic changes have an important role in regulating expression of both protein coding RNA and ncRNA. The gene promotors of miRNAs, as well as promotors for most genes, have a relatively high content of CpG islands, and these are frequently hypermethylated in tumor suppressor miRNAs, resulting in an epigenetic silencing of these miRNAs [102]. Histone modification is another epigenetic change, and has been reported to cooperate with DNA methylation to suppress the expression of tumor suppressor miRNAs in cancer [103]. Together, these epigenetic mechanisms play important parts in repressing tumor suppressive miRNAs in cancer.

In miRNA biogenesis, the two RNase III proteins Drosha and Dicer are frequently downregulated in various cancers, and this downregulation is associated with poor patient outcome [104]. For example, the expression of *DROSHA* is regulated by the proto-oncogenic transcription factor MYC, which may lead to decreased pri-miRNA processing [105]. Also, downregulation of *DROSHA* has been reported in context with hypoxia, and this process was facilitated by direct binding of the hypoxia-responsive transcription factors ETS1 and ELK1 to the promoter of *DROSHA* [106]. Likewise, the expression of DICER is also subject to dysregulation in cancer, a process that is highly diverse. The transcription factor Tap63 activates DICER expression by direct promotor binding to its promotor, but this transcription factor is frequently lost in cancer, leading to downregulation of DICER. Another pathway in which DICER is downregulated is through direct binding to its 3'UTR by specific miRNAs, an effect highly influenced by hypoxia [106, 107].

2. Aims of the study

The overall aim of this study was to explore functions and expression patterns of carefully selected miRNAs in breast cancer and lung cancer. To this end, we investigated expression in tissue samples from both breast- and lung cancer patients, alongside functional studies on cancer cell lines representing both organ systems. Expression of miRNA was evaluated using miRNA microarray, qPCR and ISH, and functional studies included experiments eligible for the study of cell proliferation and cell migration.

More specifically, the objectives of the study were to evaluate expression patterns and functional properties of the miR-143/145 cluster in BC and NSCLC. Also, expression and function of the miR-126-3p, and its passenger strand, miR-126-5p, was investigated in BC tissue and BC cell lines.

3. Summary of results

Paper I

Expression and function of the miR-143/145 cluster *in vitro* and *in vivo* in human breast cancer

Vast numbers of miRNAs are dysregulated in cancer, and the miRNA cluster miR-143/145 is among them. In this paper, we sought to investigate the functional properties and expression of the miR-143/145 cluster both *in vivo* and *in vitro* in human breast cancer. For the functional studies we used three different BC cell lines corresponding to the major subtypes of BC: ER+, HER2+ and TN. In addition to analyzing expression, we also studied how the cell lines' ability to proliferate and migrate/invade was affected when transfected with either miR-143 mimic, miR-145 mimic, or miR-143 mimic and miR-145 mimic in combination. For the *in vivo* part, the cellular and subcellular expression of miR-143 and miR-145 was evaluated in full histological slides from both benign and malignant breast tissue. Patient samples were stratified according to molecular subtype, tumor grade and receptor status, and expression patterns of miR-143 and miR-145 were analyzed accordingly.

Results from a comprehensive miRNA microarray study on breast cancer tissue revealed both miR-143 and miR-145 to be downregulated in BC tumors when compared to benign breast tissue. These results were later verified by RT-qPCR. Similar, expression of both miR-143 and miR-145 were downregulated in all tested BC cell lines.

In vitro, miR-143 promoted proliferation of the ER+ and the TN BC cell line, whereas having no significant effect on the proliferation properties of the HER2+ cell line. In contrast, all BC cell lines suffered proliferation inhibition when transfected with miR-145. The cotransfection with miR-143 and miR-145 resulted in inhibited proliferation similar to that of miR-145 alone

in all BC cell lines. All BC cell lines suffered inhibition of invasion when transfected with either miR-143, miR-145 or miR-143 and miR-145. *In vivo*, the expression of miR-143 and miR-145 was lower in malignant compared to benign breast tissue, and lower in the more aggressive tumors with higher tumor grade, loss of ER and the basal-like phenotype. The collected ISH data also contributed very interesting findings regarding cellular and subcellular distribution of the studied miRNAs, especially for the miR-145. Staining patterns of miR-143 were primarily cytoplasmatic and predominantly found in luminal cells of benign breast tissue. In contrast, miR-145 was mainly expressed in the myoepithelial cells of benign breast tissue, and sub-cellularly located to the nuclei.

