AN INVESTIGATION OF HNRNPA1 FUNCTIONS IN BREAST CANCER

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ABSTRACT

AN INVESTIGATION OF HNRNPA1 FUNCTIONS IN BREAST CANCER

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Master of Science, Molecular Biology and Genetics
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Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) is an RNA-binding protein with a broad range of functions including transcriptional and translational regulation of mRNAs, transport of mRNAs from the nucleus, alternative splicing, telomere maintenance, and miRNA processing. Among all, miRNA related functions of HNRNPA1 are the least understood. Hence, in this study, we aim to investigate potential miRNAs regulated by HNRNPA1.Using HNRNPA1-silenced MCF7 cells, we identified several notable miRNAs using NanoString technology that responded to the loss of HNRNPA1. One of these miRNAs was mir-21, an oncogenic miRNA, that is significantly downregulated in response to loss of HNRNPA1. Furthermore, CRISPR/Cas9 deletion of HNRNPA1 showed downregulated pri-mir-21 levels with little to no change in the host gene (VMP1) expression. Three different mRNA targets of mir-21(STAT3, BCL2, and PDCD4) also showed upregulation upon HNRNPA1 deletion. Mir-21 is a well-accepted oncogenic miRNA that has potential diagnostic and therapeutic roles in many different cancers. Therefore, understanding the regulatory mechanisms behind mir-21 levels in cancer is of interest. Results suggest that HNRNPA1 may regulate the mir-21 levels in breast cancer cell line MCF7. Future studies will provide a more detailed mechanistic explanation.

Keywords: HNRNPA1, miRNA, Breast Cancer

ÖZ

MEME KANSERİNDE HNRNPA1 FONKSİYONLARININ İNCELENMESİ

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Heterojen nükleer ribonükleoprotein A1 (HNRNPA1) geniş çaplı fonksiyonları olan bir RNA-bağlayıcı proteindir. Fonksiyonları arasında transkripsiyonal ve translasyonel mRNA kontrolü, nükleustan mRNA transferi, alternatif splicing, telomer bakımı, ve mikro RNA işlenmesi bulunur. Mikro RNA işlenmesi, bu fonksiyonlar arasında en az anlaşılan ve çalışan görevidir. Bu çalışmada, HNRNPA1 tarafından regüle edilen diğer potansiyel mikro RNA'ları bulmayı amaçlamaktayız. MCF7 hücrelerinde HNRNPA1 shRNA metoduyla susturulmuş ve bu hücrelere NanoString teknolojisi uygulanmıştır. Sonuç olarak çok fazla sayıda mikro RNA'nın seviyesi değişmiştir. Bunlardan biri, mir-21, bir onkomirdir ve HNRNPA1'nin hücrede kaybı bu mikro RNA'nın gen ifadesinin düşmesine yol açmıştır. Üstelik, CRISPR/Cas9 metoduyla silinmiş HNRNPA1 geni, VMP1 geninin seviyesini önemli bir şekilde değiştirmezken, pri-mir-21 seviyesinin düşmesine yol açmıştır. Aynı hücrelerde, üç farklı mir-21 hedef mRNA'sının (STAT3, BCL2 ve PDCD4) ifadelerinde artış gözlemlenmiştir. Mir-21 birden farklı kanser türünde tanısal ve tedavisel potansiyeli olan bir onkojenik mikro RNA'dır. Bundan dolayı, mir-21'in kanserde regülasyon yolaklarının açıklanması büyük önem arz etmektedir. Bu bulgular, HNRNPA1 proteininin meme kanseri hücre hattı MCF7'de mir-21 ifadesini

regüle edebileceğini göstermektedir. Gelecek çalışmalar bu mekanizmanın açıklanmasında daha çok detay sağlayacaktır.

