EXPRESSION PROFILING OF MICRORNAS AND HSA-MIR-145 TARGETS IN HUMAN PROSTATE CANCER

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“This thesis is dedicated to
to my lovely mother, father, sister
and nation”

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ABSTRACT

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Prostate cancer is one of the most significant cancers of the men and there is a need to identify novel therapeutic approaches in this disease. MicroRNAs are small, approximately 18–24 nucleotides, non-coding, endogenously synthesized new class of small RNAs as regulators of gene expression post-transcriptionally. Alteration in microRNA expression may play a critical role in pathogenesis of the disease and its gain of androgen independency. Recurrence is one of the main problems in prostate cancer therapy due to lack of understanding the mechanism and insufficient treatment options. Global microRNA expression studies revealed the key regulatory microRNAs in prostate cancer. It was shown that human prostate cancer possess a widespread deregulation of microRNA expression and hsa-miR-145 is down regulated in prostate tumors as compared to normal prostatic tissues. Hsa-miR-145 is also down-regulated in prostate cancer cell lines including PC3, DU145, LNCaP and LAPC4 but has relatively higher expression in normal prostatic epithelial cells. The microRNA expression profiling studies between recurrent and non-recurrent prostate cancer samples suggest that there is not a widespread change but expression of some microRNAs is significantly altered.

ÖZET

İNSAN PROSTAT KANSERİNDE MİKRORNALARIN VE HSA-MİR-145 HEDEFLERİNİN İFADE SEVİYESİ PROFİLLEMESİ

Prostat kanseri erkeklerde görülen en ciddi kanser türlerinden biridir ve bu kansere karşı yeni tedavi yöntemlerinin geliştirilmesi ihtiyacı vardır. MikroRNAlar kısa, yaklaşık olarak 18-24 nükleotid uzunluğunda, kodlamayan, hücrenin kendisinde üretilen, yeni keşfedilmiş bir RNA çeşididir ve yazılma sonrası gen ifadesi düzenleyicisi işlevi görür. MikroRNA ifade seviyesindeki değişiklikler, hastalık patogenezinde ve androgen duyarsızlığının gelişmesinde önemlidir. Kanser tekrarlaması, mekanizmasının çözülemediğinden ve tedavi yöntemlerinin yetersiz kalmasından dolayı, prostat kanseri terapisinde önemli bir sorundur. MikroRNA gen ifadesi çalışmaları prostat kanserinde anahtar görevi alan mikroRNAların belirlenmesini sağladı. Prostat kanserinde genişçaplı bir mikroRNA ifade değişikliği gerçekleşir ve hsa-miR-145 prostat kanserinde ciddi azalma gösteren mikroRNAlardan bir tanesidir. Hsa-miR-145 prostat kanseri hücre hatlarında da (PC3, DU145, LNCaP ve LAPC4), normal prostat epiteli hücre hattına (PNT1a) göre daha az ifade olunur. Tekrarlamış ve tekrarlamamış prostat kanseri örneklerinde yapılan mikroRNA ifade seviyesi karşılaştırmasında ise geniş çaplı bir değişme belirlenmemekle birlikte bazı mikroRNAlarda, örnek olarak miR-145*, belirgin değişiklikler belirlenmiştir.

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1. INTRODUCTION

Prostate cancer is the second most commonly diagnosed cancer, after lung and bronchus cancer, worldwide with estimated 782.647 new cases and deaths in 2007. It is the sixth leading cause of cancer mortality (253.906 deaths in 2007) worldwide [1]. The ranking of cancers in Turkiye is similar to worldwide statistics. Prostate cancer is also the second most commonly diagnosed cancer among men in Turkiye, according to statistics in 2006 by Turkish Ministry of Health [2].

Prostate tumors are formed in the multiple sites of the epithelial part of prostate tissue. The early detection of prostate cancer is possible by prostate specific antigen (PSA) and successful and early screening decreases the mortality rate. Advanced disease cause symptoms that are difficulty, pain or burning during urination [1]. There are some challenges in prostate cancer studies which are relatively small tumor size, being a multifocal disease, slow progression and recurrence, observation of less or no symptoms at early stages and etc.

MicroRNAs (miRNAs) are small, approximately 18-30nt, non-coding, endogenously synthesized RNAs that regulate gene expression post-transcriptionally. They are found in viruses, plants and animal cells [3-5]. Today, a total of 14197 miRNAs have been identified and 940 miRNAs belong to Homo sapiens. (miRBase:Release15.0: April 2010) [6-8]. Approximately 30% of total coding RNAs in human are regulated by at least one type of miRNA [9]. In order to discover the true miRNA-mRNA matches, some algorithmic software have been developed to help investigators for finding the exact miRNA-mRNA target list although these are not always accurate.

MicroRNAs have significant roles in post-transcriptional gene expression and their deregulation may cause alteration in transcriptome of the cells, tumor formation and progression. Today, it is known that tumors possess widespread deregulated miRNA levels. Over-expression or down regulation of specific miRNAs in different tumor types make them potential diagnostic markers and therapeutic targets. Up-regulated miRNAs

inhibiting tumor suppressor genes in tumor cells are commonly termed as oncogenic miRNAs or oncomirs. The miRNAs whose down regulation promotes tumor progression are tumor suppressor miRNAs. One type of mRNA may possibly be targeted by multiple different miRNAs with variable efficiencies. Conversely, a single miRNA may target more than one mRNA [10].

Today, prostate cancer profiling studies show that most miRNAs have a common deregulation pattern. The profiling studies have been performed by independent laboratories since 2006 and most showed the widespread deregulation of miRNAs in prostate cancer [11-16].

Profiling studies have been reported in cell lines with androgen independent phenotype (PC3 and DU145) and cell lines with androgen dependent phenotype (LNCaP, LAPC4, VCaP, and 22Rv1), tumor xenograft models, and clinical radical prostatectomy samples.

The mechanisms of miRNA targeting and translational inhibition have not been fully understood yet. To be able to understand the true role of miRNAs in prostate cancer pathogenesis and progression, and in therapy, more studies about functions of miRNA and regulation of both miRNAs and proteins with a role in miRNA maturation are needed.

In our study, we had focused on two subjects. First one is the identification of the targets of hsa-miR-145, one of the down-regulated microRNAs in prostate cancer. Hsa-miR-145 is expressed in most human tissues and its down-regulation cause formation of several cancer types including prostate cancer [16-18]. The possible targets of hsa-miR-145 was identified by searching putative targets in silico and investigating by qPCR on prostate cancer cell lines after transfection with miR-145 precursor molecule. The second subject is the differences of microRNA profile between recurrent and non-recurrent prostate cancer samples after radical prostatectomy. Profiling of the samples was performed via microRNA microarray.

2. THEORETICAL BACKGROUND

2.1. PROSTATE CANCER

Prostate Cancer is a malignancy occurring in prostate tissue of men. It is mostly common in elderly men and forms multifocal development. Greene et al. [19] and Villers et al. [20] showed that prostate samples possess tumors on separated sites of tissue at the rates of 80% and 50%, respectively.

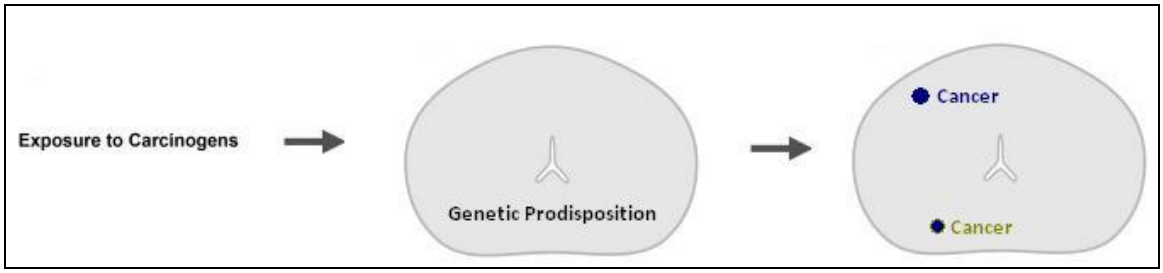

Figure 2.1. Field effect in development of prostate tumors

Prostate cancer develops by a mechanism called field effect, illustrated in Figure 2.1. Multiple factors cause the development of prostate carcinoma; mainly environmental factors/carcinogens and genetic predisposition. When the tissue have genetic predisposition for the carcinogenesis, environmental factors induce the formation of tumor precursor lesions at multiple sites at varying rates. Independent expansions of precursors would cause multifocal tumor formation [21].

The treatment for prostate cancer depends on the clinical stage, determined at diagnosis. If the disease is localized cancer than radical prostatectomy is a possible treatment method. The mortality mostly depends on the clinical stage at diagnosis and genetic alterations. If the tumor is in metastatic stage, death is the most commonly expected end [22]. The main aim for prostate cancer therapy is to determine the differences between lethal, metastatic, and the benign tumors which has little effect on

patients life. To be able to understand the general idea behind these, microRNA research is crucial as a promising treatment and diagnostic option.

2.1.1. Genetic and epigenetic alterations in prostate cancer

There are mainly two terms regarding genetic predisposition of cancers. One is hereditary and the other is familial prostate cancer. Hereditary prostate cancer means tumors with mutant genes with Mendelian inheritance that cause tumorigenesis. However, familial prostate cancer is used when at least two individual in a family have the disease with same pathology [23]. As the most of the tumors, accumulation of genetic mutations is the common reason for tumorigenesis. Deletions in one or more tumor suppressor genes and up-regulation of some proto-oncogenes may be observed in prostate carcinogenesis at different stages. For the identification of disease initiation and progression, researchers investigate molecular alterations in genetic, epigenetic, expressional and regulatory views.

The well-known most commonly deregulated proteins in prostate tumors are prostate-specific antigen (PSA), prostein (P501s), prostate-specific membrane antigen (PSMA), NKX3.1, and precursor form of PSA (proPSA) and these are also used as diagnostic tumor markers [24]. In prostate cancer diagnosis is done also by abnormality in rectal examination and histopathological examination of biopsy [25].

