Identification of MicroRNAs Responsible for Migration and Invasion of Prostate Cancer Cells

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Summary

Prostate cancer (PCa) is the second cause of cancer death among men in the Western world. Metastasis is the major reason of prostate cancer-related deaths. However, the precise mechanism that governs the cancer cells migration and metastasis is still ill-defined. New research has addressed the role of microRNAs (miRNAs) in mediating tumor migration and metastasis. Some miRNAs are associated with cancer cell migration or identified in migratory and metastatic cell lines or tissues. In the present study, by mining data from Solexa deep sequencing and literature, 6 miRNAs were selected as candidate miRNAs responsible for migration of PCa cells since they were differentially expressed in PCa metastases vs. organ-confined PCa. The expression level of these miRNAs in 8 different PCa cell lines were measured using an RT-qPCR. Migratory ability of all 8 PCa cell lines were determined utilizing a Boyden chamber assay. MiR-141 and miR-200c were selected due to a high inverse correlation between the expression of these miRNAs and migratory ability of cell lines. The expression levels of these miRNAs were manipulated in cell lines with a high (DU145 and PC3) and low (VCaP and PC346C) expression of endogenous miR-141 and miR-200c. The transfection results were confirmed using an RT-qPCR. Subsequent Boyden chamber assays were performed. Results indicated that miR-141 and miR-200c acted as suppressors of migration in VCaP and DU145 cells, respectively, whereas, in PC3 cells miR-141 acted as an inducer of migration. A growth assay was performed, indicating that the transfections did not affect cell proliferation. Moreover, the endogenous expression levels of ZEB1, ZEB2 and TGFβ2 as validated targets for miR-141 and miR-200c were monitored by RT-qPCR in different PCa cell lines and the results indicated a positive correlation between their expression level and the migratory ability of cell lines. The expression level of these genes in transfected cell lines resulted in decreased expression of ZEB1, ZEB2 and TGFβ2 by overexpression of miR-141 and miR-200c in DU145 cells. However, in PC3 cells, only overexpression of miR-141 led to decreased TGFβ2 expression. This might be due to the regulatory role of miR-141 and miR-200c at translational level rather than mRNA level. Taken together, we observed that miR-141 and miR-200c have an important role in regulating the migration ability of PCa.
cells. Based on our data, miR-141 and miR-200c have diverse effect on different cell lines. They do not affect the migratory ability of the same cell line, similarly. These findings might lead to a conclusion that based on the type of cell line, these miRNAs might regulate migration via a somewhat different route.
Table of Contents

Acknowledgements .................................................................................................................. 2
Summary .................................................................................................................................. 3
Table of Contents ..................................................................................................................... 5

1. Introduction .......................................................................................................................... 7
   1.1. The prostate ...................................................................................................................... 7
   1.2. Prostate cancer ................................................................................................................ 7
   1.3. Metastasis ......................................................................................................................... 8
   1.4. EMT and human cancer .................................................................................................. 9
   1.5. MicroRNAs .................................................................................................................... 11
   1.6. MicroRNAs biogenesis, processing and gene regulation .................................................. 12
      1.6.1. Transcription ........................................................................................................... 12
      1.6.2. Hairpin release in the nucleus ................................................................................... 12
      1.6.3. Export to the cytoplasm ........................................................................................... 12
      1.6.4. Dicer processing ...................................................................................................... 12
      1.6.5. Strand selection by RISC ....................................................................................... 13
   1.7. MicroRNAs and human cancer ....................................................................................... 13
   1.8. Role of miRNAs in metastasis ....................................................................................... 15
   1.9. MiR-200 family and EMT ............................................................................................ 16
   1.10. MiR-141 and miR-200c known regulatory cascade ...................................................... 18
   1.11. Scope of this thesis ...................................................................................................... 20

2. Materials and Methods ........................................................................................................ 22
   2.1. Cell lines and culture conditions .................................................................................... 22
      2.1.1. Cells ........................................................................................................................ 22
      2.1.2. Cell culture .............................................................................................................. 22
   2.2. Viable cell count using trypan blue .............................................................................. 23
   2.3. RNA isolation ................................................................................................................ 24
   2.4. Real-time quantitative RT-PCR to measure the expression level of ZEB1, ZEB2 and TGFβ2 .......................................................... 24
   2.5. TaqMan real time PCR microRNA assays ................................................................... 24
      2.5.1. Uniplex RT and Multiplex RT ................................................................................. 25
   2.6. MiRCURY LNA™ Universal RT microRNA PCR .......................................................... 26
   2.7. Optimization of transfection ....................................................................................... 27
   2.8. Transient transfection .................................................................................................. 28
2.8.1. Overexpression of miR-141 and miR-200c ................................................................. 29
2.8.2. Inhibition of miR-141 and miR-200c ........................................................................ 29
2.9. MTT assays ................................................................................................................ 30
2.10. Cell migration assays .............................................................................................. 31
  2.10.1. Migration assay to investigate migratory ability of PCa cell lines ...................... 31
  2.10.2. Migration assay after transfection ..................................................................... 32
3. Results .......................................................................................................................... 33
  3.1. Selection of candidate miRNAs responsible for migration of PCa cells .................. 33
  3.2. Selection of candidate miRNAs whose expression are correlated with migratory ability of PCa cell lines ............................................................ 34
    3.2.1. Investigation of migratory ability of different PCa cell lines ......................... 34
    3.2.2. Differential expression level of selected miRNAs and their correlation with migratory ability of PCa cell lines .................................................. 35
  3.3. Functional studies to identify the role of miR-141 and miR-200c in migration of PCa cells ................................................................. 37
    3.3.1. Optimization of transfection conditions ......................................................... 38
      3.3.1.1. Finding a suitable transfection reagent for each cell line ...................... 38
      3.3.1.2. Finding an efficient transfection condition for each cell line using Lipofectamine RNAi MAX ................................................................. 40
    3.3.2. Manipulating the expression level of miR-141 and miR-200c ...................... 41
  3.4. Migration of PCa cell lines upon manipulation of miR-141 and miR-200c expression .... 43
  3.5. Investigating the growth ability of PCa cell lines upon manipulation of miR-141 and miR-200c expression ................................................. 47
  3.6. Monitoring the expression level of the validated target genes for miR-141 and miR-200c in different PCa cell lines before and after transfection ......................... 48
  3.7. Summary ................................................................................................................. 50
4. Discussion ..................................................................................................................... 52
  4.1. Association of miR-141 and miR-200c expression and migratory ability of PCa cells .... 52
  4.2. MiR-141 and miR-200c affect migration ................................................................. 53
  4.3. Role of miR-141 in promoting the PC3 cell migration ............................................. 54
  4.4. MiR-141 and miR-200c act differently per cell line and among cell lines ................ 56
  4.5. The miR-141 and miR-200c cascade in PCa ............................................................ 57
  4.6. Effect of combining the miR-141 and miR-200c ....................................................... 59
  4.7. Clinical implications ............................................................................................... 59
  4.8. Conclusions ............................................................................................................. 61
References ....................................................................................................................... 62
List of Abbreviations ...................................................................................................... 67
1. Introduction

1.1. The prostate

Prostate is an exocrine male reproductive organ whose function is to secrete prostatic fluid. Prostatic fluid contains sugars, proteins and enzymes that make up 30% of the semen and regulate the motility of the sperm. A normal prostate is about 3 cm in diameter; however, its size can change over time, and in many men, the prostate gets larger when they get older. The prostate consists of two pear-shaped lobes and is located in front of the rectum, under the bladder where urine is stored (Fig. 1). The urethra, which carries both urine and semen out of the body, also passes directly through the prostate [1].

![Figure 1. Anatomy of the prostate gland (Obtained from [2]).](image)

1.2. Prostate cancer

Prostate cancer (PCa) is the most common non-skin malignancy and the second cause of cancer death after lung cancer among men in the Western world [3, 4]. From a clinical standpoint, prostate cancer will be diagnosed as local or advanced disease. In case prostate cancer is confined within the prostate, prostatectomy or radiation therapy can be performed with curative intent. In advanced prostate cancer, hormone therapy is the most common treatment to inhibit cancer growth that has spread to distant
parts in the body. Chemical castration using LHRH agonists is an example of hormonal therapy resulting in a strong decrease of circulating androgen levels. Although most patients respond favorably to the hormone therapy, ultimately all patients will relapse and the prostate tumors will at best poorly respond to other kinds of therapies, resulting in prostate cancer mortality [5].

1.3. Metastasis

While metastasis has been the subject of intense research for over a century, the underlying molecular mechanisms are still not fully understood. What is clear is that metastasis is a complex and multi-step process that involves at least four major steps (Fig. 2) [6]:

1- An epithelial to mesenchymal transition (EMT) is required for cells to undergo the first step of metastasis. EMT includes an initial cell transformation, proliferation of the transformed cells and a subsequent local invasion and destruction of paranchymal cells.

2- Migration of tumor cells form primary tumor and entering of the cancer cells in systemic circulation.

3- Extravasation

4- Embolisation of cancer cells at distant organs and formation of a secondary tumor.

Generally, lymph nodes are a most common site of metastasis in a variety of cancers. In prostate cancer for instance, 75% of patients with lymph node metastases at the time of diagnosis will develop bone metastases within 5 years [7]. It has been shown that in many cases, cancer-related deaths are caused by subsequent metastases rather than the primary tumor itself. The proposed reason for this fatal outcome is the development of therapy-resistant metastases [8-10]. Recently, it has been demonstrated that in prostate cancer a small number of cells that reside in the tumor have tumor-initiating properties. These cells putatively have a higher capacity to metastasize and survive cancer treatment. These prostate cancer initiating cells are independent of androgen for survival and are able to generate new tumors at distant sites via migration and invasion [10-17]. Therefore, an effective treatment for the eradication of PCa requires an approach in which tumor-initiating cells are targeted.
1.4. EMT and human cancer

Despite all the research that has been carried out to find the important genes and pathways involved in cancer initiation, progression and metastasis, the molecular mechanisms governing the progression from a primary tumor to lethal metastasis is still ill-defined. Emerging knowledge on expression-profiling analyses have indicated that a select group of genes involved in metastasis initiate a process, which is required for the cells to undergo the first step of metastasis [6, 18, 19]. This early and probably most critical step in metastatic conversion of tumor cells, is reported to be a cellular program called epithelial to mesenchymal transition or EMT [18]. A typical epithelium is a monolayer sheet of cells. Individual epithelial cells are bound to each other via cell-cell junctions and adhesions and form immotile sheets, which inhibits the individual cell movement. Mesenchymal cells, on the other hand, are not tight in their cell-junctions. They form irregular shapes. Cell adhesions in mesenchymal phenotype are less strong than in their epithelial counterparts. This suggests an increased in migration ability of these cells. Mesenchymal cell movement is more dynamic than epithelial cells since these cells have the ability to move individually [20]. EMT describe a series of transcriptional and protein modification events, by which epithelial tumor cells change their morphology by down-regulation of epithelial markers such as E-cadherin, lose their adhesion and obtain a mesenchymal phenotype leading to enhanced motility and invasiveness. E-cadherin is a cell adhesion protein and is thought to be a suppressor of migration [18, 21, 22]. EMT sometimes can be reversed (mesenchymal to epithelial transition (MET)). In some cases, there will be morphological similarities between primary tumor and metastatic lesions. It is likely that tumor cells reactivate certain epithelial properties at the
metastatic sites by a MET [23]. It has been reported that MET can facilitate the establishment of macro-metastases [24, 25].

Cell motility is a prerequisite to invasion of cells to distant sites in the body. Therefore, identification of molecules that control cell motility is considered to be essential for understanding cancer metastasis [10, 21, 22]. Several studies have investigated a link between a recently discovered class of non-coding RNA, so called microRNAs (miRNAs) and EMT [9, 19, 26].