Anahtar Kelimeler: HNRNPA1, Mikro RNA, Meme Kanseri

to all curious minds

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LIST OF ABBREVIATIONS

RT-qPCR	Real Time quantitative Polymerase Chain Reaction
cDNA	Complementary RNA
shRNA	short-hairpin RNA
poly(A)	Polyadenylation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
miRNA	micro RNA

LIST OF SYMBOLS

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0
HNRNPA1	Heterogeneous Nuclear Ribonucleoprotein A1
PDCD4	Programmed Cell Death 4
STAT3	Signal Transducer and Activator of Transcription 3
BCL-2	BCL2 Apoptosis Regulator

CHAPTER 1

INTRODUCTION

1.1. HNRNPs (Heterogeneous nuclear ribonucleoproteins)

When an mRNA exits from transcription machinery, it does not stay bare and alone. RNA-binding proteins interact with mRNAs and form ribonucleoprotein complexes (RNPs). One type of RNPs is called Heterogeneous nuclear ribonucleic proteins (HNRNPs), a family of multifunctional proteins with major roles in the fate of mRNAs. There are 20 well-known proteins in this family. They are named alphabetically from A1 (34 kDa) to U (120 kDa) (Gideon Dreyfuss, Matunis, Pinol-Roma, & Burd, 1993). Each of these proteins has more than one function in different cellular pathways and mechanisms. That is the reason why there are called "multifunctional proteins". HNRNPs function in both nucleus and cytoplasm (Matthew Michael, Choi, & Dreyfuss, 1995).When hNRNPs bind to the mRNA and form HNRNP complexes, they participate in processes like mRNA transport across the nuclear membrane, regulating mRNA stability, and alternative splicing (N. Han, Li, & Zhang, 2013).In addition to the roles in determining mRNA fate, HNRNPs also function in telomere biogenesis, DNA repair, cell signaling, and micro RNA maturation (Jean-Philippe, Paz, & Caputi, 2013). This wide range of functionality expresses the importance of HNRNPs in both health and diseases.

1.2. HNRNPA1 (Heterogeneous nuclear ribonucleoprotein A1)

There are common HNRNPs (A1, A2, B1, B2, C1, and C2) with high expression levels compared to other members of the family in eukaryotic cells (G Dreyfuss, Swanson, & Piñol-Roma, 1988). HNRNPA1, as one of those HNRNPs, is expressed in all types of tissues in the human body (Uhlen et al., 2010). With this conserved expression pattern in all tissues, the protein is involved in many aspects of RNA metabolism from

mRNA splicing, export, and stability to telomere maintenance and miRNA processing (Piñol-Roma & Dreyfuss, 1992).

1.2.1. Gene, Isoforms and Localization

The HNRNPA1 gene is located in the plus strand of chromosome 12 q13.13 with a size of 6896 bp in homo sapiens (GRCh38/hg38) (Kent et al., 2002). The gene is conserved across many species from yeast to primates (Burd & Dreyfuss, 1994; Lau et al., 2000).

It has two experimentally supported mRNA isoforms, called HNRNPA1-A and HNRNPA1-B. The A isoform (320 amino acid, 34 kDa) is the most abundant one with 20 times higher expression value than B isoform (372 amino acid, 38 kDa) in HeLa cells (Buvoli et al., 1990) (Figure 1.1).

The longer isoform(B) has an inclusion of an exon between 252-303 aa region (Buvoli et al., 1990; Piñol-Roma & Dreyfuss, 1992).

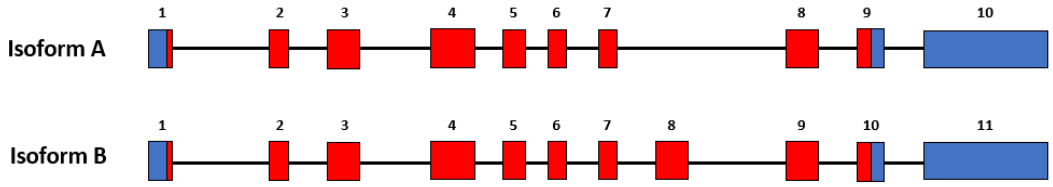

Figure 1.1. Isoforms of HNRNPA1.

HNRNPA1 has two main isoforms. Isoform A is the abundant one with 320 amino acid long and 34 kDA in size. Isoform B has an extra exon (#8) and it is 372 amino acid long and 38 kDA in size. Numbers indicate the exon number for each HNRNPA1 isoform.

HNRNPA1 protein is primarily localized in the nucleus. However, while functioning in mRNA export, HNRNPA1 protein shuttles between the nucleus and cytoplasm (Piñol-Roma & Dreyfuss, 1992). Subcellular localization of the protein depends on

the physiological state of the cell. In early mouse embryos where transcription activity is high, HNRNPA1 is primarily localized in the nucleus. When transcription levels slow down, HNRNPA1 can be found both in the nucleus and the cytoplasm (Pettit Kneller, Connor, & Lyles, 2009).