The most common and consistent chromosomal changes in prostate tumors are TMPRSS2-ERG fusion, 8p loss, and 8q gain [26].

The most commonly deregulated tumor suppressor gene in prostate cancer is p53 as well as in other tumors [27]. P53 is a transcription factor having regulatory role in cell cycle during G1/S and G0/G/S transition and apoptosis. Another role of P53 is inhibition proliferation in the case of DNA damage and driving cell into apoptosis [27]. P53 also take role in maturation of some microRNAs, selectively [28]. P53, p73 and p63 controls the miRNA processing elements for some miRNAs such as miR-143, miR-145, let-7, miR-200c and some others [28,29]. P53 gene is either deleted or mutated in prostate cancer cell lines. The p53-microRNA interaction in prostate cancer has also been investigated.

Ectopic expression of normal p53 gene causes up-regulation of miR-145 in prostate cancer cell lines [30]. This indicates the possible therapeutic target as possessing regulatory role of p53 in microRNA maturation.

Prostate gland requires zinc to alter metabolism for citrate production. The citrate is important component semen. Zinc is actively transported in to prostate gland cells by protein ZIP1. The tumor suppressor ZIP1 is silenced in prostate cancer cells epigenetically. Thus the cancer cells save energy not making citrate [31].

Some other tumor suppressor genes implicated in advanced prostate cancer are retinoblastoma susceptibility gene (RB1) [32], PTEN/MMAC1 [33], WWOX [34], Kruppel-like factor 6 (KLF-6) [35].

Myc is an important proto-oncogene in prostate cancer cells. The Myc-proto-oncogene, located in chromosome 8q24, is a transcription factor functioning normally in proliferation, differentiation and apoptotic pathways. Her-2/neu, Ras family, and PTI-1 are also other proto-oncogenes observed in prostate tumors at different ratios [36-38].

There are also some genes that were previously considered as tumor suppressor but later their oncogenic properties are shown. Early growth response-1 (EGR-1) is a transcription factor, which is one the genes described above, having regulatory function in apoptosis and proliferation [39]. EGR-1 is up-regulated in most of aggressive prostate tumors but interestingly down-regulated in breast, lung and brain tumors [40]. EGR-1 up-regulates cyclin-D2 and inhibits CD95 expression [41].

Epigenetic regulations like DNA methylation and histone modification are as important as other regulatory mechanisms of gene expression [42]. DNA methylation is addition of Methyl- groups on cytosine nucleotid mostly in DNA regions called CpG islands. The CpG islands are present in promoters of 60% of genes in human genome [43]. Abnormal epigenetic alterations may cause severe tumorigenesis and metastasis [42]. The genes, GSTpi, APC, MDR1, GPX3, and 14-3-3sigma, are most commonly

hypermethylated and inhibited during prostate cancer progression. Histone methylation and acetylations have also efect on functions of genes such as androgen receptors [43]. In prostate cancer, DNA Methylase-1 (DNMT-1) and Histone Deacetylating class of proteins (HDACs) are upregulated. Histone Acetylating enzymes (HATs) have both positive and negative effects and their deregulation effect variable cellular processes [43]. Furthermore, not only individual histone modification alterations but also global modification pattern of cells indicates clinical outcomes. Researches suggest that differences in global histone modification patterns can discriminate recurrence, tumor-stage, and invasion [44].

2.1.2. Androgen Independency

Androgen receptor (AR) in prostate tissue is the transcription factor functioning by the induction of male sex steroid hormones. The AR induce transcription of hundreds of genes functioning in proliferation, survival, differentiation and lipid metabolism [45].

Androgen depletion therapy provides inhibition of most of the prostate tumor cells but if there is a small amount of androgen-independent tumor cells or tumor stem cells then the prostate cancer become an androgen independent or androgen-depletion independent state [44].

2.1.3. Prostate Cancer Recurrence and Metastasis

When the tumor becomes aggressive and androgen independent, the last treatment method is radical prostatectomy surgery. It is complete removal of prostate gland together with surrounding tissue. Even, it has certain risks especially affecting the comfort of life; the removal of tumor is required before metastasis. Since, prostate cancer is a multifocal disease removal of specific tumor sites is not possible.

Prostate tumor cells require active androgen receptors (ARs) that are induced by dihydrotestosterone, synthesized from testosterone. Most cancer cells are eliminated by androgen deprivation therapy that is prevention of testosterone production. However some

cells stay alive and prostate tumor recur causing metastasis and death [46]. This recurrence may occur due to several reasons such as cancer stem cells, mutations on ARs, trans-activation of AR by up-regulation of other factors etc. [46,47]. Actually, it is a big challenge to find suitable predictive molecular targets of recurrence since relatively small number of cells have recurrence ability in tumor [48].

The genomic expression level analysis to find biological markers for recurrence is needed to predict the prognosis of the disease. However, recurrence time in prostate cancer is longer relative to other cancers such as breast cancer. To be able to find molecular targets against recurrence - there is not a chemotherapy agent preventing recurrence in PCa – global genomic and expression studies are done [48]. It is reported that expression level analysis could not give clue for predicting recurrence but DNA copy number profiling could [26,48]. Taylor et al. [26] propose that with extensive DNA-copy number, recurrence happens much earlier than others. Additionally, we have identified some microRNAs that are expressed differently between recurrent and non-recurrent prostate cancer.

Metastasis in prostate cancer is a specialized event and some prostate tumors are more aggressive and more likely to metastases than others. Qi et. al. identified a protein called Siah2 which triggers pathways and turns the cells into metastatic neuro-endocrine tumor. Currently, there is not a study investigating the association of metastasis and microRNAs.

2.2. MICRORNAs

MicroRNAs, transcribed as non-coding RNAs, are post-transcriptional regulators of gene expression. Their mature form, incompletely bind onto 3'- untranslated region (3' UTRs) of messenger RNAs (mRNAs) causing gene silencing. MicroRNAs (miRNAs) are short, 18-30 nucleotides long RNA molecules [9,49]. First identification of miRNAs was in 1990s but direct characterization of their regulatory role was understood in 2000s. MiRNAs are taking role in most biological processes such as development, secretion, differentiation, metabolic pathways, morphogenesis, and signaling [50-55]. All tissues

micrornome – total content of expressed microRNAs - differs from each other. Also studies show that cancer cells possess deregulated microRNA expression [56].

MicroRNAs are also small RNAs but small RNA term is a general definition of most short non-coding RNAs such as transfer-RNA (tRNA), small nuclear RNAs (snRNAs), and also bacterial short regulatory RNAs.

MicroRNAs vs. small-interfering RNAs (siRNAs) differ from each other at their sources. MiRNAs are produced endogenously and siRNAs are synthetically synthesized and exogenously transferred. Recent studies; however, revealed the discovery of endogenously synthesized siRNAs especially in plants with four main roles: trans-acting siRNAs (tasiRNAs), repeat-associated siRNAs (rasiRNAs), small-scan RNAs (scnRNAs), and piwi-interacting RNAs (piRNAs) [57,58]. Differently from others the maturation of piRNAs is independent of Dicer cleavage [59]. Endogenous siRNAs are mostly produced by RNA-directed RNA polymerases and precursor of endogenous siRNAs is double-stranded RNAs. This is the main difference between endogenous siRNAs and miRNAs, since the precursor of miRNAs is a hairpin-shaped RNA molecule [57]. The endogenous siRNAs are independent from Drosha cleavage [60]. The type of Argonaut (AGO) proteins interacting with miRNA, endogenous small interfering RNA (endo-siRNA) and pi-RNAs differ respectively [60].

2.2.1. MicroRNA Biogenesis

The microRNAs (miRNAs) have close loci in genome and can be localized on non-coding intronic and non-coding exonic regions as well as coding intronic and exonic transcription units [60]. The primary miRNA (pri-miRNA) is transcribed by RNA polymerase-II (pol-II). The pri-miRNA has one or more several hairpin structures with stem and terminal loops consisting several same or different kind of microRNAs [60]. As transcription around Alu elements is done by RNA Pol III enzyme, microRNAs inside Alu elements are also transcribed by RNA Pol III enzyme [61]. Like other messenger mRNAs, pri-miRNAs are also 5'-capped, spliced, and polyadenylated at 3'-end [60,62].

The pri-miRNAs are maturated by two processes in nucleus and cytoplasm. First, a complex of RNase III enzyme (Droscha) and DGCR8 (a double stranded RNA binding domain)(DGCR8 is DiGeorge Syndrome Critical Region Element) cleaves the pri-miRNA into ~70nt precursor microRNAs (pre-miRNAs) [60]. There may be more than one type of pre-miRNAs cropped from one pri-miRNA. The Droscha complex, or microprocessor complex, contains cofactors and heterogeneous nuclear ribonucleoproteins (hnRNPs). The cleaved, small-hairpin pre-miRNA is transported into cytoplasm by Exportin-5 with Gan-GTP mechanism [63]. The pre-miRNA is not functionally active and must be further cleaved or "diced" to mature 18-24nts-long microRNA form. This cleavage is catalyzed by Dicer that is also an RNase III enzyme forming complex with double-strand RNA binding domain (dsRBD) and TRBP/PACT [63]. The pre-miRNA hair-loop contains two mature miRNA sequences. One of them is normal miRNA or guide strand of miRNA and the other is complementary sequence or passenger strand of miRNA. The passenger strand of miRNA is indicated with "*" on guide's name such as miR-145*. It was formerly thought that miRNA* that has more stable 5' end is degraded after biogenesis but later studies indicate that they are also functional and take role in different pathways together with AGO1 or AGO2 [64,65]. The guide-strand mature-miRNA, forms a complex with Argonaut proteins and RNA-induced silencing (RISC) complex. This complex together with miRNA targets the specific mRNAs to negatively regulate the translation process [63]. The miRNAs and miRNA*'s have different targets and take role in different pathways in metabolism, and also their amount in cytoplasm alternates with a tissue dependent manner [66].