Paper II

Different functional roles and expression of miR-126-3p and miR-126-5p in breast cancer cell lines and tissues

In the literature, miR-126 is described as a tumor suppressor in various cancers, and is involved in regulation of metastatic processes in BCs. In this paper, we were interested in investigating expression and functional properties of both the miR-126-3p and its passenger strand, miR-126-5p. The study explored the effects of transfecting miR-126-3p mimic, miR-126-5p mimic, or miR-126-3p mimic and miR-126-5p mimic in combination on proliferation and invasion in BC cell lines representing the major subtypes of BC. Expression of miR-126-3p and miR-126-5p in tissue samples were investigated using *in situ* hybridization and tissue miRNA microarrays and PCR.

Results from the miRNA microarray revealed both miR-126-3p and miR-126-5p to be downregulated in BC tumors when compared to benign breast tissue. Results were verified by

qPCR. Likewise, expression of both miR-126 strands were downregulated in all our tested BC cell lines when compared to the non-cancerous breast cell line MCF-10A. Transfection of miR-126-3p resulted in decreased proliferation and invasion in all BC cell lines. Transfection of miR-126-5p decreased proliferation in the ER+ and the HER2+ BC cell lines, whereas having a strong opposite effect in the TN BC cell line, dramatically increasing proliferation potential. *In vivo*, expression of miR-126-5p was significantly higher in high grade tumors and in stroma and tumor cells of luminal B, HER2+ and TN tumors when compared to luminal A tumors. In addition, both miR-126 strands were downregulated in lymph node positive BCs when compared to tumors with no nodal involvement.

Paper III

A gender specific improved survival related to stromal miR-143 and miR-145 expression in non-small cell lung cancer

In addition to the BC cohort (paper I), the miRNA cluster miR-143/145 was investigated in a large retrospective study including 553 NSCLC patients. Tissue was collected from primary lung tumors and metastatic lymph nodes, and tissue microarrays were subsequently constructed from these. Functional studies to evaluate proliferation and migration after transfection of either miR-143 mimic, miR-145 mimic, or miR-143 mimic and miR-145 mimic in combination, were performed using different NSCLC cell lines representing adenocarcinomas, squamous cell carcinomas and large cell carcinomas. Expression of miR-143 and miR-145 in tissue samples was investigated using *in situ* hybridization and tissue miRNA microarrays.

Expression of both miR-143 and miR-145 was downregulated in all NSCLC cell lines included in this study. In contrast, ISH-results from patient samples demonstrated significantly increased

expression of miR-143/miR-145 in tumor cells and adjoining stromal cells when compared to non-malignant tissue. Migration studies were performed on the NSCLC cell lines representing adenocarcinoma and large cell carcinoma. Both cell lines displayed a notable decrease in migration potential when transfected with either miR-143 or miR-145. Proliferation was evaluated in NSCLC cell lines representing adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Both the adenocarcinoma cell line and the large cell carcinoma cell line experienced proliferation inhibition when transfected with either miR-143, miR-145, or miR-143 and miR-145 in combination. In contrast, the squamous cell carcinoma cell line displayed increased proliferation when transfected with miR-143, whilst displaying inhibition of proliferation when transfected with miR-145. When combining miR-143 and miR-145, the proliferation rate of the squamous cell carcinoma cell line was not significantly different to the negative control. *In vivo*, expression of the miR-143/145 cluster was found to correlate with expression of several sex steroid hormone receptors, including progesterone receptor, androgen receptor and estrogen receptor beta. In addition, stromal expression of miR-143 was an independent positive prognostic factor in female patients in both univariate and multivariate analysis, whereas stromal expression of miR-145 was associated with improved disease specific survival for male patients in both univariate and multivariate analysis.

4. General discussion

The main focus of this work has been to investigate functional properties and expression patterns of selected miRNAs in breast cancer tissue. In **paper III** we investigate the same miRNAs as in **paper I**, but in NSCLC. Results for each paper are discussed within the respective papers. In the first part of the discussion (4.1), selected materials and methods will be briefly presented and discussed, and in the second part (4.2) the major findings in each paper will be discussed.