1.2.2. Structure of HNRNPA1 Protein

HNRPA1 has an amino(N)-terminal RRM (RNA recognition motif) and one carboxyl-terminal Gly-rich (RGG) regulatory domain (Y. He & Smith, 2009). RRMs are evolutionarily conserved sequences shared by many different RNA-binding proteins. HNRNPA1 utilizes the RRM in alternative splicing and RNA-protein interactions (Mayeda, Munroe, Xu, & Krainer, 1998).

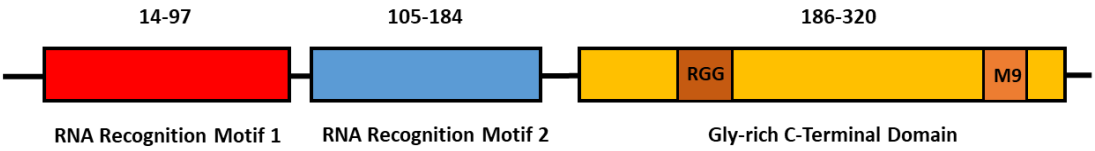

Figure 1.2. Domains of HNRNPA1.

From N to C terminus: RRM1, RRM2 and Gly-rich Domain. In the Gly-rich domain, there are RGG and M9 motifs. Numbers indicate the start and end amino acid number for each domain.

The C-terminus RGG box that contains Arg-Gly-Gly repeats to mediate protein-protein interactions, RNA binding, RNA annealing activity and alternative splicing (Nadler et al., 1991). Carboxyl-terminal is also where nuclear localization and nuclear export signals (called “M9”) are located. This M9 sequence is required for the shuttling of HNRNPA1 between nucleus and cytoplasm during mRNA transport (Siomi & Dreyfuss, 1995).

1.2.3. Disease Relevance

Given these multiple functions in gene expression and other cellular processes, HNRNPA1 has been implicated in various disease etiologies.

HNRNPA1 has key roles in neuron metabolism and any change in the abundance of HNRNPA1 leads to major effects on RNA metabolism in neurons. Therefore, HNRNPA1 has been associated with amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Alzheimer's disease (AD), spinal muscular atrophy (SMA) and several other neurodegenerative diseases (Bekenstein & Soreq, 2013).

HNRNPA1 gene is over-expressed in many different cancers like lung (Boukakis, Patrinou-Georgoula, Lekarakou, Valavanis, & Guialis, 2010), advanced gastric cancer (Y. Chen et al., 2018), and colorectal cancer (Ushigome et al., 2005). HNRNPA1 promotes tumor invasion in hepatocellular carcinoma (Zhou et al., 2013). High expression of HNRNPA1 in gastric cancer induces EMT (Epithelial to Mesenchymal Transition) and increases invasion potential (Y. Chen et al., 2018). In breast cancer, knockdown of HNRNPA1 leads to decreased cell invasion of MDA-MB-231 cells (Loh et al., 2015). Expression of HNRNPA1 has anti-apoptotic outcomes and siRNA mediated knockdown of HNRNPA1 leads to apoptosis in cancer cells (Patry et al., 2003). HNRNPA1 may regulate caspase-2 pre-mRNA alternative splicing and leading to suppression of apoptosis (Jiang, Zhang, Yi, & Wu, 1998). Overall, current findings highlight the oncogenic potential of HNRNPA1 in different cancers.

1.2.4. Functions

As a multifunctional RNA-binding protein, HNRNPA1 exhibits its activity in a wide range of cellular functions. These include transcriptional and translational regulation of mRNAs, transport of mRNAs from the nucleus, alternative splicing, telomere maintenance, and miRNA processing (Jean-Philippe et al., 2013).

1.2.4.1. Transcription

Apart from being an RNA-binding protein, HNRNPA1 can also interact with single and double-stranded DNA (Dickey, Altschuler, & Wuttke, 2013; Fukuda et al., 2002). The protein is known to interact with promoters of different genes. The effect on gene expression can be positive or negative. For instance, Vitamin D receptor (VDR), γ-fibrinogen (FGG) and thymidine kinase (TK) promoter activities are reduced while apolipoprotein E(ApoE) and Protein kinase R(PKR) promoters are activated upon HNRNPA1 association with the corresponding promoter (Campillos et al., 2003). The precise mechanism of action of HNRNPA1 is unknown; however, it is known that Hnrnpa1 has the ability to bind and destabilize G-quadruplex DNA, which facilitates transcription. In addition, HNRNPA1 has a direct impact on transcription by binding to co-regulator proteins. For example, activation of NF-κB is positively affected by the interaction of HNRNPA1 with the inhibitor of NF-κB (IκBα) leading to the degradation of the transcriptional inhibitor (Hay, Kemp, Dargemont, & Hay, 2001) (Hay et al., 2001).