In addition, the EMT process requires a complex mechanism in which EMT inducers repress E-cadherin. Due to the importance of these EMT inducers in metastases, characterization of them should uncover the molecular mechanisms that govern tumor migration and invasion [27]. There are several master genes including zinc finger E-box binding homeobox or ZEB1 (TCF8 or δEF1), zinc finger E-box binding homeobox 2 or ZEB2 (ZFXH1b or SIP1), transforming growth factor β2 or TGFβ2 and Snail factors, which have essential roles in EMT. ZEB1, ZEB2 and Snail factors act as transcription factors. As part of EMT, their expression is increased in cancer cells in response to stimulation by extracellular cytokines that are present in the tumor environment including TGFβ2. These transcription factors act as EMT inducers as well as transcriptional repressors by inverse regulation of E-cadherin. E-cadherin is a master regulator of epithelial polarity, whose functional loss is one of the EMT’s hallmarks. Furthermore, the protein expression level of EMT inducers and suppressor could potentially be used as markers for identification of cells with mesenchymal or epithelial characteristics. In this regard, these EMT targets could potentially be considered as EMT markers in clinical samples or cell lines. TGFβ2, ZEB1, ZEB2 and Snail factors are mesenchymal markers and up regulated during EMT. E-cadherin is an epithelial marker and down regulated during EMT [27-29].

Recent studies have identified that these metastasis-associated genes and more specifically EMT inducers or inhibitors have important roles in tumor metastasis [22, 26, 27, 30]. MiRNA networks can potentially act as upstream regulators of these genes [18, 30-33]. Therefore, identification of those miRNAs is essential for understanding cancer metastasis and promotes a better understanding of molecular mechanism of metastasis.
1.5. MicroRNAs

MiRNAs were first discovered from the observation in *Caenorhabditis elegans* that the RNA from lin-4 gene binds to the 3’untranslated region of the lin-14 mRNA and inhibits its translation. The importance of miRNAs became appreciated in 2001, with identification of evolutionary conserved miRNAs among different organisms including human. Since then, the list of known miRNAs has expended, now standing at approximately 800 in human and predicted to increase further [18].

MiRNAs are a class of short non-coding RNAs (about 21-24 nt in length). They are processed from a long primary miRNAs (pri-miRNA) to a shorter stem-loop structure called precursor miRNA (pre-miRNA) and subsequently to a functional mature miRNA [34].

MiRNAs regulate gene expression at posttranscriptional level either by suppressing the translation of their target mRNAs via partial base paring with 3’UTR sequence in mRNA or in case of perfect base paring by degradation of their target mRNAs. Each miRNA has the potential to target a large number of mRNAs and conversely, different number of miRNAs can target the same mRNA [35-37]. The specificity of miRNA targeting is determined by the complementarities between the miRNA seed sequence, 2-8 nucleotides from the 5’, and the 3’ UTR of the target mRNA. Many miRNA genes are conserved in closely related species and their number per species is nearly 1000. This constitutes approximately 3% of genes in the human genome and represents a major regulatory aspect of posttranscriptional and translational regulation [37-39]. MiRNA can be either encoded by known genes (introgenic) or sequences located between known genes (intergenic). It has been shown that 50% of human miRNAs are expressed from introns of protein-coding genes. Introgenic miRNAs are transcribed along with their host genes, while intergenic miRNAs are transcribed as separate units, with their own transcriptional regulatory elements. They can exist either in polycistronic clusters or as independent transcription units. In case of polycistronic clusters, miRNA genes are co-expressed from a single promoter to make a polycistronic pri-miRNA. Many of intergenic miRNAs exist as polycistronic clusters, suggesting that from the same primary transcript, more than one pre-miRNA may be processed [39-42].
It has been documented that different tissues and developmental stages are characterized by different miRNA expression patterns and estimated that more than 30% of the human genes are regulated by miRNA [43, 44]. That is why, miRNAs play important roles in various biological process including differentiation, development, apoptosis, proliferation, signal transduction and carcinogenesis[43, 45, 46].

1.6. MicroRNAs biogenesis, processing and gene regulation

1.6.1. Transcription

Initially, miRNAs are transcribed by RNA polymerase II in the nucleus as long primary miRNAs (pri-miRNA) that can be thousands of nucleotides long and might contain several precursor miRNAs. The transcript contains a 3’ poly-A and 5’-cap and likely forms a hairpin structure with signals for dsRNA-specific nuclease cleavage (Fig. 3) [43, 45-47].

1.6.2. Hairpin release in the nucleus

The RNAase III enzyme Drosha digests the pri-miRNA for release of the hairpin from the nucleus. Hairpin structures encoding miRNAs are called precursor miRNAs (pre-miRNA), have a length of ~70 nt RNAs with 1-4 nt 3’ overhangs, 25-30 bp stems and relatively small loops [43, 45-48].

1.6.3. Export to the cytoplasm

Exportin-5 (Exp5) is responsible for pre-miRNA’s transportation from nucleus to the cytoplasm, where they go through further processing. Exp5 binds specifically to the correctly processed pre-miRNAs [49, 50].

1.6.4. Dicer processing

Dicer is an endoribonuclease and a member of the RNase III family, which cleaves ds-RNAs into shorter ds-RNA fragments. Dicer cleaves the pre-miRNA and removes the loop and the resulting dsRNA is the miRNA/miRNA* duplex with the length of 19-22 nt. Only one of two strands is the
mature miRNA. Some of the mature miRNAs derive from the leading strand of the pri-miRNA transcript and others derive from the lagging strand [49, 50].

1.6.5. Strand selection by RISC

Single stranded mature miRNAs associated with the RNA-Induced Silencing Complex (RISC), bind to their target mRNA leading to direct cleavage of the complementary mRNA or inhibition of translation. Selection of the mature strand from the ds-RNA is based on the base pairing stability of the termini of the two ends. The strand with lower stability base paring is preferentially associated with RISC and will become the mature miRNA [51, 52].

![Diagram of miRNA biogenesis and processing](image)

Figure 3. MiRNA biogenesis and processing. Explanation can be found in the text (Obtained from [34])

1.7. MicroRNAs and human cancer

Protein coding genes including oncogenes and tumor suppressor genes have been identified to be involved in cancer initiation, progression and metastasis. However, discovery of non-coding RNA has shown that genomic complexity of cancer cells is greater than expected [43, 53]. Indeed, accumulating
evidence has established an important role for miRNAs as regulatory factors in formation and progression of most types of human cancers [34, 43, 54]. For example, the let-7 family regulates several oncogenic pathways in lung cancer and miR-21 suppresses the cell cycle regulators in colorectal cancer [55]. This is perhaps not surprising due to the wide range of cancer-associated processes that are regulated by miRNAs. Interestingly, more than 50% of miRNA genes have been shown to be within cancer-associated genomic regions that are frequently involved in amplification or deletion in human cancer [18]. In this regard, they may contribute as a novel class of oncogenes or tumor suppressors. A direct role for miRNAs in cancer came with an observation in the majority of Chronic Lymphocytic Leukemia (CLL) patients. A frequently deleted region was identified on chromosome 13q14, where miR-15a and miR-16-1 are encoded. Reduced expression level of miR-15a and miR-16-1 in human CLL has also been confirmed by microarray analysis and their tumor suppressive role has been proven by other studies. It has been reported that loss of miR-15a and miR-16-1 from their locus results in increased expression of BCL2 as an anti-apoptotic gene. Alternatively, those miRNAs whose expression is increased in tumors may be considered as oncogenes. In several kinds of lymphoma and lung cancer, a genomic locus at chromosome 13q31, where the members of the miR-17-92 cluster are encoded, is amplified. Further research revealed the high expression of the miR-17-92 cluster in cancer tissues compared to normal tissues and its oncogenic role has also been documented in lung, breast, colon and stomach cancer as well as myeloma and lymphoma [34, 43, 53, 56]. Alterations in miRNA expression level and functionality can also be due to several other mechanisms including epigenetic modification, mutations and SNPs located in mature miRNAs as well as defects in the miRNA biogenesis machinery [57].

Recognition of differentially expressed miRNAs between cancer and normal tissues may provide information for those miRNAs that are involved in human cancer [53]. The altered expression of several miRNAs and their target genes have been discovered in PCa, associated with the invasion and metastasis of this disease. Identification of those miRNAs can be useful as biomarkers in diagnosis, prognosis and classification purposes of PCa [58]. From the more than 50 dysregulated miRNAs in PCa identified in published profiling studies, only few miRNAs have been experimentally determined
to be involved in cancer initiation and progression [58]. Several other miRNAs such as the miR-34 cluster, miR-205 as well as the miR-200 family as tumor suppressors and miR-221/222 and miR-125b as oncogenic miRNAs have also been reported in different types of cancer [47, 58, 59].

1.8. Role of miRNAs in metastasis

Although the role of miRNAs as tumor suppressors or oncogenes has been determined in numerous articles, recent studies have addressed their specific role in mediating metastasis [60]. Different miRNAs are involved in the regulation of biological process leading to the acquisition of metastatic potential, as EMT, adhesion, migration, invasion, apoptosis, angiogenesis and colonization (Table 1) [9, 61]. In this regard, miRNAs can function as either metastatic activators or suppressors by regulating various critical steps in the metastatic cascade. These miRNAs enhance metastasis by affecting multiple signaling pathways and targeting important proteins that play a significant role in different steps of metastasis. Studies revealed the potential role of miR-21 in promoting tumor metastasis by downregulating metastasis suppressor genes in breast cancer and colon cancer [26, 61]. Conversely, other miRNAs can serve as metastasis suppressors by regulating diverse pathways to block metastasis. It has been shown that miR-145 inhibits breast cancer cell migration and invasion by affecting genes with pro-metastatic effect [9, 18, 26, 61].

Based on available data, a number of miRNAs that are strongly associated with cancer metastasis are summarized (Table 2). These miRNAs regulate different molecular mechanisms involved in metastasis including EMT [61]. One of the best described cascade which is found to be a metastasis-specific feature, is down regulation of miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) causing EMT and cancer cell migration. Therefore, we will further discuss the functional importance of miR-200 family in EMT and cell migration [18, 61].
Table 1. The pro- and anti-metastatic miRNAs, which affect the different steps of metastatic cascade are listed [9, 61].

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Adhesion migration invasion</th>
<th>Apoptosis</th>
<th>Angiogenesis</th>
<th>Colonization</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro-metastatic</td>
<td>miR-10</td>
<td>miR-29a/b/c Mir-182</td>
<td>Mir-27a/b Mir-19a/b Mir-221 Mir-222 let/7f Mir-210 Mir-130a</td>
<td>miR-31</td>
</tr>
<tr>
<td>anti-metastatic</td>
<td>miR-141</td>
<td>miR-146a/b Mir-206 Mir-335 mir-31</td>
<td>Mir-31 Mir-156 mir-16 mir-20a/b mir-126 mir-122</td>
<td>miR-31</td>
</tr>
</tbody>
</table>

Table 2. miRNA signature of cancer metastasis

<table>
<thead>
<tr>
<th>MiRNAs</th>
<th>Molecular regulation</th>
<th>Deregulation in cancer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-9 family</td>
<td>↓NF-KappaB1 ↓DNA methylation</td>
<td>Down-regulated</td>
<td>[62-64]</td>
</tr>
<tr>
<td>miR-148a, miR-148b</td>
<td>↓DNMT3b, ↓TGIFB</td>
<td>Down-regulated</td>
<td>[63, 65, 66]</td>
</tr>
<tr>
<td>miR-200 family</td>
<td>↑E-cadherin, ↓EMT, ↓Cancer cell migration/ invasion</td>
<td>Down-regulated</td>
<td>[62, 66]</td>
</tr>
<tr>
<td>miR-210</td>
<td>Hypoxia-induced, ↑angiogenesis</td>
<td>Up-regulated</td>
<td>[66, 67]</td>
</tr>
</tbody>
</table>

DNMT3B DNA (cytosine-5)-methyltransferase 3 beta; TGIFB TGFB-induced factor homeobox 2

1.9. MiR-200 family and EMT

The miR-200 family consists of five miRNAs that are encoded by two separate polycistronic transcripts within two clusters, miR-200b/a/429 and miR-141/200c, which are located on human chromosomes 1 and 12, respectively. Members of each cluster are co-regulated as a result of being processed from the same primary transcript. They can be divided into two different subgroups based on their seed sequences (Fig. 4). MiRNAs from the same group have the same seed sequence leading to a high degree of overlap in their target genes. MiRNAs from different groups differ only in one nucleotide in their seed sequence (Fig. 4). Although one nucleotide difference in seed sequence is expected to result in largely distinct target specificity, it has been shown that different members of the miR-200 family, target a large common set of genes resulting in enhanced efficiency of genetic regulation [22].
The genomic localization of the miR-200 family is shown using the UCSC Genome Browser (GRCh37/hg19) (Fig. 5) [68]. All the members are located on the human genome and mapped to the RefSeq genes and Spliced Expressed Sequence tags (ESTs). It is shown that the miRNA cluster miR-200a/b/429 is located on chromosome 1 and is intergenic. Their host gene is unknown. A search for ESTs did not find any matched ESTs for these miRNAs. The miRNA cluster miR-141/200c is located on chromosome 12. This cluster is also intergenic with an unidentified host gene, although various spliced ESTs have been described.