4.1 Materials and methods

4.1.1 Patient cohorts

Patients in the BC cohort were collected from the NOWAC postgenome cohort [108], and included participants diagnosed with BC during the years 2004-2010. A total of 102 BC surgery specimens and 38 benign breast specimens were included in the miRNA microarray, and the data from the miRNA microarray analysis were used in the planning and design of **papers I and II**. The histological grading was based on the modified criteria by Elston and Ellis [109], and the molecular subtyping of tumors were based on the expression of ER, PR, HER2 and Ki67 in consensus with guidelines provided by the St Gallen International Expert Consensus and previous publications [29, 110].

The large NSCLC cohort, which is a part of **paper III**, comprises 553 resected patients with stage I-IIIB NSCLC. The cohort includes patients who underwent surgical resection at the Nordland Central Hospital (NS), Bodø and the University Hospital of North Norway (UNN), Tromsø from 1990-2011 [111, 112], and includes complete medical records with follow-up data up until October 1, 2013 [113].

The most common histological subtype of NSCLC has shifted during the last years from SCC towards ADC. This shift contributes to our data being not quite representative of the current situation, as the SCC subtype had a higher frequency in the early material (1990-2004), which contains 335 of the 553 patients in the NSCLC cohort [111, 112]. The enrollment of patients and the collection of clinical data used in this study was done over a time period spanning more than 20 years. During this period, there have been several alterations in how lung cancer was treated, with the most important one being the implementation of adjuvant chemotherapy in 2005. Moreover, criteria for diagnosing and subtyping NSCLC have also changed during the follow up period. The changes in treatment regimens and criteria for diagnosing and subtyping that have been implemented since the start of data collection present a challenge for this retrospective study, as the longitudinal data may not have been treated adequately homogenously, resulting in skewed results. However, to counter any discrepancies due to updated criteria and to minimize test-retest variations, all tumor material was systematically examined by an experienced pathologist whenever new criteria for NSCLC classification were applied.

4.1.2 Tissue microarray

The process of constructing a tissue microarray (TMA) involves systematically transferring cylinders of small representative tissue from a ‘donor’ block, usually a whole-tissue section (WTS) block, into an empty ‘recipient’ paraffin block [114]. One ‘recipient’ block may hold several hundred cores, allowing several samples from numerous patients in the same ‘recipient’ block [115].

Compared to WTSs, TMAs saves both time and tissue, as one slide containing TMAs can hold close to 800 cores. Another very important advantage of using TMAs, is the elimination of

batch-to-batch variability. To analyze the same amount of samples using WTSs, one would have to prepare slides from hundreds of patients, and the staining process would have to be performed in numerous batches, greatly increasing the chance for batch-to-batch variability [116]. The pathologist(s) that would subsequently evaluate and analyze the samples to identify the relevant tumor compartments, would be facing a very time consuming task, complicating the standardization of staining.

In regards to challenges using TMA, the question of whether a 0.6-1.0 mm core is representative for the heterogeneity of the total tumor is often raised. Studies have, however, revealed that there can be a correlation of more than 90% when comparing the expression of certain biomarkers in TMA and WTS [117, 118], and a recent study provides additional evidence of reliability in determining biomarkers using TMA when using more than one tissue core [119]. It is, however, important to also consider the limitations of WTS, as one single WTS contains a small fragment of the total tumor volume, hence representing a small part of the tumor, and may not itself be representative for large heterogenic tumors.

4.1.3 Immunohistochemistry

IHC is a method for the detection of antigens within cells or tissue sections (usually WTSs or TMAs) using specific antibodies against the antigen of interest. Next, the antibody-antigen complex is visualized by staining. This is a versatile technique, and can be used to assess the expression of numerous antigens, including proteins, amino acids and infectious agents. IHC is an important tool in routine diagnostics, as well as in basal research [120].

The advantages of IHC are many, and include cost effectiveness and *in situ* assessment of distribution and localization of cellular components of interest. It is a well-established method around the world, and IHC analysis can be performed on archived tissue, decades old.

Factors contributing to the challenges of IHC include adequate fixation, tissue processing and antigen retrieval. If tissues are not adequately fixated, decomposition of tissue and its markers may occur, and insufficient antigen retrieval may contribute to false negative results. Other analytical factors include selecting antibody with sufficient specificity, antibody concentration for adequate visualization, incubation conditions, and selection of secondary antibodies and mode of detection.

4.1.4 *In situ* hybridization

ISH is a method used to detect specific nucleotide sequences, like DNA and RNA, in tissue samples or in individual cells. A probe with a specific nucleotide sequence is hybridized to its target RNA or DNA, and subsequently visualized microscopically.