1.2.4.2. Translation and mRNA Turnover

HNRNPA1 binds to internal ribosomal entry sites (IRES) and facilitates IRES-mediated translation of sterol-regulatory-element-binding protein (SREBP-1) (Damiano et al., 2013), human rhinovirus (HRV) (Cammas et al., 2007), and human fibroblast growth factor 2 (FGF-2) mRNAs (Bonnal et al., 2005). More importantly, it is reported that c-MYC and cyclin D1 mRNA translation is also enhanced via binding of HNRNPA1 to IRES sequences (Jo et al., 2008; Martin et al., 2011). HNRNPA1 also binds to AU-rich elements (ARE) on 3'UTR of the granulocyte-macrophage-colony-stimulating factor (GM-CSF) and interleukin 2 (IL-2) mRNAs and potentially regulates stabilities of these mRNAs (Hamilton, Burns, Nichols, & Rigby, 1997; Hamiltonll, Nagyll, & Rigbyoll, 1994).

1.2.4.3. mRNA Export from the Nucleus

As one of the HNRNP complex proteins, HNRNPA1 carries mature poly(A)+ mRNAs through nuclear pores. This transport is mediated by M9 sequence having both nuclear export and nuclear localization signals. For instance, the binding of HNRNPA1 on BCL-XL and XIAP mRNAs promote their export to the cytoplasm in HEK293 cells (Campillos et al., 2003; Roy et al., 2014).

1.2.4.4. Alternative Splicing

Alternative splicing utilizes different exon-intron acceptor and donor signals and leads to multiple mRNA isoforms with different exon numbers and/or organizations. Small ribonucleoproteins (snRNPs) and SR proteins are the core of the nuclear complexes responsible for alternative splicing (Will & Lührmann, 2011). There are also auxiliary proteins with regulatory roles. HNRNPA1 is one of those regulatory proteins. HNRNPA1 functions as an auxiliary RBP that sometimes compete with other splicing regulators and functions as an inhibitor of splicing (Daubner, Cléry, & Allain, 2013). This inhibition can be established in more than one way. In some cases, HNRNPA1 prevents SR proteins and other regulatory proteins from binding to splice sites by binding to exons. This competition between SR proteins and HNRNPA1 determine the ratio of exon inclusion and exclusion (Rooke, Markovtsov, Cagavi, & Black, 2003). Moreover, there are specific binding sites for HNRNPA/B proteins in introns when bound can block other proteins from binding regulatory regions nearby (Bruun et al., 2016). Phosphorylation of snRNPs can prevent their localization in the nucleus. HNRNPA1 is no exception. In osmotic stress conditions, p38 MAPK kinase (van der Houven van Oordt et al., 2000) and MNK1/2 kinase are known to phosphorylate HNRNPA1 (Guil & Cáceres, 2007), thus regulating the alternative splicing patterns in the cell.

Among HNRNPA1 regulated splicing events, there are insulin receptor gene (INSR, exon 11 skipping), breast cancer 1 (BRCA1, exon 6 & 18 skipping), fibroblast growth

factor receptor 2(FGFR2, K-SAM exon skipping), its own pre-mRNA (exon 7b skipping) and many others listed in Table 1.1 (Jean-Philippe et al., 2013).

Table 1.1. *List of alternative splicing events performed by HNRNPA1*

Table is adapted from Jean-Philippe et al. (2013)