The biogenesis of the miRNAs should also be kept under control by general and specific regulatory mechanisms [63]. There are some microRNAs regulating the expression of transcription factors that control transcription of microRNAs [62]. Also, one of the microRNAs transcribed from the same cluster may be repressed while other miRNAs in the same cluster continue biogenesis [63].

Furthermore, miRNAs are post-transcriptionally controlled to maintain the differentiation, signaling, tumorigenesis, cell cycling and other mechanisms [67]. The Figure 2.2 illustrates the microRNA biogenesis process.

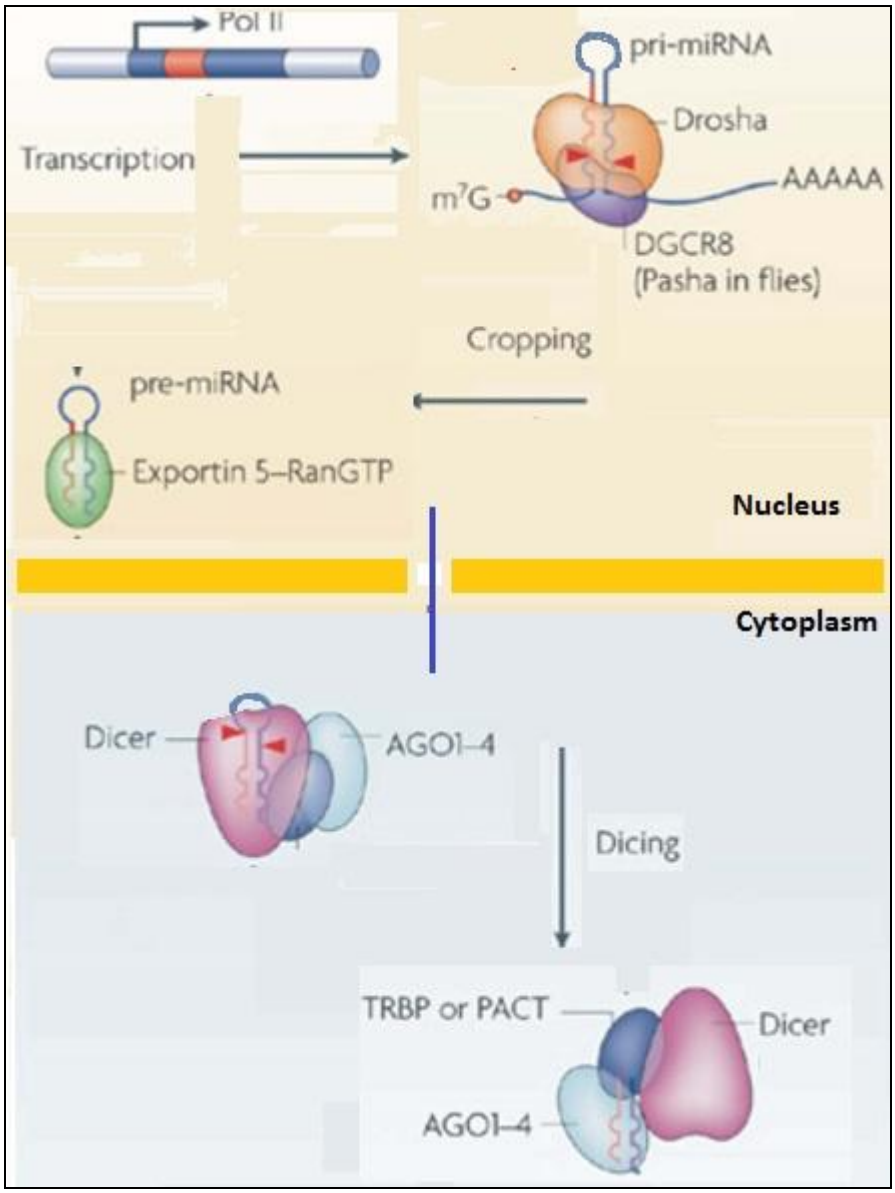

Figure 2.2. The miRNA biogenesis, derived from［60］

2.2.2. MicroRNAs in Post-Transcriptional Regulation Mechanism

The regulations of mRNA transcripts occur by incomplete hybridization of miRNA on most commonly on 3' UTR of target mRNAs and rarely on coding regions of mRNAs, during translation［68］. This hybridization of miRNA together with RISC causes degradation of the mRNA, inhibition of the protein synthesis and/or deadenylation of

targeted mRNA [69,70]. Currently it is known that incomplete hybridization includes a 5' complete binding on a 5'- seed sequence of 7-8bp long-miRNA. After the seed-match, a non-hybridized loop sequence and variable hybridization pattern is observed [9]. The Figure 2.3 illustrates the main post-transcriptional effects of miRNAs on target mRNAs. The figure shows only the general idea but miRNA-specific functions are not included.

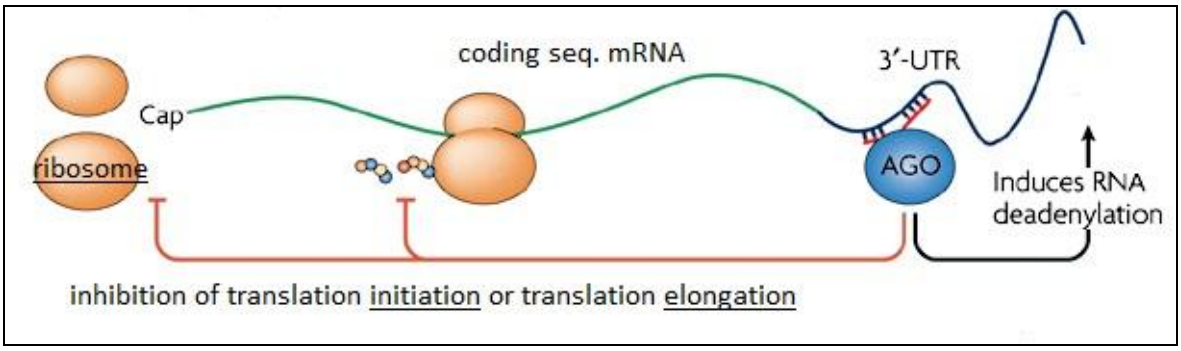

Figure 2.3. Post-transcriptinal funtions of miRNAs [51]

The Argonaut proteins serve the miRNA while binding on targets sites on mRNAs. In mammals there are four AGO proteins functioning in repression of expression. AGO2 serves both miRNAs and siRNAs [71]. In metazoan and mammals the miRNA bind on target sequence imperfectly. This imperfect binding include a complete hybridization at 2-8 nucleotides called seed sequence [71].

2.2.3. Altered expression of microRNAs in prostate cancer

The initial investigations of miRNA deregulation in tumors were performed via miRNA microarray profiling. Determination of miRNA signature is essential to solve the general alterations and specific expression changes between tumor and normal tissues, hormone refractory samples, metastases, and cultured cells. MiRNA microarray expression studies comparing cancer tissue with normal tissue samples reveal over-expressed and down regulated individual miRNAs. This deregulated expression of specific miRNAs may be explained by some problems in miRNA mechanism including tendency of miRNA

genes to be located in cancer related genomic regions; such as chromosomal instable regions. There is a direct relation between chromosomal instability and miRNA expression. Evidences reveal that in prostate cancer cell lines and xenograft samples, the miRNAs at deleted chromosomal regions show very low expression. The ones located at amplification sites, however, are highly expressed [11,12]. For example, miR-126 and its minor miR-126* is located on the intronic region of vascular endothelial EGF-like 7 gene (Egfl7) which is deleted in most of the prostate tumors. Its absence increases the protein level and its ectopic expression represses the invasiveness and migration of LNCaP cells [72]. MiRNA genes also have promoter under epigenetic regulations. MiR-126 shares the same promoter with Egfl7 gene and epigenetic therapy targeting promoter of this gene also activates the intronic miR-126 [73].

MiRNA processing genes such as Drosha and Dicer are aberrantly-expressed in cancer cells [74]. Absence of transcription factor-inducing miRNAs cause decreased production of precursor miRNAs [13]. More complex pathway may be triggered due to mutation on a single miRNA. Different cancer types have different miRNA deregulation pattern. Thus microRNA microarray profiling can also be helpful to characterize tumors and their subtypes. The aberrantly expressed miRNAs have crucial roles in pathogenesis of cancer, cell survival, apoptosis, invasion and metastasis.

Oncomirs are the miRNAs that are amplified or over-expressed in tumors and they act like oncogenes. Some miRNAs significantly over-expressed in most of the tumor types are: miR-21, miR-291, and miR-17-5p [75]. Depending on the tumor type, various other miRNA might have altered expression to act oncomirs.

Mir-17-92 cluster contains six-microRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1 on chromosome 13) processed from a single transcript by Drosha. These microRNAs are over-expressed in prostate cancer and in some other tumors [76]. MiR-20a in this cluster is over-expressed in prostate cancer samples [77] and its inhibition induces cell death and apoptosis in PC3 cell lines [78]. It is not certain that the over-expression of this cluster alone is sufficient to accelerate prostate tumorigenesis. Its over-expression and homozygous deletion in mouse models cause autoimmunity and

premature death of B-cells, respectively [79,80]. MiRNAs, originating from the miR-17-92 cluster target significant genes including E2F1, TSP1, CTGF, PTEN, BIM, RB2, and p21. E2F class contains transcription factors inducing genes having role in cell cycle regulation and apoptosis. Thus they are strictly regulated both at transcriptional and protein level, posttranslational and pre-translational manner. Members of E2F family proteins, E2F1, E2F2, and E2F3 also re-regulate and induce the expression of miR-17-92 cluster, possibly to prevent accumulation of those transcription factors [78].