A great advantage when using ISH for the detection of miRNAs, is the visualization of both expression levels and the ability to pinpoint cellular localization. However, miRNAs are small in size, the sequence between related miRNAs may be very similar, resulting in unspecific hybridization, and they may be tissue specific. Other challenges are similar to those for IHC, and include probe specificity, probe concentration, incubation conditions and mode of detection. Probe specificity is important to avoid unspecific hybridization (false positive), whereas correct probe concentration is important to ensure a representative view of expression.

4.1.5 Human cell lines

Human immortalized cancer cell lines are widely used as a substitute for primary cells to investigate the biology of human cancers. They are cost effective, easy to use, they divide practically eternally, hence producing an unlimited supply of material, and they bypass ethical concerns often associated with animal studies. In addition, they comprise a very pure population of cells, absent of stromal and immune cells, contributing to consistency and reproducibility of results. However, this also produces a challenge, as cell lines are cultivated in absence of a normal tumor microenvironment, lacking the stromal compartment. Also, cell lines are highly manipulated and their original phenotype, functions and responsiveness may, to a great extent, have been lost [121]. Additionally, high passage numbers can inflict both genotypic and phenotypic variations, and genetic drift may over time cause heterogeneity in cell cultures [121].

It is important to realize that cell lines do not mirror primary cells, which in turn do not mirror source tissue either, and caution should be made when/if conclusions are drawn from cell line experiments. It is also important to recognize that both primary cells and cell lines are usually cultured in the absence of their normal environment, again urging for the use of caution when ascribing function in the body/model based on results from cell culture experiments alone.

4.2 Discussion of main results

4.2.1 Paper I

In the first paper we present functional properties and expression patterns of the miRNA cluster miR-143/145 in BC. The findings are based on results from functional studies, miRNA microarray, RT-qPCR and ISH. A miRNA cluster is miRNAs whose genes are localized in close proximity to each other on the DNA, resulting in their simultaneous transcription under the control of a common promotor. The correlation in expression between miR-143 and miR-145 was highly significant ($R=0.88$, $p<0.001$), suggesting their cotranscription.

We found the miR-143/145 cluster to be significantly downregulated in tumor cells when compared to benign cells, both in tissue samples collected from the BC cohort and in BC cell lines. Downregulation of the miR-143/145 cluster has been previously published for both BC and other cancer tissues [113, 122-126], but together with miRNA microarray and RT-qPCR, our study also verified expression in BC by ISH.

Results from the miRNA microarray indicated that expression of the miR-143/145 cluster was higher in ER+ tumors than in ER- tumors, and this result was later verified by RT-qPCR (**paper I, table 4**). Partly due to their sensitivity to endocrine therapy targeting the ER, ER+ BCs are generally considered among the least aggressive subtypes. In fact, increased expression of both miR-143 and miR-145 was observed consistently in the least aggressive subtypes (**paper I, tables 3 and 5**). This finding is in line with previous studies describing miR-143 and miR-145 as tumor suppressor miRNAs [127-129].

Functions of miRNAs are complex, and not yet fully understood, as is exemplified by publications reporting adverse effects of miR-143 and miR-145. Dimitrova et al. reported the stromal expression of miR-143 and miR-145 to stimulate neoangiogenesis, and in turn facilitate

tumor expansion in the lung [130]. Also, Donnarumma et al. reported increased levels of miR-143 in exosomes from cancer associated fibroblasts, and that exosome mediated delivery to BC cells could promote further BC progression [131].

Results from the functional studies performed for **paper I** were interesting and somewhat surprising. The miR-143, which is conventionally considered a tumor suppressor [128, 132, 133], displayed evidence of tumor promoting characteristics. The functional studies in **paper I** included three different BC cell lines, representing the major subtypes of BC. When transfecting these with the miR-143 mimic, both the ER+ and the TN BC cell line experienced increased proliferation. Proliferation in the HER2+ cell line did not significantly change from the negative control after transfection with the miR-143 mimic. These findings confirm the presence of a dualism in the function of miRNAs, and that environment and cellular context may be more important than expected.