Gene	Organism	Spicing event
Medium-chain acyl-CoA dehydrogenase (MCAD)	Human	Exon 11 skipping
Myelin-associated glycoprotein (MAG)	Human	Exon 12 skipping
Interferon regulatory factor-3 (IRF-3)	Human	Exons 2 and 3 skipping
TNF Receptor Superfamily Member 6 (Fas)	Human	Exon 6 skipping
Ras-related C3 botulinum toxin substrate 1 (Rac1)	Human	Exon 3b skipping
Insulin receptor gene (INSR)	Human	Exon 11 skipping
Breast cancer 1 (BRCA1)	Human	Exon 18 skipping
Breast cancer 1 (BRCA1)	Human	Exon 6 skipping
Homeodomain interacting protein kinase 3 (HIPK3)	Human	Exon skipping
Bovine growth hormone (BGH)	Bovine	Exon 5 skipping
Survival of Motor Neuron 2, (SMN2)	Human	Exon 7 skipping
Fibroblast growth factor receptor 2 (FGFR2)	Human	K-SAM exon skipping
Amyloid precursor protein (APP)	Human	Exon 7 and 8 skipping
Dystrophin	Human	Exon 31 skipping
β-tropomyosin	Chicken	Exon 6B skipping
pX region	Human T-cell leukemia virus type 1 (HTLV-1)	Exon skipping
V-Ha-ras Harvey rat sarcoma viral oncogene homolog (C–H-ras)	Human	Exon IDX skipping
Proto-oncogene tyrosine-protein kinase Src (c-SRC)	Human	Exon N1 skipping
Trans-activator of transcription (Tat)	Human immunodeficiency virus type 1 (HIV-1)	Exon 3 3′ ss repression
Trans-activator of transcription (Tat)	Human immunodeficiency virus type 1 (HIV-1)	Exon 2 3′ ss repression
Carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1)	Human	Exon 7 skipping
heterogeneous ribonucleoprotein A1 (hnRNP A1)	Human	Exon 7B skipping
Pyruvate kinase (PKM)	Human	Exon 9 skipping
Viral protein R (VPR)	Human immunodeficiency virus type 1 (HIV-1)	Repression 3′ splice site A2
E6/E7	Human papillomavirus type-16 (HPV-16)	E6 exon skipping

1.2.4.5. Telomere Maintenance

Telomeres have guanosine-rich repeats at protruding 3' ends which protects chromosomes from degradation. Having a consensus RNA-binding sequence (UAGGGA/U) similar to vertebrate telomeric repeats, HNRNPA1 interacts with both human telomerase and telomeres in vitro. In fact, HNRNPA1 unwinds the G-quadruplex structures at telomere ends and facilitates the telomere elongation (Zhang, Manche, Xu, & Krainer, 2006). Binding of A1 to G-quadruplex of telomeres is shown to be both structure and sequence-dependent (X. Liu & Xu, 2018).

1.2.4.6. miRNA Processing

As of today, there are only two identified microRNAs that are regulated by HNRNPA1 by directly interacting with miRNA stem-loops. First one is mir-18a. HNRNPA1 facilitates the maturation of pri-mir-18a by binding to its terminal loop and changing the conformation of the stem-loop structure. This change leads to more effective Drosha cleavage resulting in an increase in mature mir-18a levels (Michlewski, Guil, & Cáceres, 2010). This miRNA functions as an oncogene in lung cancer, by targeting interferon regulatory factor 2 (IRF2) (Sun et al., 2017). Hence, circulating levels of mir-18a is a potential biomarker of different cancer types, like esophageal, pancreatic, hepatocellular, colorectal and others (Morimura et al., 2011). These findings correlate with the potential oncogenic features of HNRNPA1.

Second miRNA that is affected by HNRNPA1 is let-7a. Biogenesis of let-7a is negatively regulated by HNRNPA1 activity. Unlike the positive effect on pri-mir-18a processing, binding of HNRNPA1 to the conserved terminal loop of pri-let-7a-1 inhibits the miRNA processing by Drosha, leading to decreased mature let-7a levels (Michlewski & Cáceres, 2010). Let-7a also inhibits the growth and migration rates of breast cancer cells (K. Liu et al., 2015). Furthermore, through the downregulation of C-C chemokine receptor type 7 (CCR7), let-7a inhibits the migration and invasion of breast cancer cell line MDA-MB-231 (S.-J. Kim et al., 2012). These findings also support the oncogenic roles of HNRNPA1.

1.3. Micro RNAs (miRNAs)

In the central dogma, genetic information in DNA is first converted to mRNA, then ribosomes use the mRNA to synthesize proteins. This process is tightly regulated in every intermediate step.