MiR-125b deregulation has a significant effect on prostate cancer tumorigenesis and on androgen independency [81]. MiR125b is up-regulated in clinical samples and androgen independent cell lines [81-83]. Functional studies reveal that miR-125 over-expression is related to progression of androgen independence and cell survival in prostate cancer [81]. Her2 and Her3 are two important proteins over-expressed in prostate cancer and mir-125b inhibits their translation in breast cancer samples [84]. Her2 might have a role in androgen independency in prostate cancer [85]. The increase of miR-125b might be also related with the interaction of HER2-AR pathway [81]. The same group has searched for novel targets of miR-125b and suggests Bak1 as a target after in vitro expression studies and luciferase assay. Bak1, together with Bax protein regulate apoptotic signaling in prostate cancer synergistically [81].

MiR-21 is another oncomir, having function in tumorigenesis, invasion and metastasis. MiR-21 induces invasion and motility of prostate tumor cells. It also takes role as anti-apoptotic molecule triggering resistance to apoptosis. Possibly, miR-21 does not have a direct negative effect on cellular proliferation [86]. Those effects may be due to targeting and repression of tumor suppressor genes; TPM1, PDCD4, maspin, PTEN, and myristoylated alanine-rich protein kinase c substrate (MARCKS) by miR-21. Targeting the PDCD4 is a translational inhibition since the protein level of PDCD4 decreased by miR-21 while the mRNA level is stable. Down-regulation of PDCD4 and maspin by mir-21 may promote tumor invasion and metastasis. Additionally, maspin protein suppresses the invasion of tumor and inhibits metastatic action but has no known affect on cell proliferation [75,87].

Volinia et al reported that miR-106a is over-expressed in prostate cancer and some other solid tumors except breast cancer. Its putative target RB1 is found to be down-regulated in prostate cancer [77].

MiR-221 and miR-222 have been previously reported as up-regulated in several cancers and they are determined as oncomirs due to targeting p27 [88,89]. However, some studies claim those miRNAs are down-regulated in stage T2a/b prostatectomy samples and androgen dependent cell lines [90,91]. MiR-221 and miR-222, transcribed from a cluster on chromosome X, have identical seed regions and possibly common mRNA targets. MiR-221 and miR-222 are both increased in prostate tumor cells and almost absent in androgen dependent cell lines [88,90]. They act as oncomirs since miR-222 targets the p27 (Kip1) and down-regulates its translation [88,89]. Hu et al. [92] reported that thrombin down-regulates p27 (Kip1) in TRAMP mice and cancer cell lines, and interestingly miR-222 expression is increased by thrombin. This increase in miR-222 expression suggest the idea that it has an oncogenic function and stimulate tumor proliferation by inhibiting p27 (Kip1) expression [92]. Mercatelli et al. [89] also performed in vitro studies for identification of oncogenic and triggering androgen independency functions of miR-221 and miR-222. LNCaP-derived tumors in SCID mice formed tumor by ectopic over expression of miR-221 and PC3-derived subcutaneous tumor growth was inhibited by anti-miR-221 and anti-miR-222 antagomir treatment. Increased level of p27 was observed in these suppressed tumors [89].

Tumor suppressor miRNAs are the ones that are down-regulated or deleted from chromosomes. Their reduced expression cause increased level of oncogene expression. The inhibition of proliferation, apoptosis induction, reduced invasion and lack of metastatic activities in cancer cells were shown via the in vitro and in vivo over-expression of tumor suppressor miRNAs by various independent experiments.

The miR-15a/16-1 cluster has 2 microRNAs functioning as tumor suppressor. They are located at chromosomal region 13q14 which is frequently deleted in most of CLLs, in 50% of prostate cancers and some of lymphomas [93,94]. The endogenously down-regulation of this miR-15/16 cluster was found in chronic lymphocytic lymphoma (CLL),

prostate adenocarcinoma and pituitary adenomas [95]. Inhibition of miR-15a and miR-16-1 activity causes hyperplasia of prostate in mice and increases in vitro cell proliferation and invasion [96,97]. However, increased expression of miRNAs in this cluster provide inhibition of proliferation, induction of apoptotic pathways and suppression of tumor producing potential of cancer cells both in vitro and in vivo [94,95]. Additionally, increased expression via intra-tumoral delivery of this miR-15a/16-1 cluster for therapeutic purposes has a significant regression potential in prostate cancer xenografts [94]. The validated targets of miR-15a/16-1 cluster are BCL2, MCL1, CCND1, and WNT3A [95].

MiR-145, down-regulated in most of cancer types, is another tumor suppressor miRNA with decreased expression level in prostate cancer cell lines and tumor samples. MiR-145 and miR-143 form a cluster on chromosome 5 [98-100]. The direct role of miR145 in prostate cancer prognosis has already been revealed and mentioned in a separate part below. A recent article identified the miR145 and miR-331-3p as the critical microRNA in aggressive prostate cancer and found that their ectopic expression induce apoptosis and decrease proliferation [101]. They also identified CCNA2 as potential miR145 target in LNCaP and VcaP cells [101].

Lin et al. [102] reported in early studies that miR-146a is down regulated in androgen independent prostate cancer samples. In vitro insertion of miR-146a into PC3 cell line decreased the proliferation and survival. Endogenously miR-146a expressing cells have less invasive or metastatic functions [102]. Its lack of expression in androgen-independent state of prostate tumor implies that it may have a role in androgen independency.

P53, a well-known tumor suppressor, cell cycle regulator and activator of DNA repair genes, induces expression of miR-34a, a commonly reduced miRNA in various cancers that also lack p53 expression [103,104]. Lodygin et al. [104] have reported that its lower expression is probably because of irregular CpG methylation on its promoter in prostate carcinoma and some other carcinoma cell lines [104]. MiR-34a transfection into PC3 cell lines provided decreased expression of SIRT1 in both mRNA and protein levels.

SIRT1, a sirtuin class III histone deacetylases, is up-regulated in various cancers and its inhibition results in decreased proliferation and colony formation ability of prostate cancer cell lines [103,105]. Dependent to SIRT1 or not, ectopic expression of miR-34a cause cell cycle arrest in G1 phase, decrease in cell proliferation and induce apoptosis [103,104]. DNA double-strand breaks inducing agents also activates DNA-repair related protein p53 as a survival mechanism. P53, after DNA breaks recognition induces variety of genes including the ones taking role in apoptosis including miR-34a and 34b/c [106]. Rokhlin et al. [106] reported that miR-34a/b/c induce apoptosis only in the presence of AR and p53 activation by DNA double-strand break inducing agents. P53 also may induce transcription of miR-145 via p53 response element (p53RE) on its promoter region, in prostate tumor [107]. P53 represses c-Myc but the complete mechanism has not been identified yet. Sachdeva et al. [107] reported that miR-145 targets c-Myc and inhibit its translation; therefore, miR145 might involve in p53 pathway and control tumor cell growth [107]. Very recently, it has been demonstrated that p53 interacts with the Drosha processing complex via DDX5. In this report, a new function of p53 in miRNA processing has been revealed, as described above [108].

Let-7 is one of the first identified and mostly well-known tumor suppressor miRNA in human and it targets the RAS oncogene. [109,110] In different clinical tests and cancer studies increased expression of Let-7 and some of its isoforms have showed higher survival rate and decreased clonogenicity and radioresistance in vitro [110].

Cancer cells possess high level transcription at certain locuses, thus histone deacetylases (HDAC), neutralizing the positively charged amines on histones, are generally over-expressed in tumors [111]. Noonan et al. [111] identified that miR-449a targets HDAC-1 and this miRNA is down-regulated in human prostate cancer samples. MiR-449a transfection studies reveal partial triggering apoptosis and cell-cycle arrest in cancer cells [111].

Prostate cancer cells have lower expression of miR-205. Exogenous expression of miR-205 results reduced invasion of prostate cancer cells and caused mesenchymal-to-

epithelial transition of cells possibly due to translational inhibition of miR-205 of N-chimaerin, ErbB3, E2F1, E2F5, ZEB2, and protein kinase Cepsilon [112].

MiR-200 family generally triggers Epithelial-Mesenchymal transition (EMT). Kong et al. [113] reported that miR-200 family is down-regulated in prostate cancer PC3 PDGF-D cell lines and transfection of miR-200b results in gaining epithelial phenotype and decreased migration and invasion activity [113].

The epigenetic changes in cancer cells cause silencing of tumor suppressor genes by methylation of their promoters. Enhancer of zeste homolog 2 (EZH2), histone methyltransferase, is up-regulated in most solid tumors including prostate cancer and its link with miR-101 was reported by Varambally et al [114]. Prostate tumor tissues have decreased expression of miR-101 mostly due to genomic loss and increased expression of EZH2 causing cancer progression [114].

2.3. EXPRESSION OF MICRORNAs ANDROGEN INDEPENDENCY

The most common therapy for metastatic prostate cancer is androgen-ablation. However, the tumors ultimately become independent of androgen and become clinically progressive. Although there are several hypotheses in the literature, the true mechanism of this transition has not been revealed yet in all cases. Androgen-independent prostate cancer possesses over-expression of AR or mutated form of it functioning without androgen induction. To be able to eliminate AR and convert the tumor into androgen-dependent state, targeting AR is a possible strategy. Moreover, altered miRNA expression between androgen dependent and androgen independent prostate cancer samples and cell lines could give a clue about the exact mechanism of this transition. DeVere R.W. et al. [115] identified the differentially expressed miRNAs between androgen responsive and independent cell lines including 10 up-regulated and 7 down regulated miRNAs. One of them is miR-125b which functions in altering LNCaP cell lines into androgen independent state. BAK1, targeted by miR-125b, takes role in apoptotic pathway [115]. They have also reported that AR positive cell lines possess higher expression of miR-125b than AR negative cell lines. Additionally, Sun et al. [116] found that miR-221 and miR-222 have

increased expression level in androgen independent (or castration-resistant prostate cancer) cell lines, LNCaP-Abl, relative to androgen dependent cell lines, LNCaP and LAPC-4. Functional studies of miR-221 and/or miR-222 reveals over-expression in LNCaP and LAPC-4 triggers androgen-independent growth and their inhibition converts LNCaP-Abl into androgen-dependent phenotype [116].