Interestingly when considering the proliferation experiments, the miR-143 mimic had inhibitory effect on the cells' ability to invade in all three BC cell lines. In addition, all three BC cell lines suffered inhibition of both proliferation and invasion when transfected with the miR-145 mimic. The inhibitory effect on proliferation presented as very potent (**paper I, figures 2a-c**), and these results are in accordance with most of previous publications on miR-145 [134-139]. Cotransfecting miR-143 and miR-145 in equal concentration, resulted in an inhibition of both proliferation and invasion in all three BC cell lines, which is in line with previous publications [140-142]. The magnitude of the effect was similar to that of the cells transfected with miR-145 alone. This translates into two deductions: 1) the proliferation promoting properties of miR-143 in the ER+ and the TN BC cell lines were not able to significantly halt the inhibitory effects of miR-145, and 2) the cotransfection of miR-143 and miR-145 did not in synergy contribute to significantly lower the cells invasive potential when

compared to cells transfected with either miR-143 or miR-145 alone (**paper I, figure 2d**). Different results in different BC cell lines may be explained by the target genes that are active in operating the oncogenic phenotype at any given time, and the miRNAs that are there to regulate them.

A very interesting finding in **paper I** was the subcellular miR-145 distribution in the nuclei of myoepithelial cells (**paper I, figure 7**). Mature miRNAs located to the nucleus are gaining more and more interest, and in a very recent review by Liu et al. they summarize existing evidence of nuclear miRNAs [143]. The list of nuclear mature miRNAs is ever growing, and a relevant selection is presented in table 1.

In addition to nuclear enrichment of mature miRNAs, which presence is in divergence to earlier beliefs, new and surprising functions of miRNAs are also starting to emerge. Recent publications have reported that nuclear miRNAs probably are involved in upregulation of transcription via interactions between miRNAs and gene promotors and enhancers [144]. Interestingly, Xiao et al. recently published a research article describing miRNAs as epigenetic gene activators, and that a subset of miRNAs is capable of activating transcription by means of association with active genetic enhancers [145]. Further, they demonstrated that miR-24-1 function as an alternative mediator for transcriptional gene activation by facilitating the remodeling of chromatins at enhancer regions [145].

Table 1: Profiling of nuclear microRNAs. Adapted from [143].

Cell line	Method	Result
Human nasopharyngeal carcinoma (NPC) 5-8F cell line	Deep sequencing	Among 339 nuclear and 324 cytoplasmic miRNAs, 300 of them overlap.
HCT116 human colorectal carcinoma cell	Microarray RT-PCR Northern blot	The overall average of nuclear ratio of miRNAs is 0.471 ± 0.15. MiR-16, miR-19b, miR-200b, miR-222, miR-29b, miR-29c are highly expressed in the nucleus. MiR-19b, miR-195 are highly expressed in the nucleus.
HeLa	RT-qPCR array In situ Hybridization	11 miRNAs are highly expressed in the nucleolus. MiR191, miR-484, miR-574-3p and miR-193b are highly expressed in the nucleolus
The human breast cancer cell line MCF-7, MDA-MB-231 and the human mammary epithelial cell line MCF-10A (normal breast cells)	Microarray	Nuclear/cytoplasmic ratios of numerous miRNAs vary considerably across different cell lines

4.2.2 Paper II

Based on results from miRNA microarray on the BC cohort and previous work performed by our research group, we wanted to investigate the miR-126. Downregulation of miR-126 is previously reported in malignant breast tissue, and is associated with metastatic progression in BC cells, mainly through upregulation of key functions such as cell proliferation, migration, and survival [146, 147]. In order to gain more insight into functions and expression, we decided to include the passenger strand, miR-126-5p, into our inquiry.

We found endogenous expression of both miR-126-3p (lead strand) and miR-126-5p (passenger strand) to be significantly downregulated in tumor cells when compared to benign cells, both in tissue samples collected from the BC cohort and in BC cell lines representing the major subtypes of BC: ER+, HER2+ and TN.