One way of regulation is through non-coding RNAs. These RNA molecules do not code proteins, but rather have various functions in cells. Only transfer RNAs(tRNA) and ribosomal RNAs(rRNA) are ncRNAs with direct involvement in protein synthesis. Other non-coding RNAs like long non-coding RNAs (lncRNAs), Piwi-interacting RNAs (piRNAs), Small interfering RNAs (siRNAs), micro RNAs(miRNA), and many others are involved in different cellular processes. Their roles cover RNA splicing (ncRNAs U1-U6 of spliceosome), DNA replication (Y RNAs), gene regulation (enhancer RNAs,miRNAs), genome defense(piRNAs), chromosome(ncRNA of XIST) and telomere structure(Telomere RNA) (O'Brien, Hayder, Zayed, & Peng, 2018).

In this thesis, we are interested in micro RNAs(miRNA), their regulation and functions. Micro RNAs are short (18-22 bp) RNA molecules that regulate the translation of mRNA transcripts.

1.3.1. History

Micro RNAs were first discovered in the early 1990s. However, their significance on gene regulation mechanism was not fully recognized until the early 2000s. First miRNAs were discovered in C. elegans (a nematode) in the early 1990s (Lee, Feinbaum, & Ambros, 1993; Wightman, Ha, & Ruvkun, 1993). Two Lin-4 gene transcripts (22 and 61 nucleotides) were found to be complementary to 3'UTR of lin-14. The lin-14 gene is known to be downregulated in nematode developmental stages. Twenty-two nucleotide long products of the lin-4 gene were proposed to silence the lin-14 gene with direct RNA-RNA interaction. However, this discovery was regarded

as a unique mechanism in Nematodes. Seven years later, another miRNA, let-7 was discovered with a role of inhibition of C. Elegans development (Reinhart et al., 2000). Comparison of let-7 expression in different species revealed that let-7 is conserved from C. Elegans to Homo sapiens (Pasquinelli et al., 2000). After this discovery, miRNA research quickly accelerated. Different sets of miRNAs are found to be expressed in different types of cells and tissues in mouse (Lagos-Quintana et al., 2002) and zebrafish (Wienholds et al., 2005) with functions in different biological processes (C. Z. Chen, Li, Lodish, & Bartel, 2004; Cuellar & McManus, 2005; Wilfred, Wang, & Nelson, 2007). Now, miRNAs are accepted as one of the main gene regulation mechanisms in eukaryotes. There are over 2000 different micro RNAs (2300 true human mature miRNAs, 1115 of which are currently annotated in miRBase V22) (Alles et al., 2019). in humans. They are implicated in various diseases (cancer, liver, autoimmune, neurodevelopmental, cardiovascular and inflammatory diseases) and considered as potential drug targets and diagnostic measures for clinical purposes (Rupaimoole & Slack, 2017).

1.3.2. Biogenesis

Biogenesis of miRNAs starts with the processing of RNA polymerase II/III transcripts (Ha & Kim, 2014). About half of currently discovered miRNA are intragenic, most of them originate from introns and few of them from exons. Remaining miRNAs are intergenic. These miRNAs are transcribed independently from their host genes and are regulated by their own promoters (de Rie et al., 2017). Interestingly, there are miRNAs encoded within long non-coding RNAs (S. He et al., 2008). Some of the intergenic miRNAs are transcribed as clusters in long transcripts and are characterized as miRNA families (Tanzer & Stadler, 2004). There is also a unique type of miRNAs which are located in 3'UTR of protein-coding genes. Examples of this type of miRNA are mir-198 (located on 3'UTR of FRP gene) (Tanaka et al., 1998), mir-BHRF1-1 and mir-BHRF1-2(located on 3'UTR of Epstein-Barr virus BHRF1 gene) (Chien et al., 2004) and mir-21 that is located in immediate downstream of VMP1 gene 3'UTR (Cai et al., 2004).

There are canonical and non-canonical ways of miRNA biogenesis. The canonical pathway starts with the transcription by RNA polymerase II, leading to the synthesis of primary miRNA (pri-miRNA) with a 60-80 bp stem-loop structure (Cai et al., 2004). Drosha-DGCR8 (DiGeorge syndrome critical region 8) recognizes this pri-miRNA and processes it into shorter precursor miRNA(pre-miRNA) (Denli, Tops, Plasterk, Ketting, & Hannon, 2004; Gregory et al., 2004; J. Han et al., 2004; Landthaler, Yalcin, & Tuschl, 2004).This precursor is then shuttled from nucleus to cytoplasm by Exportin 5 (Hutvágner et al., 2001; Lund, Güttinger, Calado, Dahlberg, & Kutay, 2004; Yi, Qin, Macara, & Cullen, 2003). Inside the cytoplasm, by the action of Dicer–TRBP (TAR RNA-binding protein 2), pre-miRNA is cleaved into 18-22bp double-strand mature RNA. One strand of this double-strand miRNA/miRNA* is preferentially associated with Argonaute 2 (AGO2) containing RNA-induced silencing complex (RISC) (Figure 1.3). At this step, while the active strand is associated with AGO2, the other strand (*) is degraded (Gregory et al., 2004; Ruby, Jan, & Bartel, 2007). In the non-canonical pathways, pre-miRNAs are synthesized either via Drosha/DGCR8-independent or Dicer-independent pathways (O'Carroll et al., 2010; Ruby et al., 2007).