2.3.1 Mir-145 in Prostate Cancer

As discussed above, hsa-miR-145 is one of the tumor suppressor microRNAs that is down-regulated in prostate cancer [16]. The initial research step before using miR-145 as a therapeutic agent is investigating its functional effects after its ectopic expression and discovering its potential targets in prostate cancer cells.

The miR-143/miR-145 cluster is located on chromosome 5. The host gene encoding these microRNAs namely pri-miRNA has three transcripts at different lengths and its expression is also down regulated in prostate cancer [117].

BNIP3 is a gene, up-regulated in prostate cancer, and has a binding site for miR-145 in its 3'UTR. Chen et al. [30] showed that ectopic expression of miR-145 cause increase in cell death together with decrease in cancer cell proliferation and BNIP3 expression. Ectopic expression of TP53 gene cause the same phenotype since it also up-regulates the miR-145 biogenesis. miR-145 also down-regulates the expression of c-Myc by the p53 induction [118]. The tumor repression role of miR-145 is also shown by other investigations [119,120] and thus we have focused on understanding its repression mechanism in prostate cancer.

3. MATERIALS

3.1. CELL CULTURE SUPPLEMENTS

The cell culture media and other reagents including Trypsin-EDTA together with antibacterial and antifungal supplements are purchased from GIBCO (USA).

3.2. SAMPLES

All samples were obtained in accordance with approved ethical standards of the responsible committee of either Yeditepe University Hospital or Baylor College of Medicine where appropriate. Prostate Cancer cell lines (PC3, Du145, LNCaP, and LAPC4), immortalized prostate epithelial cell line (PNT1a) and RNA isolates radical prostatectomy samples (which are grouped as recurrent and non-recurrent) were obtained from Baylor College of Medicine, Texas, USA. Prostate cancer cell lines were cultured with RPMI (GIBCO, USA) containing 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin/Antifungal (100 µg/ml streptomycin and 10,000 units/ml penicillin) solutions. The environmental conditions for culturing is in a 5% CO_2 incubator with temperature set in 37 °C. The cell lines were passaged after reaching 70% confluency.

3.3. PRECURSOR MICRORNAs AND TRANSFECTION

The precursor microRNA miR-145 and anti-miR-145, inhibiting the miR-145 functions, molecules were purchased from Ambion (Foster City, CA, USA). The transfection of miR-145 precursor and/or anti-miR-145 molecule was performed by X-treme Gene Transfection Kit of Roche (Switzerland) after optimizing the protocol for our cell lines.

3.4. RNA ISOLATION MATERIALS AND GENE EXPRESSION ANALYSIS

For RNA isolation, total RNA was extracted by using Trizol reagent of Invitrogen Inc. (San Diego, CA). Equal amounts of RNA from isolates were used in cDNA synthesis by using Transcriptor HighFidelity Reverse Transcription kit from Roche (Switzerland). Quantitative PCR analysis was performed by SYBR Green Master Mix of Roche (Switzerland). Quantitative gene expression assay for microRNAs and microRNA specific primers were purchased from Exiqon Inc. (Denmark).

3.5. MICRORNA MICROARRAY ANALYSIS

The microarray analysis for microRNA profiling was performed with Agilent (USA) chips (Agilent Human miRNA Microarray (V2) Kit, 8x15K). Recurrent and non-recurrent radical prostatectomy samples were compared according to their microRNA content by microRNA microarray chips of Agilent. All the related reagents were also purchased from Agilent Inc. The chips were scanned by Axon GenePix 4300A array scanner (Molecular Devices, CA, USA).

4. METHODS

4.1. In Silico MicroRNA Target Prediction

Before wet lab experiments, the potential targets of hsa-miR-145 were predicted by bioinformatics algorithms. There are some computational algorithms that have been developed for prediction of putative microRNA targets. These algorithms are quite useful but further experimental validation of the predictions is necessary. The Table 4.1 below is the list that we have used for determining putative targets of hsa-miR-145.

Table 4.1. The list of miRNA target prediction algorithms

Name	Web Address	Reference
miRanda	http://www.microrna.org	[121]
miRBase	http://microrna.sanger.ac.uk	[7]
TargetScan	http://www.targetscan.org	
PicTar	http://pictar.mdc.berlin.de	
miRecords	http://mirecords.biolead.org/	[122]
TarBase	http://diana.cslab.ece.ntua.gr/tarbase/	[123]
MirDB	http://mirdb.org/miRDB/	[124,125]

Those algorithms calculate the miRNA binding sites on 3'-UTR of genes algorithmically. This is the easy way of discovering potential targets but true results would be found by gene expression profiling.

4.2. TRANSFECTION OF PROSTATE CANCER CELL LINES

In order to be able investigate the effects of miR-145 on prostate tumor; the miR-145 precursor molecule was transfected into the prostate cancer cell lines. The lipofection method was chosen for the transfection since it is a simple, relatively cheap and efficient

method. However, optimization of transfection reagent and initial cell amount for each of the cell line is required.

Before transfection, the number of cells was calculated for each group of incubation time. The approximate number of cells is 450000, 350000, 300000, 250000, and 200000 for incubation times of 8, 16, 24, 48, and 72 hours, respectively. The cells were grown in 6-well plate for 1 day and washed with PBS just before transfection. The transfection of prostate cell lines and the amount of the reagent and initial cell amounts used were given below. For one well of 6-well plate; 2,5 µL transfection reagent was mixed with 250 µL of Opti-MEM and 10 pg of precursor microRNA was mixed with 250 µL of Opti-MEM. These mixes were incubated for 5 min at room temperature. Then the transfection reagent mix was added into precursor microRNA mix and the transfection mix was incubated for 20 minutes at room temperature. The total 500 µL of transfection mix was applied on the cells. After 4 hours another 500 µL of Opti-MEM medium was added on plates. At the end of growth the cells were trypsinized and collected by centrifuge at 2500 g for 5 minutes.

The transfected cell lines were prepared to grow in 5 groups for different time periods as 8, 16, 24, 48, and 72 hours period. Thus we can understand the correct time for observation of miR-145 effect.

4.3. RNA ISOLATION AND cDNA SYNTHESIS

To be able to perform qPCR and microarray studies total RNA isolation from every sample is needed. Transfected cell lines were collected by tyrpsinization and homogenization with 0,75 mL Trizol reagent by pipetting rapidly. The radical prostatectomy samples, previously stored in -80C, were crumbled in liquid nitrogen and then, homogenized with 1,0 mL Trizol reagent. Homogenized samples were incubated at room temperature for 5 minutes for the complete dissociation of nucleic acid binding proteins. After the dissociation, 200 µl of chloroform per sample was added and continued to pipette rapidly for another 5 minutes. Samples were centrifuged at 12.000 x g for 25 minutes at 4º C. After the centrifugation three phases were observed. The bottom phase contains extracellular membranes, and polysaccharides. The middle phase contains high

molecular weight DNA, while the colorless supernatant contains RNA. The cleared phase which contains RNA was transferred into a new micro tube. 500 µl of isopropyl alcohol per sample was added into the tubes containing the cleared homogenate. Samples were incubated for 30 minutes in ice and centrifuged at 12,000 x g for 30 minutes at 4º C. After this step RNA is visible as whitish precipitate. Resultant supernatant was discarded and the pellet was washed by 500 µl 75% ethanol and centrifuged at 7,500 x g for 10 minutes at 4º C. In the final step the RNA pellet was left to air dry until the ethanol disappeared entirely that is approximately 5 minutes. The RNA pellet was re-dissolved in molecular grade nuclease free water (mostly 30 µL). 3 µL of the RNA sample was used for measurement of the concentration.

The volumes of the RNA samples were further calculated for getting equal amount of RNA molecule for cDNA synthesis. The cDNA synthesis was performed according the manufacturers protocol given below in the Table 4.2.

Table 4.2. Reagents and proper amount for cDNA synthesis

Reagent	**Volume (µL)**	**Final Concentration**
Total RNA	Variable	0,5 µg
Anchored-oligo(dT) Primer	1	2.5 mM
PCR Grade Water	Variable	To make total volume upto 11.4 µl
Transcriptor RT Reaction buffer, 5x	4	1x(8mM $MgCl_2$)
Protector RNase Inhibitor (40 U/µl)	0.5	20 U
dNTP Mix 10mM	2	1mM each
DTT	1	5 mM
Transcriptor Reverse Transcriptase	1.1	10 U

4.4. MicroRNA Microarray

The total RNA samples of recurrent and non-recurrent prostate cancer groups were adjusted to 50 ng/ µL concentration. All the microRNA microarray procedure was

performed by the instructions according to “Agilent miRNA microarray system with miRNA complete labeling and Hyb Kit protocol”. By getting 2,2 µL (110ng) of total RNA, the dephosphorylation mix was prepared at total volume of 4.0 µL as given in Table 4.3, then incubated at 37ºC for 30 minutes.

Table 4.3. Dephosphorylation mix of microRNA microarray

Components	**Volume (µL) per rxn**
10x calf intestinal phosphatase buffer	0.4
Nuclease free water	0.9
Calf intestinal phosphatase	0.5

The mix was incubated at 100 ºC for 10 minutes following the addition of 2.0 µL DMSO for denaturation and immediately transferred into water-ice bath for prevention of re-annealing.

Ligation of the samples with cyanine3 dye was performed by T4 RNA Ligase enzyme. The Ligation master mix was prepared as the Table 4.4 and 4.5 µL of it was added to each sample tube for a total reaction volume of 11,3 µL.