Endogenous expression of miR-126-3p is considered to be tumor suppressive [148-151], and this is supported by our findings in the functional experiments, where all three BC cell lines suffered both reduced proliferation and reduced invasive capacity when transfected with the miR-126-3p mimic (**paper II, figures 3 and 4**). Surprisingly, the TN BC cell line experienced a very potent increase in proliferation when transfected with miR-126-5p, whilst the ER+ and the HER2+ BC cell lines suffered inhibition of proliferation when transfected with the same miRNA. Invasion potential was also increased in the TN BC cell line when transfected with miR-126-5p, but the magnitude of the response was not as obvious as it was for the proliferation experiment. These findings are not in line with previous publications, where the passenger strand, miR-126-5p, is reported to work in synergy with the lead strand, miR-126-3p, to facilitate a tumor suppressor phenotype [151, 152]. However, there are several reports supporting our findings in the TN BC cell line, and they put miR-126-5p in association with tumor supporting properties such as drug resistance and poor prognosis in acute myeloid

leukemia (AML) patients [153], promotion and protection of endothelial proliferation by inhibition of Dlk1 and SetD5 [154, 155], and induction of proliferation and angiogenesis in non-tumorigenic cells via the PI3K/AKT and MAPK/ERK pathways [156]. Other pathways described in association with miR-126-5p and enhanced tumor progression, include the NOTCH pathway, the Akt signaling pathway and the IGF-1 signaling pathway [157-159].

Expression analysis using TMA revealed that stromal levels of miR-126-5p were significantly associated with both molecular subtype and histological grade, and the highest levels of miR-126-5p were found in the more aggressive subtypes of BC. In this context it is also relevant to point out the correlation between miR-126-5p and the proliferation marker Ki67, at R=0.24, p=0.055. Although not statistically significant at the $p \leq 0.05$ level, it is worth considering in this setting. In the clinical tissue material analyzed by microarray and RT-qPCR, expression of miR-126-3p and miR126-5p was lower in BCs with nodal involvement (**paper II, figure 5**). As previously described, miR-126 is associated with metastases, and has been demonstrated as a negative regulator of the metastatic process in BC, in part by suppressing tumor growth *in vitro* using highly metastatic BC cell lines [146]. Knockdown of miR-126 has also been proven to lead to formation of metastases with high blood vessel density due to increased recruitment of endothelial cells to the metastatic cells [160].

The passenger strands of miRNAs are typically degraded after processing, and are consequently less abundant compared to their lead strand [161], and this is also evident in our study when analyzing results from microarray and PCR. Interestingly, when comparing endogenous levels of lead strand miRNA with endogenous levels of passenger strand miRNA in the non-cancerous breast cell line MCF-10A, the ER+ BC cell line, the HER2+ BC cell line and the TN BC cell line, we discovered an incremental shift in the miR-126-3p/miR-126-5p expression pattern, revealing miR-126-5p to be the more abundant strand in the TN BC cell line (**paper II, figure**

2). It is possible that mechanisms responsible for targeting the passenger strand for degradation are either corrupted, or in some way modified, allowing the accumulation of the passenger strand (**paper II, figure 8**). Consequently, a larger part of the mature passenger strand is eligible to interact with the RISC-complex to exhibit a more potent biological response in the TN BC cell line, which represents the most aggressive BC subtype.

There was a strong link between molecular subtype, tumor grade and expression of miR-126-5p in the tumor stromal compartments. Several studies have described considerable crosstalk between tumor and stroma via exosomal transfer of miRNAs [162-164] where microvesicles containing miRNAs derived from cancer cells convert fibroblast into cancer associated fibroblasts (CAFs) with tumor-promoting properties. Together with functional studies on miR-126-5p, the increased expression in stroma of more advanced BCs tells an interesting story. This work has provided valuable insight into the duplicity of miRNA function, emphasized by mature miR-126 having both potent tumor suppressor and tumor driver functions with opposite effects of the two different miR-126 strands in TN BC.

4.2.3 Paper III

Herein, we present results on functional studies, expression patterns, and prognostic significance in regards to the same miRNA cluster as in **paper I**, namely the miR-143/145 cluster. The work in this paper was performed on a NSCLC cohort comprising 553 patients and NSCLC cell lines. My involvement in this work was primarily on the design and implementation of the functional studies, and the subsequent interpretation and discussion of the results obtained from these studies.

The results from this work revealed stromal expression of miR-143 to be a positive prognostic marker in the female population, and it was also demonstrated that stromal expression of miR-145 is a positive prognostic marker in the male population. Previous publications have reported comparable findings, where low expression of miR-145 was associated with poor outcome in NSCLC and prostate cancer [165, 166]. However, there are reports of miR-143/145 expression having a negative impact on survival for patients with esophageal cancer [167] and bladder cancer [168], suggesting that impact of the expression patterns is tissue-specific. There are indications implying that expression of certain miRNAs may be gender specific, in addition to tissue-specific, and Duttagupta et al. have observed a subset of miRNAs to be differentially expressed in men and women [169]. This is interesting, and in line with our results, where stromal expression of miR-143 was associated with positive prognosis in women, and stromal expression of miR-145 was associated with positive prognosis in men.