1.3.3. Target Recognition and Silencing

miRNAs direct RISC to target mRNAs and suppress target gene expression either by imperfect base pairing leading to translational inhibition or by perfect base pairing inducing mRNA cleavage (Behm-Ansmant, Rehwinkel, & Izaurralde, 2006). Almost all micro RNAs regulate gene expression by binding to specific sequences on 3'UTR of their target mRNAs (Huntzinger & Izaurralde, 2011). However, rare cases of miRNAs targeting coding sequences, 5'UTRs and even on promoter regions have been reported (Xu, San Lucas, Wang, & Liu, 2014). However, the result of 5'UTR and coding sequence binding is still downregulation of target mRNA (let-7 and DICER genes), while promoter binding (miR-324-3p and RelA genes) leads to transcriptional activation (Dharap, Pokrzywa, Murali, Pandi, & Vemuganti, 2013;

Forman, Legesse-Miller, & Coller, 2008). There are very few examples of gene regulation by miRNAs by other means than 3'UTR binding.

Mature miRNAs have a 'seed sequence' at their 5' end that is responsible for binding to target 3'UTR. This seed sequences are 2-8 nucleotides long and their complementarity to the target UTR determines the fate of the target mRNA. If there is perfect complementarity, with the presence of AGO2, target mRNA is recognized and cleaved. However, in imperfect complementarity, which is more common, instead of mRNA cleavage, inhibition of translation occurs. In either way, target binding leads to decreased output from an mRNA (Figure 1.3).

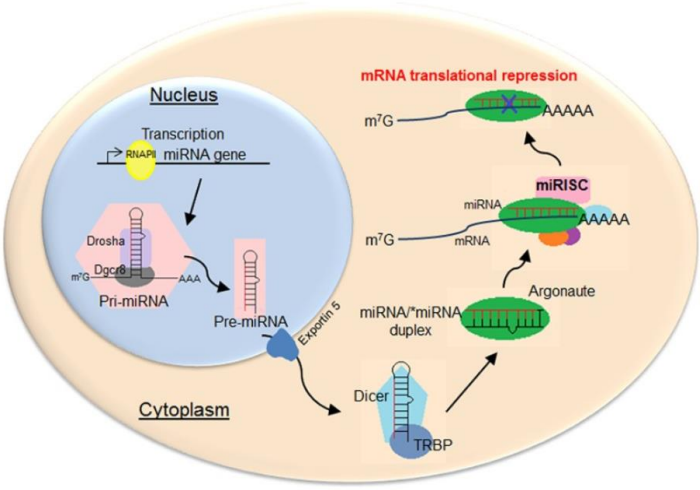

Figure 1.3. Overview of miRNA biogenesis and function.

The biogenesis begins in nucleus with RNA-polymerase II transcribing the capped and polyadenylated pri-miRNA. After that, Drosha and its cofactor Dgcr8 cut pri-miRNA into precursor miRNAs. Pre-miRNAs are transported into cytoplasm by Exportin 5, then Dicer processes precursor miRNAs and generate mature miRNA. This mature miRNA associates with miRNA-induced silencing complex and binds to its target sequence on mRNAs, resulting in post-transcriptional gene silencing. The figure is taken from (Hajarnis, Lakhia, & Patel, 2015).