Figure 4. Two subgroups of miR-200 family based upon their seed sequences. The underlined nucleotides show the seed sequence and the red nucleotide shows the differences in seed sequence between the two subgroups.

![Figure 4. Two subgroups of miR-200 family based upon their seed sequences. The underlined nucleotides show the seed sequence and the red nucleotide shows the differences in seed sequence between the two subgroups.](image)

Figure 5. Genomic localization of miR-200 family using UCSC genome browser [68]. Explanation can be found in the text.

![Figure 5. Genomic localization of miR-200 family using UCSC genome browser [68]. Explanation can be found in the text.](image)

Increasing evidence strongly indicates that the miR-200 family inhibits EMT by maintaining the epithelial phenotype and inhibiting the EMT activators. As was mentioned before, ZEB1, ZEB2, Snail factors and TGFβ2 are EMT activators and regulate the expression of E-cadherin. These proteins are
of particular interest since they are expressed at the invasive front of the tumor and correlate with occurrence of distant metastasis. Thereby, loss of the miR-200 family may have the potential to promote metastasis by loss of downregulation of ZEB1, ZEB2, Snail factors and TGFβ2 and hence activating EMT and cell migration [18, 22, 27].

In contrast to the targeting of ZEB1 and ZEB2 by the miR-200 family, ZEB1 and ZEB2 themselves can suppress the expression of both miR-200 gene clusters by binding to the highly conserved sites on their promoter regions. A number of validated targets for miR-200 family members are shown bellow (Table 3). All the studies so far have described ZEB1 and ZEB2 as direct and crucial targets for all miR-200 family members, explaining the fact that a negative correlation exists between ZEB factors and miR-200 family expression [18, 22, 27, 69]. According to Target Scan 4.1 [70], ZEB1 has 5 putative miR-200b/c/429 and three miR-200a/141 sites in its 3’ UTR. ZEB2 carries two predicted miR-200a/141 and five miR-200b/c/429 seed matches in its 3’ UTR.

<table>
<thead>
<tr>
<th>Target</th>
<th>MiR-200 family members</th>
<th>Origine of the cancer cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZEB1</td>
<td>All family members</td>
<td>Diverse tissues</td>
<td>[27, 30, 31, 71, 72]</td>
</tr>
<tr>
<td>ZEB2</td>
<td>All family members</td>
<td>Diverse tissues</td>
<td>[27, 31, 72]</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>miR-141, miR-200c</td>
<td>Breast, Pancreatic, Colorectal</td>
<td>[30]</td>
</tr>
</tbody>
</table>

### 1.10. MiR-141 and miR-200c known regulatory cascade

Here, we address a mechanism by which the miR-200 family is known to be regulated and focus mainly on one cluster of miR-200 family including miR-141 and miR-200c. However, this mechanism is still not defined in prostate cancer.

Both miR-141 and miR-200c map within a 500 bp region on human chromosome 12 and are regulated by a common promoter [30]. There are putative binding sites for ZEB and Snail transcription factors within the promoter. In non-invasive tumors, epithelial cells express high levels of miR-141 and miR-200c and low levels of ZEB and Snail factors (Fig. 6). High expression level of miR-141 and miR-
200c and hence low expression level of ZEB factors result in high expression of E-cadherin. On the contrary, during the EMT process, when the cells obtain the mesenchymal phenotype, certain cytokines are stimulated via a key signaling pathway including TGFβ. In response to TGFβ, the expression of Snail factors and subsequently ZEB factors are increased [22]. ZEB factors repress the expression of miR-141 and miR-200c and directly inhibit the expression of E-cadherin, which stimulates a phenotypic change. Depending on the extracellular signals, such a loop can easily switch from one state (EMT) to the other state (Mesenchymal to Epithelial Transition or MET) and specifies epithelial or mesenchymal status of cells and hence their migratory capacity [18].

The recently described role of miR-141 and miR-200c in regulating the EMT process was validated using animal experiments. Studies have shown the role of miR-141 and miR-200c in migration of cancer cells and EMT upon the stable gain or loss of miR-141 and miR-200c expression in tumor cell lines and injection of those cells into the mice [73]. In addition, in a study on human colon cancer, high expression of ZEB factors were detected and proposed to be associated with tumor migration and invasion. In this study, it was shown that the stable knockdown of ZEB factors in tumor cell lines and injection of the cell lines into the mice lead to the reduction of primary tumor in size and almost no distant metastasis formation [33]. Based on these studies, miR-141 and miR-200c might provide us with novel therapeutic interventions by introducing these miRNAs in cancer cells and hence reversing tumor progression. In this regard, miRNAs might also have the potential to be prognostic indicators of cancer progression.
1.11. Scope of this thesis

In this study, we are interested in investigating miRNAs, which are assumed to be responsible for migration of PCa cells. It is our hypothesis that differentially expressed miRNAs in metastases vs. organ-confined PCa cells may have the potential to activate or inhibit the process of migration and by doing so, uncover parts of the molecular mechanisms behind the process of metastasis. Identifying responsible miRNAs for PCa cell migration and the exact mechanism behind it is of paramount importance since it is still unknown for PCa cells.

To assess our hypothesis, the first aim was to select for candidate miRNAs, which were differentially expressed in metastases vs. organ-confined PCa cells utilizing two approaches. First, by using data from Solexa deep sequencing, we selected miRNAs, which were differentially expressed in metastasis vs. organ-confined PCa cells. Second, a literature search was performed and a list of miRNAs, which were differentially expressed in metastases vs. organ-confined cancer cells were selected. Then, a comparison was carried out between the two lists. Finally, we came up with a number of miRNAs, which had the similar change in expression level in both lists and were not yet known for their role in PCa migration and metastasis.
The second aim of our study was to select for the candidate miRNAs, whose expression level correlated with migratory ability of the PCa cell lines. We investigated the migratory ability of different prostate cancer cell lines using the Boyden Chamber assay. Then, expression levels of all selected miRNAs were measured using RT-qPCR in the panel of PCa cell lines. Two miRNAs were selected for further analyses based on their expression correlation with the migratory ability of PCa cell lines.

The third aim of our study was to perform a functional study to investigate the role of miRNAs in the migration of PCa cells. We manipulated their expression level by transfection of mimics and antagomiRs in selected cell lines and analyzed the effect by RT-qPCR, migration and growth assays. Cell lines were selected based on the endogenous expression level of miRNAs; two cell lines with high expression of those miRNAs and two cell lines with their low expression. In the next step, transient overexpression in cell lines with low expression of those miRNAs and transient inhibition, in cell lines with their high expression were performed using free oligos as mimics and LNA oligos as antagomiRs. Effect of transfection was confirmed by RT-qPCR. Migration and growth assays were performed upon manipulation of the miRNAs expression levels. With a subsequent migration assay, we checked the migratory ability of the selected cell lines after transfection. By a growth assay, we checked the growth rate of the selected cell lines after the transfection.

The fourth aim of our study was to monitor the expression level of some validated miRNA target genes in selected cell lines, upon manipulation of the miRNA expression level. The purpose of this aim was to find out whether ectopic transfection of miRNAs in the selected cell lines, resulted in changing the expression level of their target genes at mRNA level. To do so, RT-qPCRs were performed on the validated target genes for those miRNAs.
2. Materials and Methods

2.1. Cell lines and culture conditions

2.1.1. Cells

In our experiment, 8 PCa cell lines were used, representing different stages of androgen dependency and migration ability as is shown below (Table 4):


<table>
<thead>
<tr>
<th>Prostate cancer In vitro cell lines</th>
<th>Established in:</th>
<th>Origin</th>
<th>Androgen response</th>
<th>Migratory ability</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>1978</td>
<td>Brain</td>
<td>AI</td>
<td>Highly migratory</td>
</tr>
<tr>
<td>PC3</td>
<td>1978</td>
<td>Bone</td>
<td>AI</td>
<td>Highly migratory</td>
</tr>
<tr>
<td>PNT2C2</td>
<td>1980</td>
<td>Non-tumorigenic epithelial prostate cells (Immortalized)</td>
<td>AI</td>
<td>Highly migratory</td>
</tr>
<tr>
<td>LNCaP</td>
<td>1980</td>
<td>Lymph node</td>
<td>AD/AS</td>
<td>Poorly migratory</td>
</tr>
<tr>
<td>22RV1</td>
<td>1999</td>
<td>Primary, from CWR22R xenograft</td>
<td>AI/AS</td>
<td>Poorly migratory</td>
</tr>
<tr>
<td>LAPC4</td>
<td>1997</td>
<td>Lymph node</td>
<td>AD</td>
<td>Non-migratory</td>
</tr>
<tr>
<td>PC346C</td>
<td>2000</td>
<td>Primary, from PC346P xenograft</td>
<td>AS</td>
<td>Non-migratory</td>
</tr>
<tr>
<td>VCaP</td>
<td>2001</td>
<td>Vertebra</td>
<td>AI/AS</td>
<td>Non-migratory</td>
</tr>
</tbody>
</table>

2.1.2. Cell culture

DU145, PC3, PNT2C2 and LNCaP cell lines were cultured in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). VCaP cells were grown on RPMI 1640 with 10% FCS and 1% P/S. 22RV1 cells were cultured in RPMI 1640 + DMEM F-12 (Bio Whittaker, Verviers, Belgium) with 7.5% FCS and 1% P/S. The LAPC4 cell line was grown in IMDM (Bio Whittaker, Verviers, Belgium) supplemented with androgen analogue R1881 (NEN DuPont, Boston, USA) with final concentration of 10^{-8}M, 7.5% FCS and 1% P/S. The basic culture medium used in the maintenance of PC346C cell lines consisted of DMEM-F12.
(BioWhittaker, Verviers, Belgium) supplemented with 2% FCS (PAN Biotech, Aidenbach, Germany), 1% insulin-transferrin-selenium (GIBCO BRL, Gaithersburg, MD), 0.01% BSA (Boehringer-Mannheim, Mannheim, Germany), 10 ng/ml epidermal growth factor (Sigma-Aldrich, Milan, Italy) and penicillin/streptomycin antibiotics (100 U/ml penicillin, 100 lg/ml streptomycin; BioWhittaker) plus the following additions: 100 ng/ml fibronectin (Harbor Bio-Products, Tebu-bio, the Netherlands), 20 mg/ml fetuin (ICN Biomedicals, Zoetermeer, The Netherlands), 50 ng/ml cholera toxin, 0.1 mM phosphoethanolamine, 0.6 ng/ml triiodothyronine and 500 ng/ml dexamethasone (all from Sigma-Aldrich). Cell lines in culture were passaged when they reached almost 80% confluency. To do so, medium was removed from the flasks and cells were rinsed with DPBS (Bio Whittaker, Verviers, Belgium). The purpose of this is to remove residual serum proteins, which could inhibit trypsin. The DPBS was removed and the cells were trypsinised with a pre-warmed trypsin/EDTA (Bio Whittaker, Verviers, Belgium) for 5-10 min at 37°C, until cells were detached. The trypsin was deactivated by adding the medium with serum. The entire solution was transferred to a sterile tube and centrifuged at 1000 rpm for 5 min. The supernatant was removed, the collected cell pellet was resuspended in fresh complete medium, counted (section 2.2) and used to re-seed in a new flask with the desired confluency or to set up an assay.