The functional experiments were performed on NSCLC cell lines representing adenocarcinoma, squamous cell carcinoma and large-cell lung cancer. Cell lines were transfected with either miR-143 mimic, miR-145 mimic or miR-143 and miR-145 in equal concentrations. All cell lines suffered a significant loss in their capacity to both proliferate and migrate when introduced to miR-145. The adenocarcinoma cell line and the large-cell lung cancer cell line displayed

similar behavior when introduced to either miR-143 alone, or miR-143 and miR-145 in combination and in equal concentrations, although the effect was less prominent for the miR-143 transfected cells. When the squamous cell carcinoma cell line was transfected with miR-143, the proliferation was significantly increased, indicating miR-143 to promote tumor growth in this cell line. This is a very interesting observation when considering the results from the BC study on miR-143 and miR-145. In **paper I** we demonstrated that miR-143 was a potent tumor promoting factor in the ER+ and the TN BC cell lines (paper I, figures 2a and 2c). We have jet to explain why miR-143 has this effect on these cell lines, but it is noteworthy that basal cells and squamous cells share many cellular signatures [45, 55]. It is possible that the proliferation promoting properties of miR-143 in these two cell lines from different organs are a direct result of their cellular similarities, perhaps arguing for cell-type specific miRNAs rather than, or in addition to, tissue-specific miRNAs. Unfortunately, we were not able to get the squamous lung cancer cell line to migrate, so there are no results for this experiment. It would have been interesting to observe if the miR-143 has an inhibitory effect on migration in the squamous cell lung carcinoma cell line, like it had in the TN BC cell line, or if it would promote migration as it promotes proliferation.

As we observed for BC cell lines in **paper I**, the miR-143/145 cluster has a two-faced function in NSCLC cell lines as well, probably ascribed to cellular context. In order to increase our understanding into this duplicity, future experiments should focus on deciphering cellular pathways and miRNA targets in cells with different subtype, but within the same tissue, and also from cells in various tissues.

5. Conclusions and future perspectives

The discovery of miRNAs in the early 1990s was groundbreaking, and a MeSH search (medical subject heading search) today, revealed close to 60 000 entries regarding miRNAs in the PubMed database. In the majority of publications their described function appears to be pretty obvious: To act as post-transcriptional inhibitors of translation, targeting specific mRNAs via interactions with endonucleases. Today, however, we have come to realize that the actual picture is far more complicated. New pathways and mechanisms of action are frequently being described for both well-known and newly discovered miRNAs. Very recently (August 2018), Dragomir et al. published a SnapShot in Cell, describing unconventional functions of miRNAs, including miRNAs directly activating transcription and miRNAs coding for proteins [170]. These are modes of action quite opposite to the central/old dogma of miRNA biogenesis and function, and will likely maneuver future miRNA research into new directions.

The main focus of this thesis has been on investigating functions and tissue expression of selected miRNAs identified as dysregulated in BC. We have determined that the miR-143/145 cluster generally function as a tumor suppressor, which is in accordance with the general consensus, but that this function is dependent on (at least) cellular context. The functional dualism observed for the miR-143 in both BC cell lines and NSCLC cell lines, emphasizes the need for more specific research into pathways and functions of miRNAs. Adding to this story, are the results from the paper describing miR-126. Again, we encounter results contradicting the general consensus, and again we observe a dualism in function which is unsuspected.

One of the major questions of the future is whether or not miRNAs can be effective players in targeted therapy, e.g. as miRNA replacement therapy in cancer treatment. Indeed, interesting research has depicted certain miRNAs to prevent development of drug resistance when used in multi-targeted treatment [171, 172], and the first miRNA treatment eligible for clinical trial,

MesomiR-1, quite recently completed phase I, and preliminary results are promising [173]. But as this thesis accentuates, caution should be exercised when navigating the miRNA landscape in search for new therapeutics, as their functional duality may become a concern.

Hopefully, the future development of new technologies will help shed light on the complex role of miRNA function in human cells. It is my sincere belief that, guided by miRNAs, the future holds promise of increased accuracy of diagnosis and treatment options for many malignancies.

6. References

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Paper I

Paper II

Paper III