Table 4.4. Ligation mix of the microRNA microarray

Components	**Volume (µL) per rxn**
10x T4 RNA Ligase buffer	1.0
Cyanine3-pCp	3.0
T4 RNA Ligase	0.5

The ligation mixture was incubated at 16 ºC for 2 hours. After the 16 ºC labeling reaction, the samples were dried in vacuum concentrator for 2 hours at 60 ºC. The samples were eluted in 19 µL of nuclease-free water and 1 µL of it was used for measuring the dye incorporation rate via spectrophotometer. The hybridization mix was prepared as the Table

4.5 below for a total volume of 45 µL. The mixture was incubated at 100 ºC for 5 minutes and immediately transferred into ice-water bath for 5 minutes.

Table 4.5. Hybridization mix of the microRNA microarray

Components	Volume (µL) per rxn
Labeled miRNA sample	18.0
10x GE Blocking agent	4.5
2x Hi-RPM Hybridization Buffer	22.5

Totally 40 samples were tested by 5 chips including 8 arrays. With each chip 4 recurrent and 4 non-recurrent sample were tested to minimize the experiment specific errors. The chips were at 55 ºC for 20 hours. The washing steps of chips were also performed according the manufacturer's instructions.

4.5. Q-RT-PCR FOR MICRORNA EXPRESSION LEVEL ANALYSIS

After every transfection assay, to confirm the proper transfection with microRNAs and the presence of mature microRNA molecules, miRNA qPCR was performed. Aliquots of 15 ng/µL concentration from the total RNA of samples were used for specific cDNA synthesis with Exiqon Inc. primers. The control housekeeping gene for quantifying the relative expression was 5S-rRNA. Specific cDNA synthesis was performed for both 5S-rRNA and microRNAs separately and the qPCR was performed with SYBR green method. cDNA synthesis and qPCR mixes were given in the Table 4.6 and Table 4.7.

Table 4.6. cDNA synthesis mix for microRNA quantification by Exiqon miRCURY Kit

Components	Volume (µL) per rxn
Template RNA (15 ng/µL)	1.0
5X RT- reaction buffer	2.0
miR-specific or control-specific reverse primer	1.0
dNTP mix	0.5
RNase inhibitor	0.5
Reverse Transcriptase	0.5
Total volume	10.0

Table 4.7. rt-qPCR mix for microRNA quantification by Exiqon miRCURY Kit

Components	Volume (µL) per rxn
Diluted cDNA template (1/5 dilution)	2
SYBR green master mix	5.0
LNA PCR primer (microRNA) specific	0.5
Universal PCR primer	0.5
Nuclease free water	2.0
Total volume	10.0

4.6. Q-RT-PCR FOR MICRORNA TARGET'S EXPRESSION LEVEL ANALYSIS

The same RNA samples from transfected PC3 cell line extracts were further used for target gene expression analysis. CDNA synthesis was performed with 500 ng RNA and the reaction mixture is given in the Table 4.8. The reaction conditions are performed according to manufacturer's quantitative rt-PCR protocol in "Transcriptor High Fidelity cDNA Synthesis Kit" by Roche.

Table 4.8. cDNA-synthesis mix for target gene quantification

Components	Volume (µL) per rxn
Total RNA (500ng)	depends on sample
Random hexamer primer	2.0
Nuclease-free water	Up to 9.4
5 X RT-reacition buffer	4.0
Protector RNase inhibitor	0.5
dNTP mix	2.0
DTT	1.0
RT – enzyme	1.1
Total Volume	20.0

The expressional analysis was performed with real-time PCR SYBR Green method. SEMA3A and SOX9 genes as more possible putative targets were analyzed with absolute expression real-time PCR. Other genes which are CTNNBIP1, ITGB8, OCT4, SOX2, and KLF4 were analyzed by relative quantification. The housekeeping gene HPRT1 was used as internal control gene. Before real-time PCR experiment all the primers for the quantifications of these genes were tested by gradient PCR to find the most efficient annealing temperature and it is determined as 60ºC. The real-time PCR mixture and conditions were given in Table 4.9.

Table 4.9. rt-qPCR mix for target gene quantification by Roche SYBR Green 1 Master

Components	Volume (µL) per rxn
Diluted cDNA template (1/5 dilution)	2.5
Nuclease-free water	1.5
Primer Forward and Reverse	1.0
2X Master Mix	5.0
Total volume	10.0

5. RESULTS AND DISCUSSION

5.1. IN SILICO MICRORNA TARGET PREDICTION

The common target genes, predicted by the algorithms, are given in Table 5.1 below. The "*" marked ones are also validated in embryonic stem cells as target genes of miR-145 [126]. The genes underlined are investigated and the results are provided.

Table 5.1. The common putative target genes of hsa-miR-145 predicted in silico by algorithms

CTNNBIP1	RNF170	ATXN2
HHEX	RTKN	C6orf115
ITGB8	SCAMP3	CAPZB
LENG8	SEMA3A	SOX9
LOX	SEMA6A	KLF4*
PLCL2	SOCS7	OCT4*
RGS7	SRGAP1	SOX2*
ZBTB10	ACTG1	ACTNB

From the list, CTNNBIP1, ITGB8, SEMA3A, SOX9, KLF4, OCT4, and SOX2 were chosen for further qPCR analysis. The main reason for choosing them is about their functions that would be associated with cancer genetics. Moreover, KLF4, OCT4, and SOX2 are stem cell markers and their down-regulation during embryonic stem cell differentiation was associated with up-regulation of miR-145 [126].

CTNNBIP1 protein binds CTNNB1 and prevents its interaction with TCF family members. It also regulates Wnt signaling pathway. ITGB8 is a membrane protein forming an integrin complex to mediate cell-to-cell and cell-to-extracellular matrix interaction. SEMA3A protein has immunoglobin-like domain and semaphorinE domain. SEMA3A protein is both associated with human tumor cells and also progression of Alzheimer's

disease. Semaphorine3a pathway is also differentially expressed in prostatic cancer [127]. SOX9 is a transcription factor and its elevated expression is associated with prostate cancer [128]. For these reasons, we have analyzed these genes' transcript level after miR-145 transfection in vitro.

5.2. EXPRESSION OF HSA-MIR-145 IN PROSTATE CANCER CELL LINES

Before initiating the investigation, the down-regulation of miR-145 in prostate cancer cell lines was validated. The Figure 5.1 shows the relative expression of miR-145 in prostate cancer cell lines. Hsa-miR-145 has almost similar levels - independent of androgen dependence – in prostate cancer cell lines and its expression is strictly higher in normal prostate epithelial cell line.

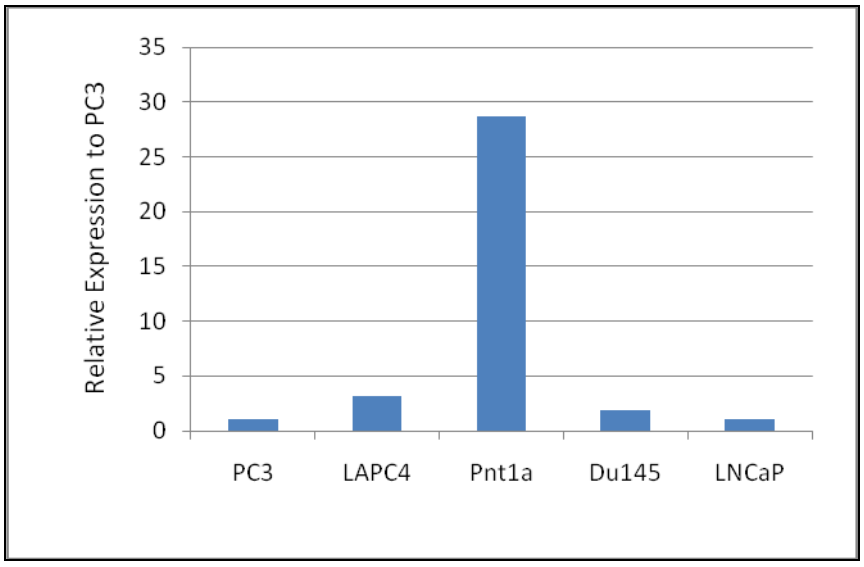

Figure 5.1. The relative expression of hsa-miR145 between prostate cancer cell lines and immortalized prostate epithelial cell line (PNT1a).

The transcript level of the miR-143, which is expressed from the same cluster with miR-145, was also measured with qPCR, but no difference was noted. The Figure 5.2 shows the relative miR-143 expression in prostate cancer cell lines.

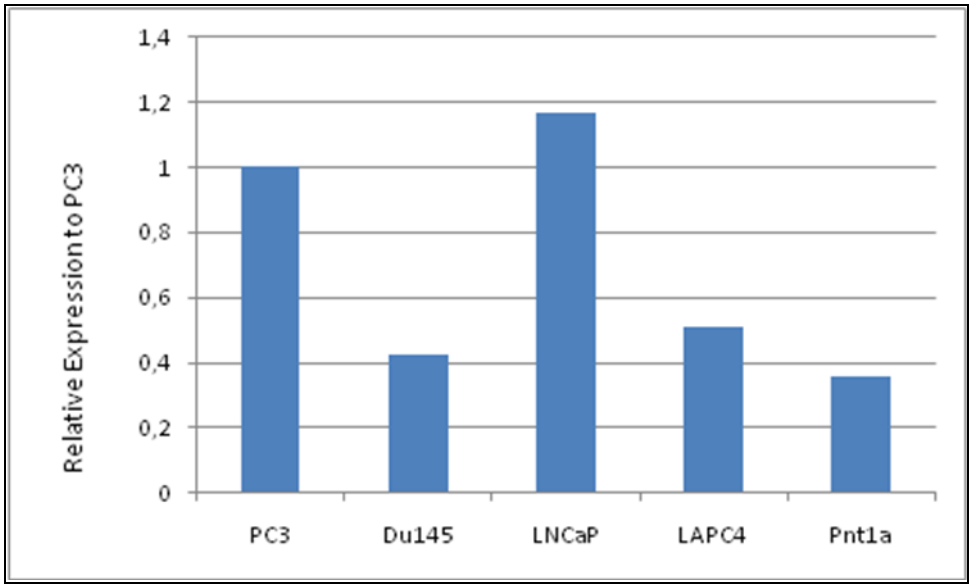

Figure 5.2. The relative expression of hsa-miR143 between prostate cancer cell lines and immortalized prostate epithelial cell line (PNT1a).