2.2. Viable cell count using trypan blue

After harvesting the cells and collecting the cell pellet, cells were counted using trypan blue (1.4 mM Trypan blue, 154 mM NaCl, 500 mM EDTA (ethyleendiaminetetra-acid), pH=8). 20 µl of cell suspension was mixed with 20 µl of trypan blue. By using trypan blue, we counted for viable cells, which are white in color. The dead cells absorb the blue color due to their damaged membrane and were not counted. Viable cells were counted on both chambers of a hemocytometer (Neubauer-Improved, Marienfeld, Germany) and multiplied by $2 \times 10^3$ to find the number of the cells/ml.
2.3. RNA isolation

Total RNA including the miRNA was isolated from cell lines including DU145, PC3, PNT2C2, LNCaP, 22RV1, LAPC4, PC346 and VCaP using miRNAeasy kit (Qiagen) according to the manufacturer’s instructions. The RNA was eluted in 30 μL of RNase free water. Concentration and purity of RNA were assessed using NanoDrop ND 1000 spectrophotometer by absorption measurements at 260 nm. The same method for RNA isolation was also performed on cell lines DU145, PC3, VCaP and PC346 after overexpression and inhibition of miR-141 and miR-miR-200c.

2.4. Real-time quantitative RT-PCR to measure the expression level of ZEB1, ZEB2 and TGFβ2

cDNA was synthesized using 1 μg total RNA from all cell lines, M-MLV RT enzyme and Oligo (dT) primer (Invitrogen) according to the manufacturer’s protocol. Reverse transcription was performed at 37°C for 60 min and 95°C for 10 min. First strand cDNA was subsequently diluted 20 times in nuclease-free water before addition to the RT-PCR reaction mixture. mRNA quantification of selected genes (ZEB1, ZEB2, TGFβ2) was performed using TaqMan Gene Expression Assays (order number HS00232783_m1*, HS00207691_m1* and HS00234244_m1*, respectively, Applied Biosystems, CA, USA). For normalization of the results GAPDH (Applied Biosystems, CA, USA) with Forward primer: ATG GCA TGG ACT GTG GTC and reverse primer: ATG GCA TGG ACT GTG GTC and PBGD (Invitrogen), Syber green assay with forward primer: CAT GTC TGG TAA CGG CAA TG and reverse primer: GTA CGA GGC TTT CAA TGT TG, were used as endogenous controls. Real time PCR was performed in 40 cycles in thermocyclers 9600 or 7900HT Fast Real-Time PCR system.

2.5. TaqMan real time PCR microRNA assays

In order to detect the expression of mature miRNAs, two different approaches were utilized including TaqMan real time PCR (Applied Biosystems, CA, USA) and MiRCURY LNA™ Universal RT (Exiqon, Denmark). In this section, the first approach is discussed. By this approach, we detected the expression level of mature miR-183, miR- 205, miR- 222 and miR-210. To this end, a two-step RT-
qPCR was performed. In the reverse transcription step, specific stem-loop primers for each miRNA from TaqMan MiRNA assay and TaqMan MicroRNA RT Kit (Applied Biosystems, CA, USA) were used (Fig. 2). Specific stem-loop primers are highly specific for their targets and hence increase the specificity of RT step. Therefore, they also can be used for multiplexing of RT primers. This step was performed using one RT-primer for each miRNA (uniplex RT) or RT-primer from multiple miRNAs (multiplex RT) in the same reaction.

2.5.1. Uniplex RT and Multiplex RT

The uniplex RT step was performed following the manufacturer’s protocol. Only one RT primer was used in this step. The multiplex RT step was performed in the following procedure (Table 5):

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Volume (μL)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTPs, 100 mM</td>
<td>0.40</td>
<td>2 mM</td>
</tr>
<tr>
<td>MultiScribe Reverse Transcriptase, 50 U/μL</td>
<td>2.00</td>
<td>5 U/μL</td>
</tr>
<tr>
<td>10x RT buffer</td>
<td>2.00</td>
<td>1x</td>
</tr>
<tr>
<td>RNase Inhibitor, 20 U/μL</td>
<td>0.25</td>
<td>0.0125 v/v</td>
</tr>
<tr>
<td>Total RNA, 20 ng/μL</td>
<td>2.00</td>
<td>1 ng/μL</td>
</tr>
<tr>
<td>5 different RT primer, each at 5 x 250 nM</td>
<td>10 (5 x 2.00)</td>
<td>Each, 25 nM</td>
</tr>
<tr>
<td>Nuclease free H2O to final volume of 20 μL</td>
<td>3.35</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20.00</strong></td>
<td></td>
</tr>
</tbody>
</table>

The RT reaction (either multiplex or uniplex) was subsequently diluted 10 times in nuclease-free water before addition to the real-time PCR reaction mixture. Real time PCR reactions were performed with 20 ng/μL total RNA and Taqman 2X Universal PCR Master Mix (Applied Biosystems, CA, USA) according to the manufacturer’s protocol. These reactions were performed in triplicate with the final volume of 10 μL for both uniplex and multiplex RT. TaqMan probes contained FAM™ dye as a reporter, linked to the 5’end of the probe (Fig. 7). A minor groove binder (MGB) is linked to the 3’end of the probe to increase the melting temperature, while the probe length is short. A non-fluorescent
quencher (NFQ) is attached at the 3’end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the FAM reporter fluorescence. During PCR, the TaqMan MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites. Cleavage by the DNA polymerase separates the reporter dye from the quencher dye resulting in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Two quality controls and one internal control were taken along in each PCR run. RNU48 was used as an internal control to compensate for differences between RT efficiencies by normalization. The relative expression of miRNAs was determined with the $2^{\Delta\Delta Ct}$ method.

A comparison was performed based on the normalized value of each miRNA between uniplex and multiplex RT among different cell lines (data not shown). This resulted in higher expression level of miRNAs in uniplex RT compared to multiplex RT. One explanation might be that the amount of RT primer added to Q-PCR experiment is less in multiplex RT compared to uniplex RT. Therefore, the threshold cycle (Ct) in multiplex RT is higher than the uniplex RT and subsequently the relative expression level for each miRNA is higher in uniplex RT (data not shown). We continued working with uniplex reactions in subsequent experiments.

![Figure 7. TaqMan MicroRNA assay chemistry (Applied Biosystems). Explanation can be found in the text. F: FAM dye, Q: quencher dye.](image)

### 2.6. MiRCURY LNA™ Universal RT microRNA PCR

This is the second approach that we utilized to detect the expression of mature miR-141, miR-200c and miR-222. In this approach, a two-step RT-qPCR was performed. In the reverse transcription step,
universal cDNA synthesis kit (Exiqon, Denmark) was used according to the manufacturer’s protocol. Based on this protocol, a single reaction step was performed for polyadenylation by adding a poly-A tail to the mature miRNA template and reverse transcription for all miRNAs into cDNA in one reaction. cDNA is synthesized by using a poly-T primer with a 3’ degenerate anchor and a 5’ universal tag. Therefore, the same RT reaction for all miRNAs is used in the real-time PCR reactions. This step was carried out using microRNA-specific and Locked Nucleic Acid (LNA)-enhanced reverse and forward primers. LNA-primers secure specificity by increasing the hybridization properties of oligonucleotides and enable accurate quantification of very low amounts of miRNAs. The Sybr Green was used for detection of PCR reaction (Fig. 8). The real time PCR reaction was performed with 25 ng/μL total RNA, LNA primer set for each miRNA and SybR Green master mix (Exiqon, Denmark) according to the manufacturer’s protocol. These reactions were performed in duplicate. Two quality controls and one internal control were carried along in each PCR run. RNU48 was used as an internal control for data normalization. The relative expression of miRNAs was determined with the $2^{-\Delta\Delta C_t}$ method. A comparison was performed for the expression level of miR-222 and RNU48 (internal control) between two different protocols from Applied Biosystems and Exiqon. The result showed that there is no significant difference between the relative expression level of MiR-222 and RNU48 using two different protocols (data not shown).

![Figure 8. Schematic outline of the MiRCURY LNA Universal RT microRNA PCR system (Exiqon, Denmark). Explanation can be found in the text.](image)

### 2.7. Optimization of transfection

To optimize the transfection efficiency of different transfection reagents on 4 different cell lines, a constant concentration of SiGLO Red (Thermo Scientific Dharmacon) as a transfection indicator was
used. SiGLO Red is a fluorescent oligonucleotid. The ratio between SiGLO Red and transfection reagent differed per cell line. The optimization experiments were performed in 96-well plates (Greiner Bio-One, CELLSTAR). One day before transfection, different numbers of cells for each cell line were plated in a final volume of 100 µl growth medium without antibiotics (Table 6). For each well, different concentrations of transfection reagents together with SiGLO Red and serum free medium were mixed gently in clean eppendorfs and incubated for 10 min at room temperature (Table 6). The medium was removed from the wells and transfection mix was added to each well containing cells. The final volume per well was 100 µl. Cells were viewed using a fluorescent microscope after 48-72 hrs. The optimal transfection efficiency was determined for each cell line by comparing the number of red fluorescent cells.

### Table 6. Recommended concentration of SiGLO Red, the ratio of transfection reagents vs. SiGLO Red and the number of cells per well in 96-well plates. ND: not determined.

<table>
<thead>
<tr>
<th>Transfection Reagents → (Companies)</th>
<th>FuGENE HD (Promega)</th>
<th>Lipofectamine RNAi MAX (Invitrogen)</th>
<th>DharmaFECT (Darmacon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines ↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU145</td>
<td>0.03</td>
<td>1 : 3</td>
<td>5*10⁴</td>
</tr>
<tr>
<td>VCaP</td>
<td>0.03</td>
<td>1 : 2</td>
<td>6*10⁴</td>
</tr>
<tr>
<td>22RV1</td>
<td>0.03</td>
<td>1 : 3</td>
<td>6*10⁴</td>
</tr>
<tr>
<td>PC3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

2.8. Transient transfection

DU145, PC3, VCaP and PC346 were transiently transfected using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer’s protocol.
2.8.1. Overexpression of miR-141 and miR-200c

To overexpress the miR-141 and miR-miR-200c in the cell lines DU145 and PC3, Pre-miR™ miRNA Precursor Molecules for miR-141 and miR-200c (Applied Biosystems, Ambion) were used. Pre-miRs are synthetic small RNA duplexes that mimic endogenous precursor miRNAs. They include one strand that can effectively mimic a known mature miRNA. Scrambled control #1 (Applied Biosystems, Ambion) was used as negative control. This RNA duplex is designed in a way that it is not identical to any mature miRNAs and expected not to mimic any miRNA. The transfection experiments were performed in 25 cm² tissue culture flasks (Greiner Bio-One, CELLSTAR). 300,000 DU145 or PC3 cells were seeded in 4 ml of growth medium without antibiotics per each flask for different conditions. 24 hours later, the medium was replaced with the transfection mix containing a final concentration of 30 nM for pre-miRs or Scrambled control and 12 µL of Lipofectamine RNAi MAX according to the manufacturer’s protocol in the final volume of 4 ml medium without serum. 24 hours after the transfection, the medium was replaced with the growth medium and incubated at 37°C for 48 hours. This experiment was repeated at least 6 times. For transfecting PC3 cells using Darmafect # 3 (Thermo Scientific Dharmacon), the same experimental setup was used. However, based on our optimization study, 8 µL of Darmafect # 3 was used for all conditions.

2.8.2. Inhibition of miR-141 and miR-200c

To inhibit the miR-141 and miR-miR-200c in the cell lines VCaP and PC346, miRCURY LNA microRNA inhibitor for miR-141 and miR-miR-200c (Exiqon, Denmark) were used. LNA miRNA inhibitors are synthetic single stranded LNA RNA molecules, which are designed in a way that specifically bind to and inhibit mature miRNAs with high specificity and stability. Scrambled control A (Exiqon, Denmark) was used as a negative control. This is designed in a way that is not complementary to any known mature miRNAs. The transfection experiments were performed in 6-well cell culture plates (Greiner Bio-One, CELLSTAR). 350,000 PC346 and VCaP cells were seeded in 2.5 ml of growth medium without antibiotics per well for different conditions. 48 hours later (these cell lines need more than one day to attach properly to the well) the medium was replaced with the
transfection mix with final concentration of 50 nM for anti-miRs and Scrambled control, 7.5 and 5 µL of Lipofectamine RNAi MAX for PC346 and VCaP cell lines, respectively, according to the manufacturer’s protocol (for VCaP cells less transfection reagent was used to keep the efficiency as high as possible and toxicity as low as possible). The transfection mix was made in the final volume of 2.5 ml medium without serum. 24 hours after the transfection, the medium was replaced with the growth medium and incubated at 37°C for 48 hours. This experiment was performed 2 times for PC346 and 4 times for VCaP cell line.

2.9. MTT assays

Cell proliferation after transfection was determined using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. DU145, PC3 and VCaP cell lines were trypsinized after the transfection. DU145 and PC3 cells were plated in complete medium in 96-well culture plates (Greiner Bio-One, CELLSTAR) at the density of 2.5 x 10^3 and VCaP cells at the density of 5 x 10^3 cells per well. The final volume per well was 200 µL. Each cell line was plated in different plates for different time points. For each time point, 30 µL of MTT reagent was added to each well and incubated at 37°C for 4 hours. In this step, viable cells had the ability to convert a soluble salt into insoluble precipitate. Thereafter, the medium was replaced with 100 µL dimethyl sulphoxide (DMSO) buffer in which the insoluble precipitate was dissolved and its concentration was measured by absorbance at 570 nm using a microplate reader (Bio-RAD, 550). This measurement determined the viable cells concentration at each time point. Different time points for different cell lines are determined based on their growth rate (Table 7). This experiment was repeated at least 2 times for each cell line.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>day 0, day 2, day 3</td>
</tr>
<tr>
<td>PC3</td>
<td>day 0, day 3, day 5</td>
</tr>
<tr>
<td>VCaP</td>
<td>day 0, day 5</td>
</tr>
</tbody>
</table>

Tabel 7. Different time points for different cell lines in MTT assay.
2.10. Cell migration assays

2.10.1. Migration assay to investigate migratory ability of PCa cell lines

To check the migratory ability of cells, a Boyden Chamber assay was performed, which is based on a chamber of two medium-filled compartments separated by a 0.8 µm pore size membrane (Fig. 9). In this assay, cells in the serum free medium are plated in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment, in which FCS is present. The FCS acts as a chemoattractant. After an appropriate incubation time, the membrane between two compartments is fixed and stained.

Migration assays were performed in duplicate using MILLIPORE QCM™ 24-well Migration Assay (8 µm pore size; Falcon). First, different PCa cell lines (DU145, PC3, PNT2C2, LNCaP, 22RV1, LAPC4, VCaP and PC346C) were plated in tissue culture flasks, 25 cm², (Greiner Bio-One, CELLSTAR). 24 hours later, the medium was replaced with starvation medium and incubated for 20 hours at 37°C prior to the assay. Thereafter, cells were trypsinized according to the manufacturer’s protocol. Different cell lines were plated at the density of 1 x 10⁶ cells per Transwell. The cells were allowed to migrate towards their specific growth medium containing FCS serum as attractant for 24 hours for VCaP cells and 18 hours for the rest. Non-migratory cells on top of the filter were removed with cotton swabs and migratory cells at the bottom of the insert were fixed and stained using cell stain (MILLIPORE) for 20 min.

Figure 9. Principle of Boyden Chamber assay (MILLIPORE).
2.10.2. Migration assay after transfection

48 hours after transfection of cell lines DU145, PC3, VCaP and PC346 with miRNA mimics and inhibitors, medium was replaced with starvation medium and incubated for 18 hours (for cell lines DU145 and PC3) or 24 hours (for cell lines VCaP and PC346) at 37°C prior to assay. Thereafter, cells were washed by PBS, and Quenching medium containing medium without serum + 5% BSA (growth medium of the cells is full of chemoattractant so that cells will not go through the chamber) was used in trypsinization step. Transfected cells were plated 3 days post-transfection in serum free medium. Migration assays were performed in duplicate using MILLIPORE QCM™ 24-well Migration Assay (8 µm pore size; Falcon). DU145, PC3 and VCaP cell lines were plated at density of 6 x 10⁴ cells per Transwell and PC346 cell line was plated at density of 12 x 10⁴. The cells were allowed to migrate towards their specific growth medium for 18 hours for cell lines DU145 and PC3 and 24 hours for cell lines VCaP and PC346. Non-migratory cells on the top of the filter were removed with cotton swabs and migratory cells at the bottom of the insert were fixed and stained using cell stain for 20 min. Then the inserts were used for quantification of migrated cells. Whole filters were manually counted for migrated cells. The inserts were placed under the microscope and cells were counted in 5 fields of view on each membrane, covering approximately 14% of the total membrane area. There were no overlaps among 5 different selected fields per membrane. Average of the cell numbers among 5 fields were calculated per membrane. Cell counts were performed by Standard 25 ICS microscope using objective with magnification of 10X (CP-ACHROMAT). The average number of the cells per insert was corrected for the number of the cells on the entire membrane. This number shows the number of the cells migrated through the whole membrane. This experiment was repeated at least 4 times for each cell line.
3. Results

3.1. Selection of candidate miRNAs responsible for migration of PCa cells

In order to select candidate miRNAs responsible for migration of PCa cells, two approaches were utilized including Solexa deep sequencing data and literature search. Finally, miRNAs were selected based upon the comparison between these two approaches. First, a list of miRNAs from Solexa deep sequencing data, which had already been performed by our research group, was generated. In this list, differentially expressed miRNAs in PCa metastases vs. organ-confined PCa were included. For Solexa deep sequencing, freshly frozen clinical samples were obtained from the tissue bank of Erasmus University Medical Center. Two sample pools each comprised of four individual patient samples were used for Solexa deep sequencing. 4 samples derived from organ-confined PCa and the other 4 samples derived from lymph node metastasis. Samples were obtained by radical prostatectomy. Solexa deep sequencing was performed twice independently. Therefore, 2 different lists of differentially expressed miRNAs were available. The output of Solexa deep sequencing is the number of reads for the sequence of a specific miRNA. In order to analyze the Solexa deep sequencing data for each specific miRNA, the number of reads in PCa metastasis was divided by the number of reads in organ-confined PCa and the results were log2 transformed. This calculation was performed for each list and the result from Solexa deep sequencing was reported based on the average number between the two lists for each miRNA (Table 8).

In the next step, for confirmation and limiting the previous list, a literature search was performed to select miRNAs, which were differentially expressed in metastases vs. organ-confined cancers. “MiRNAs and Metastasis” are the key words of our literature search. By doing so, almost 20 different articles were selected, in which 55 different miRNAs were shown to be differentially expressed in metastases vs. organ-confined cancer with respect to different types of cancer including liver, breast, colon, bladder, brain, colon, kidney, gastric, lung, esophageal and prostate cancers.
Based on the Solexa deep sequencing data, miR-183 and miR-210 are up regulated in PCa metastases vs. organ-confined PCa, whereas, miR-141, 200c, 205 and 222 are down regulated (Table 8).

Similarly, based on the literature, the same pattern is seen for differential expression of candidate miRNAs comparing metastases vs. organ-confined cancers. MiR-183 and miR-210 are up regulated in metastases vs. organ-confined cancer cells; whereas, miR-141, miR-200c and miR-205 are down regulated. MiR-222 has been shown to be either up or down regulated during cancer progression in different types of cancers (Table 8).

Therefore, 6 miRNAs including miR-141, miR-183, miR-200c, miR-205, miR-222 and miR-210 were selected as candidate miRNAs responsible for migration of PCa cells.

Table 8. Solexa deep sequencing and Literature search for differentially expressed miRNAs in metastases compared to organ-confined cancer. Data shown in Solexa deep sequencing are log2 transformed.

<table>
<thead>
<tr>
<th>MiRNAs</th>
<th>Solexa deep sequencing</th>
<th>Literature search</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCa metastases / organ-</td>
<td>Cancer metastases /</td>
<td></td>
</tr>
<tr>
<td></td>
<td>confines PCa</td>
<td>organ-confined cancer</td>
<td></td>
</tr>
<tr>
<td>Mir-183</td>
<td>0.9</td>
<td>Up</td>
<td>[77-79]</td>
</tr>
<tr>
<td>Mir-210</td>
<td>1.0</td>
<td>Up</td>
<td>[78, 80]</td>
</tr>
<tr>
<td>Mir-141</td>
<td>-0.5</td>
<td>Down</td>
<td>[33, 62]</td>
</tr>
<tr>
<td>Mir-200c</td>
<td>-0.6</td>
<td>Down</td>
<td>[33, 62]</td>
</tr>
<tr>
<td>Mir-205</td>
<td>-9.5</td>
<td>Down</td>
<td>[31, 62, 81]</td>
</tr>
<tr>
<td>Mir-222</td>
<td>-2.0</td>
<td>UP Down</td>
<td>[82-84]</td>
</tr>
</tbody>
</table>

3.2. Selection of candidate miRNAs whose expression are correlated with migratory ability of PCa cell lines

3.2.1. Investigation of migratory ability of different PCa cell lines

In order to examine the migratory ability of PCa cell lines, a Boyden Chamber assay was performed. Results are presented in the following figure (Fig. 10).
Figure 10. Overview of migratory ability of 8 different PCa cell lines using the Boyden Chamber assay. The purple color is representative of cells migrated through the filter and stained with cell stain. In not migratory cell lines, small dots are the pores of the insert.

3.2.2. Differential expression level of selected miRNAs and their correlation with migratory ability of PCa cell lines

The expression level of the 6 candidate miRNAs was measured by RT-qPCR in the 8 different PCa cell lines. To do so, RNA was isolated from 8 different prostate cancer cell lines including: DU145, PC3, PNT2C2, LNCaP, 22RV1, LAPC4, VCaP and PC346C. Then the expression level of selected miRNAs and an internal control (RNU48) were measured. To calculate the relative expression level of selected miRNAs in these cell lines, data were normalized to RNU48.

Results of this experiment showed a correlation between the expression level of some miRNAs and migratory ability of PCa cell lines (Fig. 11). MiR-141 and miR-200c are very lowly expressed in cell lines DU145, PC3 and PNT2C2, which are highly migratory cell lines. MiR-141 and miR-200c are highly expressed in rest of the cell lines, which are poorly or non-migratory. Conversely, miR-222 is highly expressed in highly migratory cell lines and low expressed in poorly or non-migratory cell lines (Fig. 11). This correlation was not observed for other miRNAs. For further validation, expression of the miRNAs of interest were retrieved from a microarray database, kindly provided by prof. T. Visakorpi, University of TURKU, Finland (Fig. 12). The microarray data confirmed the low expression of miR-141 and miR-200c and high expression of miR-222 in highly migratory cell lines.

MiR-141 and 200c were selected for further research based on their expression correlation with migratory ability of different PCa cell lines. We did not pursue further investigations on miR-222, since the role of this miRNA in metastases has already been described extensively in literature.
Figure 11. Relative expression level of selected miRNAs in 8 different PCa cell lines using RT-qPCR. Relative expression level of miRNAs compared to U48 as internal control (± SEM). Expression level of miR-141, miR-200c and miR-222 correlates with the migratory ability of cell lines.
3.3. Functional studies to identify the role of miR-141 and miR-200c in migration of PCa cells

In the next step, we directly determined whether the correlation between the expression level of miR-141 and miR-200c, and migration ability of PCa cell lines plays a role in conferring aggressive and migratory traits to PCa cell lines. For this, we assayed how gain or loss of function using transient overexpression or knockdown for miR-141 and/or miR-200c affected cell migration, a hallmark of cancer metastasis. Before starting the transfection experiments, transfection conditions were optimized. The optimization study was performed on those cell lines, in which the expression level of miR-141 and miR-200c were highest and lowest. The DU145 and PC3 cells had a very low expression of miR-141 and miR-200c (Fig. 11). Therefore, they were chosen for overexpression of these miRNAs. The VCaP and PC346C cells had a very high expression of miR-141 and miR-200c (Fig. 11). They were chosen for inhibition of these miRNAs. Cell line 22RV1 with moderate expression of miR-141 and miR-200c was also taken along in an optimization study.
### 3.3.1. Optimization of transfection conditions

Before starting transfection experiments, transfection conditions were optimized per cell line to achieve highest efficiency for each cell line. Generally, there are a number of important factors that influence transfection efficiency. These factors include type and quantity of transfection reagent, quantity of RNA (mimics or antagomiRs), ratio between the transfection reagent and RNA and duration of the incubation time of cells with transfection mix. With respect to the aforementioned parameters, our aim was to keep the cell death as low as possible and achieve high transfection efficiency.