5.3. VERIFICATION OF MICRORNA TRANSFECTION

After the transfection of miR-145 precursor in prostate cancer cell lines, presence of mature miR-145 molecules and the duration of their stability were analyzed with microRNA qPCR. The results are given in the Figure 5.3 below.

It was observed that after transfection of precursor-miR-145, mature miR-145 molecules are produced and degraded gradually in 3 days. After 72 hours of incubation, mature miR-145 molecules are very few, thus possibly have less functions on cell metabolism. It is interesting that PC3 and DU145 cell lines degrade mature-miR-145 more rapidly than LNCaP or the miR-145 molecules are more stable in LNCaP cell lines.

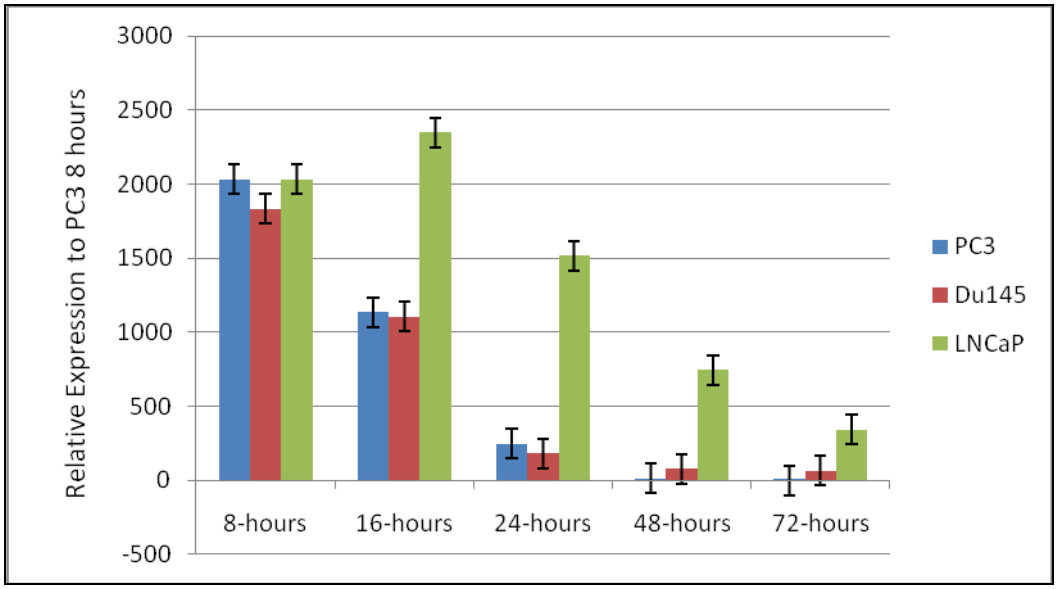

Figure 5.3. The relative expression of miR-145 in transfected prostate cancer cell lines in terms of growth time

5.4. MICRORNA MICROARRAY FOR PROFILING IN RECURRENCE

The recurrence of prostate cancer mostly dependent on small number of cells, since it is observed after removal of tumor cells via chemotherapy or radical prostatectomy. Thus quantifying the expressional alterations is quite difficult as mentioned by Trotman et al. [48].

Our results suggest that there is not a widespread alteration between recurrent and non-recurrent prostate cancer samples. However some microRNAs have significant alteration in expression and they are given in Table 5.2. The number of miRNAs that has altered expression is lower than the chance expected but it is logical that small number of microRNAs is enough for recurrence in cancer.

The analysis of the microarray data was performed by bioconductor. The results include 9 microRNAs. All are indicated in the Table 5.2 . The array data are processed with quantile normalization using bioconductor. The log2 expression values were used for each microRNA and between recurrent and non-recurrent sample groups t-test was

performed. Then the microRNAs that have p-values lower than 0.01 were selected. Out of 15714 spots or features, 93 of them have p-value <0.01. The heat map was shown in Figure 5.4.

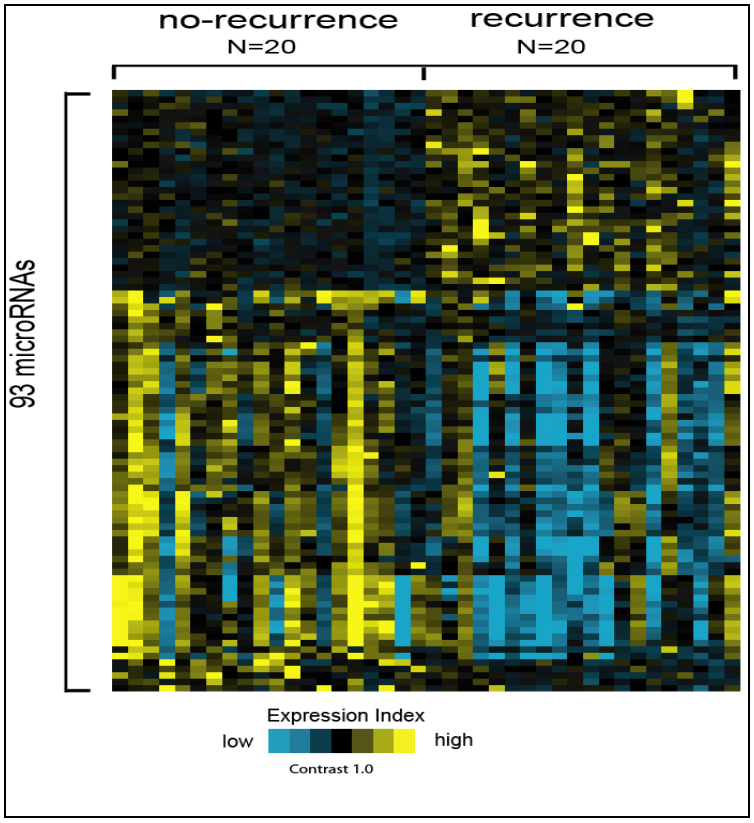

Figure 5.4. MiRNA spots significant with nominal P-value <0.01

When the log-transformed values are averaged and 9 microRNAs were significant with P-value <0.01 (The result is lower than chance expected) The heat map of the miRNAs are given in the Figure 5.5. Some miRNAs are represented by multiple spots. As in the Figure 5.4 the blue color indicates lower expression and the yellow indicates the higher expression.

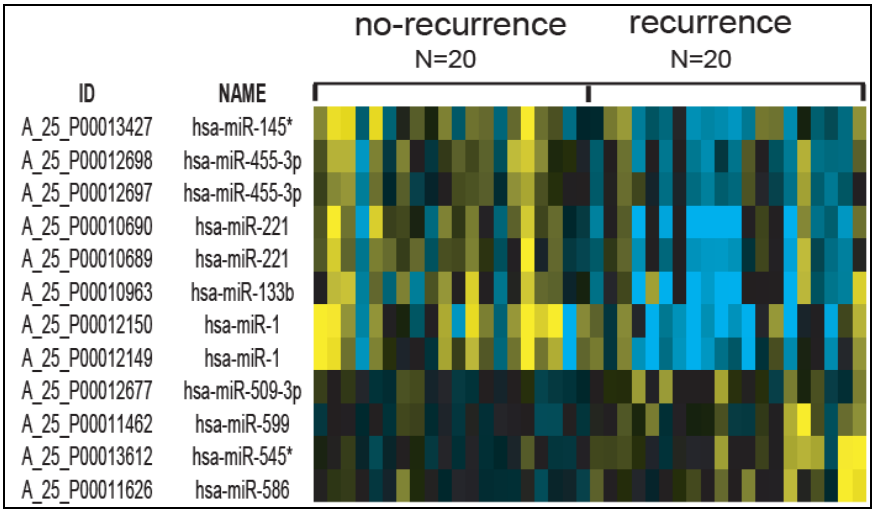

Figure 5.5. Average array spots by probe. The miRNAs that are nominally significant between recurrent and non-recurrent prostate cancer sample groups

Table 5.2. The microRNAs that are differentially expressed in recurrent prostate cancer samples, taken from microarray data

By Bioconductor	Alteration
Hsa-miR-145*	Lower in recurrent
Hsa-miR-455-3p	Lower in recurrent
Hsa-miR-221	Lower in recurrent
Hsa-miR-133b	Lower in recurrent
Hsa-miR-1	Lower in recurrent
Hsa-miR-509-3p	Higher in recurrent
Hsa-miR-599	Higher in recurrent
Hsa-miR-545*	Higher in recurrent
Hsa-miR-586	Higher in recurrent

5.5. EXPRESSION CHECK AFTER ECTOPIC EXPRESION OF MIR-145

Identification of target genes of hsa-miR-145 is crucial for understanding of its role in prostate cancer prognosis and possible treatment options. Interestingly, miR-145 is down-regulated in various tumor types and as described above it may target different genes

in different tissues and cell types. Here we tried to identify the transcripts targeted by hsa-miR145 in prostate cancer cell line PC3 by transfection of miR-145 precursor into the cell line. The transfection and maturation was validated on cell lines PC3, DU145 and LNCaP. The putative targets of miR-145 were determined by bioinformatics algorithms. The Table 5.3 shows the alteration status of predicted genes after ectopic expression miR-145 precursor. The genes that are not altered are also shown in the Table 5.3 and stated as unaltered.