#### 3.3.1.1. Finding a suitable transfection reagent for each cell line

To find the best transfection reagent for each cell line with respect to efficiency and cytotoxicity, 3 transfection reagents including FuGENE HD, Lipofectamine RNAi MAX and DharmaFECT were tested on 4 cell lines including DU145, PC3, 22RV1 and VCaP. Transfection efficiency of all cell lines was not tested by all 3 transfection reagents (Table 9). Hence, the most sensitive cell lines, such as 22RV1, with respect to their growth rate had the priority to be tested by all different transfection reagents. SiGLO Red was used as an indicator of transfection, which is a specially modified, fluorescent RNA duplex for transfection assessment. If successfully transfected, its signal localizes in the nucleus. A constant and low concentration of SiGLO Red (0.03 µM) was used in all different conditions. With respect to the type of cell line and transfection reagent, different ratios (SiGLO Red: Transfection reagent) were used (see Materials and Methods, section 2.7). The results of this experiment are summarized bellow (Table 9). Taken together, Lipofectamine RNAi MAX was the most suitable transfection reagent with respect to the transfection efficiency and cytotoxicity in all tested cell lines including DU145, VCaP and 22RV1. As an example, transfection efficiency of cell line 22RV1 using 3 different transfection reagents is shown (Fig. 13). The same result was observed for other cell lines, which were tested by Lipofectamine RNAi MAX including DU145 and VCaP (data not shown).
In addition, duration of the incubation time of the cells with transfection reagent (Lipofectamine RNAi MAX) was also taken into consideration. Transfection efficiency of the cells was investigated at 2 different time points, 48 hrs and 72 hrs. The results showed that the number of transfected cells after 72 hrs is higher than the number of transfected cells after 48hrs. Cell line 22RV1 is shown as an example (Fig. 14). The same result was observed for other tested cell lines including DU145 and VCaP (data not shown).

Table 9. Overview of the transfection efficiency of 3 different transfection reagents on different PCa cell lines with respect to cytotoxicity and transfection efficiency. Lipofectamine RNAi MAX worked as the best transfection reagent compared to other transfection reagents with respect to its low cytotoxicity and high transfection efficiency in 3 different PCa cell lines. ND: Not Determined.

<table>
<thead>
<tr>
<th>Transfection Reagents →</th>
<th>FuGENE</th>
<th>Lipofectamine RNAi MAX</th>
<th>DharmaFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td>Cytotoxicity</td>
<td>Transfection efficiency</td>
<td>Cytotoxicity</td>
</tr>
<tr>
<td>DU145</td>
<td>Very low</td>
<td>Very low</td>
<td>Low</td>
</tr>
<tr>
<td>VCaP</td>
<td>High</td>
<td>Not transfected</td>
<td>High</td>
</tr>
<tr>
<td>22RV1</td>
<td>Very low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>PC3</td>
<td>Very low</td>
<td>Low</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figure 13. Transfection efficiency of 3 different transfection reagents including Lipofectamine RNAi MAX, FuGENE and DharmaFECT on cell line 22RV1.
3.3.1.2. Finding an efficient transfection condition for each cell line using Lipofectamine RNAi MAX

To further optimize the transfection condition for Lipofectamine RNAi MAX, 3 different concentration of Lipofectamine RNAi MAX in combination with a constant concentration of SiGLO Red were tested on 5 different cell lines. The ratio (SiGLO Red: Transfection reagent) with highest transfection efficiency and lowest cytotoxicity was determined per cell line (Table 10, Fig. 15). In this experiment very low concentration of SiGLO Red (0.03 µM) was used. The amount of Lipofectamine RNAi MAX was altered between a suggested range (0.1 µl - 0.3 µl) depending on the well size (96 wells). The initial optimization study revealed that using a concentration of Lipofectamine RNAi MAX, which is above the suggested range of, 0.1 µl - 0.3 µl, cause cytotoxicity to some extent.

Table 10. Selecting a ratio (SiGLO Red: Transfection reagent) with highest transfection efficiency and lowest cytotoxicity for 5 different PCa cell lines using Lipofectamin RNAi MAX. ✓: Selected ratio for each cell line.

<table>
<thead>
<tr>
<th>SiGLO Red (µM): Lipofectamine RNAi MAX (µl)</th>
<th>DU145</th>
<th>PC3</th>
<th>VCaP</th>
<th>22RV1</th>
<th>PC346C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03: 0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03: 0.2</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03: 0.3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
3.3.2. Manipulating the expression level of miR-141 and miR-200c

Manipulation of the expression level of miR-141 and miR-200c in selected cell lines (DU145, PC3, VCaP and PC346C) was performed using mimics and antagomiRs. Mimics are synthetic double stranded RNAs that can mimic the function of miRNAs after entering the cell. AntagomiRs are synthetic single stranded RNA molecules, which are complementary to the target miRNAs. Mimics were transfected into cell lines DU145 and PC3 that express relatively low level of miR-141 and miR-200c. AntagomiRs were transfected into cell lines VCaP and PC346C that express high level of miR-141 and miR-200c.

In order to choose a suitable type of mimic and antagomiR for the selected miRNAs, different technologies were considered. The most commonly used technologies include viral expression system and free oligos. Free oligos are available in three different types, including RNA oligos, DNA oligos and LNA (Locked Nucleic acid) oligos. Although a viral expression system has an advantage of long-term expression, in our experiment RNA type free oligos were used as mimic and LNA type oligos were used as antagomiRs. LNA oligos are very specific for their targets and bind to their targets rigidly.

DU145 and PC3 cells were transfected under 5 different conditions (Table 11). In conditions 1, 2 and 3 they were transfected with 3 different combinations of Pre-miR sequences, with the final concentration of 30 nM. In condition 4, they were transfected with Scrambled (30 nM) as a negative
control, to check for non-specific effects of transfection. The Scrambled sequence does not target any known mRNA within the human transcriptome. In condition 5, a no transfection control was taken along.

For VCaP and PC346C, a similar setup was used, but now instead of the pre-miR mimics, we used 50 nM LNA Anti-miRs. In addition, the Scrambled was transfected with the final concentration of 50 nM (Table 11).

Table 11. Different transfection conditions for transient overexpression and inhibition of miR-141 and miR-200c.

<table>
<thead>
<tr>
<th>Transfection Conditions</th>
<th>Transient overexpression (DU145, PC3) Final concentration : 30 nM</th>
<th>Transient inhibition (VCaP, PC346C) Final concentration : 50 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pre-miR-141</td>
<td>Anti-miR-141 (LNA)</td>
</tr>
<tr>
<td>2</td>
<td>Pre-miR-200c</td>
<td>Anti-miR-200c (LNA)</td>
</tr>
<tr>
<td>3</td>
<td>Pre-miR-141 and 200c (15 nM+15 nM)</td>
<td>Anti-miR-141 and 200c (LNA) (25 nM+25 nM)</td>
</tr>
<tr>
<td>4</td>
<td>Scrambled</td>
<td>Scrambled</td>
</tr>
<tr>
<td>5</td>
<td>No transfection</td>
<td>No transfection</td>
</tr>
</tbody>
</table>

Before proceeding further, we confirmed the actual level of transfected and endogenous miRNAs. To do so, RT-qPCR analyses was performed to measure the expression level of miR-141 and miR-200c in the cells transfected with pre-miR and anti-miR sequences. Results of this experiment showed that the expression level of the miR-141 and miR-200c after transfecting the Scrambled is approximately the same as no transfection in all cell lines (Fig. 16). MiR-141 and miR-200c are abundantly present in cell lines DU145 and PC3, when transfected with pre-miR-141 and pre-miR-200c, respectively, as well as with the pre-miR combination. Conversely, miR-141 and miR-200c are very low expressed in cell lines VCaP and PC346, when transfected with anti-miR-141 and anti-miR-200c, respectively, as well as anti-miR combination (Fig. 16). 4 days after the transfection, RNA was isolated and this assay was performed 15 days after the transfection and was repeated 2 times individually.
It is important to note that the miR-141 and miR-200c RT-qPCR detection assay are highly specific for the tested miRNAs. Although miR-141 and miR-200c differ only in one nucleotide in their seed sequence, the miR-141 detection assay does not detect miR-200c (Fig. 16a), and vice versa (Fig. 16b).

![Graph](image)

Figure 16. Expression level (± SEM) of a) miR-141 and b) miR-200c after overexpression and inhibition of miR-141 and miR-200c, alone or in combination in suitable cell lines. Controls: 1- Scrambled 2- No transfection.

### 3.4. Migration of PCa cell lines upon manipulation of miR-141 and miR-200c expression

Next, we monitored whether the overexpression or inhibition of miR-141 and miR-200c, alone or in combination plays any role in conferring migratory traits to the selected PCa cell lines. After transfection of the cell lines with mimics or antagomiRs, the migratory ability of each cell line was evaluated using a Boyden Chamber assay (Fig. 8). Cell migration was quantified by counting the number of the migrated cells on the bottom of the filter.

**Cell line DU145:** In no transfection control, cells displayed a high migratory capacity (Fig. 17, DU145, lane 5). Migration ability of the cells reduced after transfection with Scrambled (Fig. 17, DU145, lane 4). As assayed with a Boyden Chamber assay, increased miR-200c and the combination (miR-141 and miR-200c) expression, using mimics, reduced migration of this cell line, whereas
overexpression of miR-141 had no effect on migration ability compared to the Scrambled (Fig. 17, DU145, compare lane 1, 2, and 3 with lane 4).

**Cell line PC3:** A high migration ability was observed in no transfection control (Fig. 17, PC3, lane 5). Transfection of the Scrambled led to highly reduction of this cell line migration ability (Fig. 17, PC3, lane 4). A subsequent Boyden Chamber assay indicated that increased miR-200c expression, using mimics, reduced migration ability of this cell line compared to no transfection, whereas, overexpression of miR-141 extremely promoted the migration ability (Fig. 17, PC3, compare lane 1 and 2 with lane 5). In addition, overexpression of both miRNAs had no effect on migration ability of the cells compared to no transfection (Fig. 17, PC3, compare lane 3 with lane 5).

**Cell line VCaP:** A similar setup was used for this cell line, but now the endogenous level of miR-141 and miR-200c was inhibited. In no transfection and Scrambled control, cells displayed no migratory capacity (Fig. 17, VCaP, lane 4 and 5). Applying a Boyden chamber assay showed that inhibiting miR-141 and the combination (miR-141 and miR-200c) using antagomiRs promoted migration of these cells, whereas, inhibition of miR-200c had no effect on their migration ability (Fig. 17, VCaP, compare lane 1, 2, and 3 with lane 4).

**Cell line PC346:** The same experiment was also performed on this cell line. In no transfection and Scrambled control, cells did not migrate. No migration was observed after the transfection with antagomiRs in any condition, as well (data not shown).

In the next step, we determined the problem of the migration inhibition by transfection of the Scrambled oligo in the PC3 cells. To do so, two different approaches were utilized including 1- testing a new experimental condition 2- changing the type of transfection reagent. First, we tested weather the transfection reagent only has an effect on migration ability of the PC3 cells. No transfection and scrambled were also taken along as controls. Results of this experiment showed that indeed the transfection reagent affects migratory characteristics of the cells (Fig. 18). The Number of migrated
cells decreased by using only the Lipofectamine RNAi MAX compared to no transfection. Similar result were obtained for the Scrambled control.

Second, to address the issue of the transfection reagent (Lipofectamine RNAi MAX), another transfection reagent, was tested. For this, the same experimental set up was performed with DarmaFECT number 3 (Fig. 19). The same results were obtained using Scrambled control and only transfection reagent compared to no transfection, showing that DarmaFECT did not affect the migratory ability of these cells. Overexpression of miR-200c and the combination (miR-141 and miR-200c) did not affect the cell migration compared to the Scrambled, whereas, overexpression of miR-141 strongly increased the migration ability of this cell line (Fig. 19). These findings confirm the migration promoting effect of miR-141 in the PC3 cells observed with Lipofectamine RNAi MAX.
Figure 17. MiR-141 and 200c play a role in conferring migratory ability to PCa cell lines. Pictures of the migration assay are representative of cells migrated through the filter, stained with cell stain. Graphs (± SD) and the table for VCaP show the absolute quantifications of cells migrated through the transwell.
3.5. Investigating the growth ability of PCa cell lines upon manipulation of miR-141 and miR-200c expression

In order to exclude the effect of transient expression or inhibition of miR-141 and miR-200c on cell growth and cell viability in cell lines DU145, PC3 and VCaP, an MTT assay was performed. To do so, transfected cells were plated at the appropriate cell number per well and cultured for several days. At each time point, the proliferation rate was measured by an MTT assay. Results of this experiment for all cell lines clearly showed an increase of cell proliferation for all conditions at different time points compared to no transfection condition (Fig. 20). Therefore, transfection did not affect cell proliferation and change in growth does not explain the migration changes observed in the Boyden chamber assay.
3.6. Monitoring the expression level of the validated target genes for miR-141 and miR-200c in different PCa cell lines before and after transfection

To further define a molecular mechanism behind the process of miR-141/200c-dependent migration of PCa cells, expression levels of their validated target genes were measured. The role of ZEB1, ZEB2 and TGFβ2 as target genes for miR-141 and miR-200c and their involvement in migration of different types of cancer cells has extensively been described [22, 33, 69]. Therefore, ZEB1, ZEB2 and TGFβ2 were selected as validated target genes for miR-141 and miR-200c. RT-qPCR was performed to measure the expression level of these genes in all PCa cell lines and in DU145, PC3, VCaP and PC346C cells after the transfection with mimics or antagomiRs. By measuring the expression level of ZEB1, ZEB2 and TGFβ2 in all PCa cell lines, a correlation was observed between their expression level and migratory ability of the cells (Fig. 21). In highly migratory cell lines including DU145, PC3 and PNT2C2, these genes are higher expressed than in the cell lines with lower migratory ability. These
findings fit with the inverse correlation between the expression level of miR-141 and miR-200c with the expression level of their validated target genes. Furthermore, the expression levels of these genes were measured in cell lines DU145, PC3, VCaP and PC346C after the transfection.

**Cell line DU145:** High expression of ZEB1, ZEB2 and TGFβ2 was observed in the Scrambled control, where the expression of endogenous miR-141 and miR-200c is very low (Fig. 22). Overexpression of miR-141, miR-200c and their combination reduced the expression level of ZEB1 and ZEB2 compared to the Scrambled. The expression level of ZEB1 and ZEB2 after overexpression of both miRNAs was not lower than with each miRNA alone, indicating that the effect of combining the miRNAs was not synergistic. In addition, overexpression of miR-141 and miR-200c, alone and in combination resulted in reduced expression level of TGFβ2 compared to Scrambled, however, this reduction was not always observed and highly variable.

**Cell line PC3:** ZEB1 and ZEB2 displayed a very low expression in the Scrambled control, whereas, TGFβ2 was highly expressed (Fig. 22). Overexpression of miR-141, miR-200c and the combination did not change the expression level of ZEB1 and ZEB2. The expression level of TGFβ2 did not change after the overexpression of miR-200c and the combination, whereas it was reduced after the overexpression of miR-141.

**Cell line VCaP and PC346:** ZEB1, ZEB2 and TGFβ2 were very low expressed in Scrambled where the expression of endogenous miR-141 and miR-200c are very high. Inhibition of miR-141, miR-200c and the combination did not affect the expression level of these genes, in both cell lines (data not shown).
3.7. Summary

Summary of the results for the selected cell lines is shown below (Table 12):
Tabel 12. Summary of the results

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Migration ability</th>
<th>MiRNA expression level</th>
<th>Validated target genes expression at mRNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MiR-141</td>
<td>MiR-200c</td>
</tr>
<tr>
<td>DU145</td>
<td>No transfection</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pre-miR-141</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Pre-miR-200c</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC3</td>
<td>No transfection</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pre-miR-141</td>
<td>+ + + +</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Pre-miR-200c</td>
<td>Not clear yet</td>
<td>-</td>
</tr>
<tr>
<td>VCaP</td>
<td>No transfection</td>
<td>- -</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Anti-miR-141</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Anti-miR-200c</td>
<td>- -</td>
<td>+ +</td>
</tr>
<tr>
<td>PC346C</td>
<td>No transfection</td>
<td>- -</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>Anti-miR-141</td>
<td>- -</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Anti-miR-200c</td>
<td>- -</td>
<td>+ +</td>
</tr>
</tbody>
</table>
4. Discussion

Until today, data regarding the alteration in expression level of miRNAs, which are responsible for migration of PCa cells, is limited. Identification of those miRNAs might be essential for understanding cancer metastasis and promote a better understanding of molecular mechanisms of migratory pathways and cancer progression. In our study, we measured the expression levels of several candidate miRNAs, which were assumed to influence migration of PCa cell lines with different migratory ability. MiR-141 and miR-200c were selected based on their tight expression correlation with the migratory ability of cell lines. A functional role for miR-141 and miR-200c was characterized upon manipulation of their expression level in PCa cell lines with a high and low expression of these miRNAs and a subsequent migration assay. Our findings indicate miR-141 and miR-200c play an important role in suppression of the migration in VCaP and DU145 cells, respectively. Whereas a pro-migratory role for miR-141 is observed in PC3 cells. Interestingly, upon manipulation of miR-141 and miR-200c expression in DU145 cells, an inverse correlation was observed between the expression of ZEB1, ZEB2 and TGFβ2 at mRNA level and miR-141 and miR-200c. However, for other PCa cell lines this was not always the case. These findings provide us with a starting point for exploration of molecular mechanisms behind the process of migration in PCa.

4.1. Association of miR-141 and miR-200c expression and migratory ability of PCa cells

Dysregulation of miR-200 family members has been observed previously. It has been shown that this group of miRNAs is upregulated in some, but downregulated in other types of cancer. The majority of studies have focused on the downregulation of different members of miR-200 family during tumor progression [22, 27, 30, 31]. However, upregulation of miR-200 family has also been identified in various cancers including ovary [85], cervix [86] and prostate [87].

In our experiments, we observed a strong inverse correlation between the expression levels of miR-141 and miR-200c and the migratory ability of different PCa cell lines. We observed a high expression
of miR-141 and miR-200c in non-migratory cell lines (VCaP and PC346C), and very low expression of these miRNAs in highly migratory cells (DU145 and PC3). As PCa cell lines are representative of different stages of PCa progression [88] (the non-migratory VCaP and PC346C cells indicate the androgen-dependent stage of PCa, whereas, the highly migratory DU145 and PC3 cells indicate the androgen-independent or metastatic stage), we hypothesized a potential role for these miRNAs as suppressors of cancer progression. This hypothesis can be support by other studies that focused on the downregulation of miR-200 family members during cancer progression, in particular during EMT [22, 27, 30, 31, 33, 69].

Conversely, we found a positive correlation between the expression level of miR-222 and migratory ability of PCa cell lines, meaning that cell lines with highly migratory ability showed a high expression of miR-222 and vice versa. This might suggest a role for miR-222 as promoter of PCa cancer progression. This can be support by Galardi et al. who showed that overexpression of miR-222 in PC3 cells contributes to the progression of prostate carcinoma [82].

4.2. MiR-141 and miR-200c affect migration

In the current study, we found that ectopic overexpression of miR-200c strongly inhibited the migration of DU145 cells. Ectopic inhibition of miR-141 partially promoted the migration of VCaP cells since only a few numbers of cells migrated. Although the high efficiency of inhibition of miR-141 in VCaP cells was confirmed by RT-qPCR, a strong promotion was not observed in the migratory ability of cells. This might be due to:

1- The effect of other miR-200 family members since they can target the same gene [27, 33].

2- Heterogeneity of cells per cell line. In this regard, different cells in the same cell line, obtain a different phenotypes by mutations in a specific genes. This observation has been reported previously [89].

3- Other involved mechanisms that regulate the migration ability of the cells.
Taken together, these findings indicate that miR-141 and miR-200c may play an important role in suppression of the migration and cell motility as key steps in the metastatic cascade in VCaP and DU145 cells, respectively. These findings are in agreement with other studies, particularly in breast cancer, which have identified the role of miR-200 family members including miR-141 and miR-200c in suppression of cancer progression and EMT [22, 27, 30, 31].

To our surprise, the migratory ability of PC346C cells did not change by inhibition of miR-141 and miR-200c. This might suggest:
1- Lack of chromosome 12 or a deletion in part of this chromosome in PC346C cells, since these miRNAs and their target genes are located on this chromosome, however by using our unpublished data, we found that this was not the case.
2- Due to the effect of other miR-200 family members that regulate the migration using the same target genes.
3- There might be other factors that affect migration.

4.3. Role of miR-141 in promoting the PC3 cell migration

Surprisingly, although PC3 cells are inherently migratory, overexpression of miR-141 resulted in their enhanced migration ability. In addition, Lipofectamine RNAi MAX transfection of the Scrambled control, highly decreased migratory ability of the PC3 cells compared to no transfection. These findings could be explained by transfection issues in the PC3 cells. Selection of a proper transfection reagent for each cell line can be essential. To test whether the migration of PC3 cells was affected by the type of transfection reagent, two different transfection reagents were tested. In the latest experiment, DarmaFECT transfection of the Scrambled control did not have a significant effect on migration of PC3 cells compared to no transfection. However, transfection of the miR-141 still increased the migratory ability of PC3 cells. Thus, opposite to our hypothesis and to the VCaP and DU145 observations, miR-141 acts as a migration inducer in PC3 cells. This finding suggests different possibilities:
1- MiR-141 in PC3 cells may regulate its validated target genes (ZEB1, ZEB2 and TGFβ2) via a more complex mechanism than suggested in literature. It has been reported that some miRNAs can bind to open reading frame or 5’ UTR of the target genes and, in some cases they have been shown to activate the gene regulation rather than inhibition [37]. If this would be the case in our miR-141 observation, we would expect to see the increased expression of validated target genes for miR-141 at translational level, which subsequently results in increased migration ability.

2- MiR-141 might regulate the migration ability of PC3 cells via targeting different genes responsible for pro-migration.

3- Overexpression of miR-141 promotes a MET by inhibiting its target genes, unexpectedly enhances the migration of PC3 cells. Therefore, epithelial nature of the cells does not predict migration ability. Based on most previous reports, miR-200 family members have a significant role in downregulating tumor migration and EMT as the first step of metastasis. However, others reported that miR-200 family members enhance mouse breast cancer cell colonization to form distant metastases via reestablishing an epithelial like phenotype (MET) [73]. This finding would fit our PC3 observations.

In a study by Elson-Schwab et al., has been shown that overexpression of miR-200c in melanoma cells increase migration by knock down of MARCKS [90]. MARCKS is a peripheral membrane protein and its overexpression in cancer cells results in decreased proliferation and migration [91], which supports our PC3 observations.

A future study needs to be carried out to address the pro-migratory ability of the miR-141 in PC3 cells, in which treated PC3 cells (overexpressing miR-141 in PC3 cells) are injected subcutaneously into SCID mice and monitor tumor metastasis. Similar experiment was previously reported in which using in vitro inhibition of miR-221 and miR-222 reduces tumor growth of PC3 derived tumors in SCID mice [83].

On the other hand, PC3 cells might not be inherently migratory and their migratory capacity might be due to a mutation in E-cadherin gene CDH1 or hypermethylation of the E-cadherin promoter, which is a well-described mechanism in tumors [92, 93].
Considering the issue of transfection reagent in the PC3 cells, overexpression of miR-200c inhibited the PC3 cells migration ability in the first experiment, whereas, in our second study with the DarmaFECT transfection reagent, almost no effect was observed in migration ability of this cell line. These different observations could be explained if the transfection reagent would have an effect on migration ability of the PC3 cells. Therefore, the new experiment needs to be repeated to support our findings. In addition, transfection conditions for the new transfection reagent need to be optimized in the PC3 cells.