Table 5.3. The expression results of putative target genes after ectopic expression of miR-145 into PC3 cell line

Gene Name	**Expression Analysis**	**Alteration**
SEMA3A	Absolute qPCR	Up-regulated
SOX9	Absolute qPCR	unaltered
CTNNBIP1	Relative qPCR	unaltered
ITGB8	Relative qPCR	unaltered
SOX2	Relative qPCR	Down regulated
KLF4	Relative qPCR	unaltered
OCT4	Relative qPCR	unaltered

The absolute expression analysis for sema3a in transfected PC3 cell line relative to standards is given in the Figure 5.6. The transfection control samples were also included in the qPCR assay and no significant change was observed in their data. As seen in the figure the sema3a transcript level gradually increases until the 48th hour of proliferation and then decreases.

Actually, decrease in sema3a was expected since it might be targeted by miR-145; however, analysis of the un-transfected prostate cancer cell lines and prostate epithelial cell line showed that sema3a level is also higher in PNT1a cell lines in which miR-145 level is also higher relative to prostate cancer cell lines. This data is given in Figure 5.6 This result proposes that sema3a level may be up-regulated together with the increased level of miR-

145 in prostate cancer and vice versa. The up-regulation of sema3a at high levels of miR-145 may be due to two possible mechanisms that are; Mir-145 may target another gene that negatively regulates the sema3a expression thus its down-regulation by miR-145 may cause increase in sema3a level. Another less possible mechanism is about "RNA activation" that is an unproven hypothesis [129]. This hypothesis suggests that microRNAs may bind on promoter regions of some genes and act like transcription factors. In this case miR-145 may bind on SEMA3A promoter and cause induction of its transcription. However, our search with algorithms did not provide positive result. Those algorithms mentioned in section 4.1 search the presence of microRNA binding sites in provided sequences.

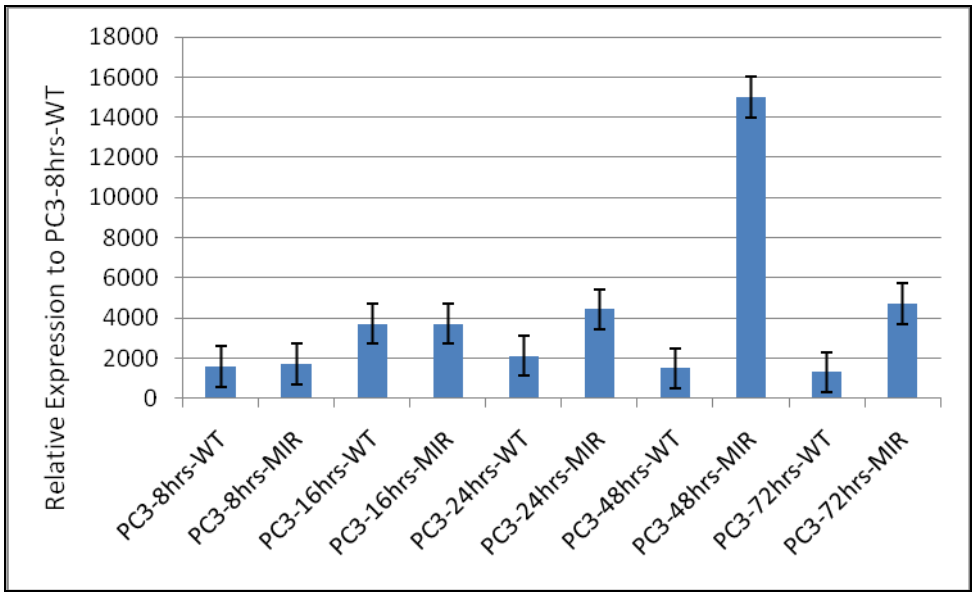

Figure 5.6. The relative expression of sema3a in miR-145 transfected PC3 cell lines

The sox2 is the only predicted positive result which is down regulated in PC3 cell lines after miR-145 transfection. The Figure 5.8 shows the results and since relative expression data for the miR-145 transfected samples are very low their numeric values are given on the bars.

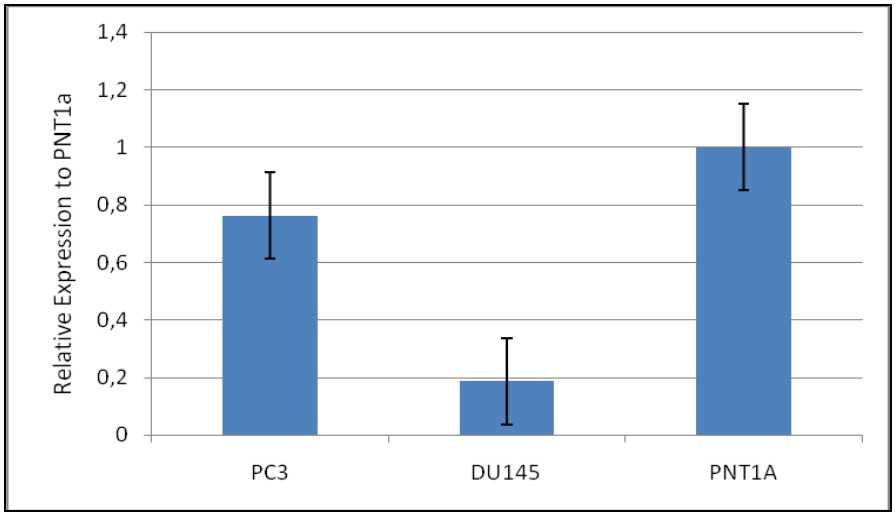

Figure 5.7. The relative expression of sema3a in un-transfected cell lines PC3, DU145, and PNT1a

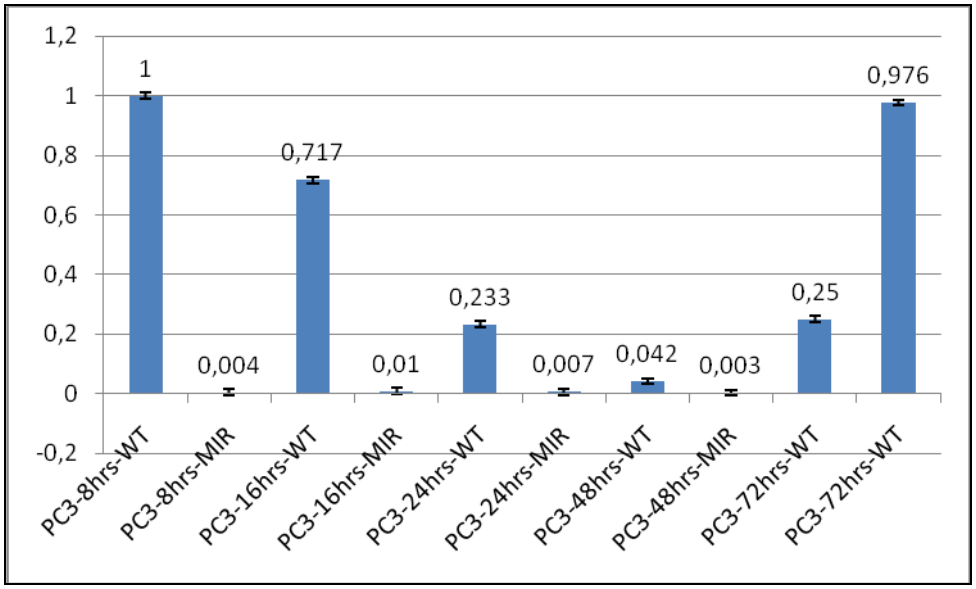

Figure 5.8. The relative expression of sox2 in miR-145 transfected PC3 cell lines

Both the sema3a and sox2 data indicate that 48 hours after transfection is the time of maximum effect. At 72 hours of proliferation after transfection, the cellular functions turn to normal.

6. CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSION

There are limited therapeutic methods against prostate cancer due to its complicated background. MicroRNAs are hopeful targets for prostate cancer therapy but identification of their mechanisms in prostate cancer prognosis is crucial. Additionally, recurrence of prostate cancer is the main problem after surgery. Due to minor group of cells are responsible for recurrence, studies investigating the genetic alterations on the recurrent cells are limited.

In this study, one possible target (SOX2) of miR-145 that is significantly down-regulated in prostate cancer was identified. It has lower expression in miR-145 transfected cell line PC3. Interestingly, sema3a, which was given as a putative target of miR-145 was up-regulated together with the increased level of miR-145 in prostate cancer. Theoretically, one type of microRNA may target hundreds of mRNAs. However, these targets may be cell specific and that miRNA may target different genes in different tissues. Although, our study is not sufficient to understand the whole picture of micrornome, it is significant to identify stem cell marker sox2 as target of tumor suppressor microRNA in prostate cancer. Our findings about miR-145 targets are preliminary data and limited to one cell line of prostate cancer. Thus the investigation will be developed to cover all other prostate cancer cell lines and expression microarray technique will be used to determine global effect of miR-145 ectopic expression.

The second project in our study is determination of differentially expressed microRNAs between recurrent and non-recurrent prostate cancer samples. The radical prostatectomy samples are grouped as recurrent and non-recurrent according to patient's history. The patients mostly suffer from recurrence of cancer after radical prostatectomy surgery. If the tumor initiating cells are understood well, better and more efficient supportive therapies might be achieved to increase one's survival. We have determined 11 micrRNAs which have possible have role in recurring cancer. Any of them have not been

previously proposed in prostate cancer profiling. Thus their putative role in recurrence is logical. MiR-145* is the most interesting one among them since we have already know that miR-145 decreases in prostate cancer cells. The validation of these microRNAs will unravel the idea about functional passenger strands of microRNAs – in this case miR-145* - and mysterious recurrence of prostate cancer.

6.2. RECOMMENDATION

For the further studies expression microarray can be performed on miR-145 transfected prostate cancer cells together with PNT1A cell to determine the cancer specific targets. Furthermore expression array can also be performed for determination of other microRNAs' global effect after transfection. For therapeutic applications, multi-microRNA transfection studies can be done and functional effects can be observed.

To be able to determine the microRNAs having role in recurrence, prostate cancer stem cells or circulating tumor cells can be isolated and their microRNA profiles can be compared with initial prostate tumor. Thus, the microRNA content of the cells that have tumor initiating capacity can be determined.

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