4.4. MiR-141 and miR-200c act differently per cell line and among cell lines

It has been reported that different members of miR-200 family including miR-141 and miR-200c have a similar effect on cancer progression and migration through direct targeting of ZEB1 and ZEB2 by the entire miR-200 family [72]. To our surprise, this was not the case in our study. However, our finding is in agreement with a study in which it is shown that different subgroups of miR-200 family, regulate the proliferation and invasion of breast cancer cells, differentially [94]. Although we found that inhibition of miR-141 resulted in promoting metastasis ability of VCaP cells, inhibition of miR-200c did not affect its migration ability. Similarly, overexpression of miR-200c led to inhibition of DU145 cell migration, whereas, its migration capacity was not affected by overexpressing miR-141. This might suggest several alternative explanations:

1- MiR-141 and miR-200c target different genes in VCaP and DU145 cells. A microarray analysis can be performed using treated VCaP (inhibition of miR-141) and DU145 (overexpression of miR-200c) cells. Those genes, which are differentially expressed between two cell lines will be our gene(s) of interest [94].

2- MiR-141 and miR-200c target similar genes, but migration program is different in VCaP and DU145, which involves different genes that are controlled by other factors, maybe other miR-200 family members. To check for the effect of other miR-200 family members, a functional analysis need to be done in which DU145 cell line is co-transfected with mimics for all members of miR-200 family and VCaP cell line is co-transfected with antagomiRs of all family members.
MiR-200c was observed to act as a suppressor of migration in the DU145 and possibly PC3 cells; however, inhibition of endogenous miR-200c did not affect migration ability of the VCaP cells. MiR-141 was also shown to act as a suppressor and inducer of migration in the VCaP and PC3 cells, respectively, whereas no effect was observed on migration ability of the DU145 cells by overexpression of miR-141.

The different effect of miR-141 and miR-200c on migration ability of different cell lines might suggest that although all three cell lines are epithelial PCa cell lines, they are phenotypically distinct and unrelated. Therefore, different behavior in different PCa cell lines would be expected to be in association with cell-type specific regulatory mechanisms for miRNA actions. This fact is in agreement with findings by Lowery et al. who showed that dysregulation of miR-183 in different breast cancer cell lines inhibits migration, but is not observed in all breast cancer cell lines [95]. Moreover, a study by Elson-Schwab et al. has demonstrated that the origin of the tissue may account for the functional differences of miRNAs, however this can also be the case in the same type of cancer [90].

### 4.5. The miR-141 and miR-200c cascade in PCa

In the majority of functional studies, transcriptional factors ZEB1 and ZEB2 have been suggested to be targets of miR-200 family [96, 97]. TGFβ2 has also been identified to be targeted by miR-141 and miR-200c [30]. Different functional studies have focused on the downregulation of miR-141 and miR-200c during metastasis, in particular EMT. They found that these miRNAs prevent cancer progression by negatively regulating ZEB transcriptional repressors and TGFβ2, and consequently preventing EMT by maintaining the high level of E-cadherin [22, 27, 30, 31].

By measuring the expression level of *ZEB1, ZEB2* and *TGFβ2* in PCa cell lines before the manipulation of miR-141 and miR-200c expression, we showed a positive correlation between the expression level of these genes and migratory ability of the cell lines. In other words, consistent with other findings, we found a somehow inverse correlation between the expression level of these genes
and expression level of miR-141 and miR-200c in PCa cell lines. In DU145 cells, overexpression of miR-141 and miR-200c resulted in decreased expression level of these genes. This suggests that the 3’UTR of ZEB1, ZEB2 and TGFβ2 might be targeted by these miRNAs in the DU145 cells. In the PC3 cells, overexpression of miR-200c had no effect on expression level of these genes at mRNA level, whereas, overexpression of miR-141 led to decreased expression level of TGFβ2. These findings can be explained by several reasons:

1- ZEB1 and ZEB2 might not be target genes for miR-141 and miR-200c in the PC3 cells and these miRNAs regulate the migration of this cell line via a different mechanism. Different factors can bind to the 3’ UTR of these genes and prevent miR-141 and miR-200c from binding.

2- TGFβ2 is not a target gene for miR-200c, whereas, it can be targeted by miR-141 in the PC3 cells. This supports the finding from Burk et al. who demonstrated the strongest reduction of TGFβ2 after overexpression of miR-141 than miR-200c in breast cancer cell lines and predicted TGFβ2 as a putative target gene for miR-141 [30].

3- Another possibility would be that these genes might be regulated at translational level. In this case, these genes might be target genes for miR-141 and miR-200c in the PC3 cells and show their inversre correlation with miR-141 and miR-200c expression, but at protein level. To validate these possibilities, Western blots can be utilized to check the protein expression levels. Different studies have shown that the regulation of these genes by miRNAs is mediated largely at the level of translational inhibition [31, 33]. In addition, the 3’ UTR of ZEB1, ZEB2 and TGFβ2 can be cloned after the cDNA of luciferase and luciferase activity measured after miR-141 and miR-200c manipulation [27].

In the VCaP and PC346C cells inhibition of endogenous miR-141 and miR-200c had no effect on the mRNA expression level of these genes, probably due to the effect of other miR-200 family members or other possibilities, which is discussed for the PC3 cells. ZEB1 and ZEB2 are considered as putative target genes for the entire miR-200 family due to the overlap in the seed sequences of miR-200 family members [27, 33]. Therefore, the implication of this study can be further explored by co-transfection of VCaP and PC346C cells with all miR-200 family members.
Some future studies need to validate if miR-141 and miR-200c by targeting their validated genes, directly regulate EMT in PCa cell lines. Therefore, the expression level of E-cadherin, which is encoded by CDH1 needs to be measured at mRNA and protein level in PCa cell lines after the manipulation of miRNAs expression level [22, 27, 33]. It is reported that ectopic expression of miR-200 family members lead to upregulation of E-cadherin in cancer cell lines and reduction of the cancer cell motility. Conversely, inhibition of miR-200 family members reduces the E-cadherin expression and induces EMT [27].

Finally, to identify the role of ZEB1, ZEB2 and TGFβ2 in migration of PCa cells, an experiment in which the expression level of these genes are manipulated and a subsequent migration assay is required.

**4.6. Effect of combining the miR-141 and miR-200c**

Although miR-141 and miR-200c affect the expression level of different molecules, similarity of their seed sequences leads into high degree of overlap in their target genes [30]. Gregory et al. reported that combining the different members of miR-200 family has a greater effect on their target genes [31]. However, in our study, a significant synergistic effect of overexpression or inhibition of both miR-141 and miR-200c was not observed neither on the migratory ability of cell lines, nor on the expression level of ZEB1, ZEB2 and TGFβ2. This indicates that manipulation of either miR-141 or miR-200c is sufficient to generate the phenotype. The synergistic effect of combining both miRNAs might be observed in a concentration-dependent manner [31].

**4.7. Clinical implications**

It is clear from this work and other studies that miR-141 and miR-200c are differentially expressed in different stages of cancer. Loss of miR-141 and miR-200c expression during the migration of cancer cells indicates the potential role of these miRNAs as a marker for cancer progression and metastasis.
Therefore, alteration in expression level of miR-141 and miR-200c might act as a prognostic marker in cancer cells.

Detection of these miRNAs in tumor samples, using In Situ Hybridization (ISH), can provide us with a better understanding of the function of these miRNAs in tumor cells. ISH addresses the general expression pattern and cellular localization of these miRNAs. This information is essential to interpret the role of these miRNAs in the tumor samples since a cellular expression in basal, endothelial, stromal or vascular smooth muscles brings about different suggestions for miRNAs functional features [98]. Detection of miR-141 and miR-200c can be used to distinguish the different stages of PCa in formalin-fixed paraffin-embedded samples from PCa patients. In this case, we expect to see the low expression of miR-141 and miR-200c at the edge of the tumor, representing the invading front of the tumor, whereas the non-invading part of the tumor shows their high expression.

To show the value of miR-141 and miR-200c as suppressor of PCa progression, synthetic miR-141 and miR-200c mimics can be delivered systematically to prostate tumors in mice, which showed decreased level of endogenous MIR-141 and miR-200c. The delivery of miRNAs might result in an accumulation of miRNAs in tumor tissue, suppression of their target genes, and importantly inhibition of tumor migration and metastasis. The promise of replacement therapy has recently been shown for some miRNAs [99]. Therefore, restoring these miRNAs expression in PCa cells might be a novel therapeutic approach.

Data available to date demonstrates the experimental bases for the use of miR-141 and miR-200c in medical applications. However, there are still some problems to address these fundamental experiments into medical practice, such as development of engineered animal models and improvement of the efficiency of miRNAs delivery in vitro and in vivo.
4.8. Conclusions

In conclusion, our work addresses the question for a role of miR-141 and miR-200c in migration of PCa cell lines. Our findings regarding the strong inverse correlation between the expression level of miR-141 and miR-200c and the migratory ability of PCa cell lines, indicates the differentially expression of these miRNAs in different stages of the PCa. Our functional studies show that miR-141 and miR-200c have diverse effect on different cell lines. MiR-141 and miR-200c play an important role in suppression of the migration in VCaP and DU145 cells, respectively. Whereas a pro-migratory role for miR-141 was observed in PC3 cells. These findings might lead to a conclusion that based on the type of cell line, these miRNAs might regulate migration via a somewhat different route. Overexpression of miR-141 and miR-200c in DU145 cells resulted in decreased expression of ZEB1, ZEB2 and TGFβ2. However, in PC3 cells overexpression of miR-141 resulted in decreased expression of only TGFβ2, which might be due to the regulation of those genes at translational level. On the other hand, although miR-141 and miR-200c have almost the same seed sequences, they might bind and repress different target mRNAs.
References

1. www.instantanatomynet.
2. www.cambridgeurologypartnership.co.uk/prostate.php.
3. www.ikcnet.nl/.


68. www.genome.ucsc.edu/.
70. www.targetscan.org.


### List of Abreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µM</td>
<td>micro Molar</td>
</tr>
<tr>
<td>3' Poly A</td>
<td>3' Poly Adenylate</td>
</tr>
<tr>
<td>3' UTR</td>
<td>3' Untranslated region</td>
</tr>
<tr>
<td>5' UTR</td>
<td>5' Untranslated region</td>
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<tr>
<td>AD</td>
<td>Androgen Dependent</td>
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<tr>
<td>AI</td>
<td>Androgen Independent</td>
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<tr>
<td>AS</td>
<td>Androgen Sensitive</td>
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<tr>
<td>BSA</td>
<td>Bovin Serum Albumin</td>
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<tr>
<td>cDNA</td>
<td>Copy DNA</td>
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<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
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<tr>
<td>Ct</td>
<td>Threshold Cycle</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>ds RNA</td>
<td>Double Stranded RNA</td>
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<tr>
<td>EDTA</td>
<td>ethyleendiaminetetra-acid</td>
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<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
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<td>ESTs</td>
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<tr>
<td>Exp5</td>
<td>Exportin-5</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>hrs</td>
<td>Hours</td>
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<tr>
<td>LHRH</td>
<td>luteinizing hormone-releasing hormone</td>
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<tr>
<td>LNA</td>
<td>Locked Nucleic Acid</td>
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<tr>
<td>MET</td>
<td>Mesenchymal to Epithelial Transition</td>
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<td>MGB</td>
<td>Minor groov binder</td>
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<tr>
<td>MiR</td>
<td>MicroRNA</td>
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<td>Abbreviation</td>
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<tr>
<td>MiRNA</td>
<td>Micro RNA</td>
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<tr>
<td>M-MLV RT</td>
<td>Moloney Murine Leukemia Virus Reverse Transcriptase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
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<td>ND</td>
<td>Not Determined</td>
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<td>NFQ</td>
<td>Non-fluorescent quencher</td>
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<td>nM</td>
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<td>nt</td>
<td>Nucleotide</td>
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<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
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<td>PBGD</td>
<td>Porphobilinogen deaminase</td>
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<td>PCa</td>
<td>Prostate cancer</td>
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<td>Pre-MiR</td>
<td>Precursor microRNA</td>
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<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNU48</td>
<td>Small nucleolar RNA, C/D box 48</td>
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<tr>
<td>RNU6</td>
<td>U6 small nucleolar RNA</td>
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<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
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<td>RT</td>
<td>Reverse transcriptase</td>
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<td>Standard Deviation</td>
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<td>TGFβ2</td>
<td>Transforming Growth Factor 2</td>
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