1.3.4. Regulation

The important role of miRNAs in control of gene expression and organismal development give rise to tight control of miRNA biogenesis. Indeed, many different steps of miRNA biogenesis can be subject to regulation. While changes in gene expression and hypermethylation of promoters are considered as transcriptional level regulation methods, change in miRNA stability and processing are considered as a post-transcriptional regulation mechanism (V. N. Kim, Han, & Siomi, 2009; Krol, Loedige, & Filipowicz, 2010). Any significant change in the expression level and activity of proteins in miRNA maturation machinery (DROSHA, DICER, Exportins and Argonaute proteins) has also a significant effect on global miRNA activity (Y. K. Kim, Kim, & Kim, 2016). The complex multilevel miRNA regulation mechanisms depend on the type of the cell, cellular state and even on internal (cytokines, hormones) and external stimuli (Gulyaeva & Kushlinskiy, 2016). Deregulation of miRNA synthesis can change global gene expression patterns and thus the state of the cells, leading to diseases, particularly cancer. For example, degenerate processing of miRNAs induces tumorigenesis and cellular transformation (Kumar, Lu, Mercer, Golub, & Jacks, 2007). It is also known that global deregulation of miRNAs is considered as one of the hallmarks of cancer (Hata & Lieberman, 2015).

1.3.4.1. Transcriptional Regulation

At the transcriptional level, miRNA expression changes depending on the regulation of host gene (intragenic miRNAs), or their own promoter sequences (intergenic miRNAs).

In the human genome, genes hosting a miRNA likely to have more introns. These genes also have longer 5’ introns (where 60% of intronic miRNAs are located) and sized three times longer than randomly sampled genes. Furthermore, their 3’UTRs are longer and have more AU-rich elements (ARE) compared to genes with no miRNA genes. It is also predicted that almost 20% of intragenic miRNAs target their host transcripts and that they may be functional in the same pathways (Hinske, Galante,

Kuo, & Ohno-Machado, 2010). All these data suggest that the miRNA and host gene expression might be co-regulated.

Transcription of intragenic miRNAs is in parallel with their host gene expression (Rodriguez, Griffiths-Jones, Ashurst, & Bradley, 2004). Potential co-regulation of miRNAs and their host genes is still open to question. In any case, it is apparent that the regulation of miRNA host gene expression directly affects the availability of those miRNAs in the cell.

Having their own promoters, intergenic miRNA genes are directly regulated by transcription factors and other regulatory proteins. Just like protein-coding genes, miRNA promoters have CpG islands, increased H3K4me3 and decreased H3K27me3 marks when miRNAs are transcribed (Corcoran et al., 2009; G. Wang et al., 2010). Supporting the role of promoter sequences, any slightest change in the promoter region of miRNAs may significantly affect the miRNA expression. For example, an SNP (rs767649) in the promoter region of mir-155 dramatically reduces the expression rate thus decrease the chance of intracranial aneurysm rupture in the brain (Yang, Peng, Pang, Wan, & Chen, 2019). Another example would be regulation over the mir-17-92 cluster, as a potential oncogene. Two different polymorphisms (rs9588884 and rs982873) in the promoter region of mir-17-92 decrease the risk of colorectal cancer (Sun et al., 2017). SREBF1(Sterol Regulatory Element Binding Transcription Factor 1) and PPARG (Peroxisome proliferator-activated receptor gamma) binding to mir-26b promoter dramatically upregulate the mir-26b expression through DNA methylation in mammary epithelial cells (H. Wang et al., 2017).

In addition to promoters, enhancer sequences do exit for miRNA genes as well. For example, miR-200b~200a~429 has an upstream enhancer when stimulated it increases the transcription rate by 27-fold in breast epithelial cells (Attema et al., 2013).

For both intragenic and intergenic miRNAs, there is also regulation over promoter methylation. Hypermethylation of mir-132 decreases its expression in colorectal cancer, leading to an increase in cell invasion (Qin et al., 2015). Again, the mir-210

promoter is hypermethylated and downregulated. Mir-33b acts as a tumor suppressor in gastric cancer, yet increased methylation in the upstream CpG islands decreases its expression (Yin et al., 2016). The promoter region of mir-31 is hypermethylated in TNBCs (Triple-Negative Breast Cancer) which is normally inhibits migration and invasion (Augoff, McCue, Plow, & Sossey-Alaoui, 2012).

Following transcription, there are additional ways of controlling miRNA function.

1.3.4.2. miRNA Stability and Turnover

Stability of miRNAs is one of the methods to control the miRNA activity in cells. Depending on the developmental stage and cell type, mature miRNA stabilities change. One way to establish this control is through the addition of a few nucleotides to 3' end of miRNAs. These nucleotides are generally adenosines and increase miRNA stability just like a poly(A) tail for mRNAs (Towler, Jones, & Newbury, 2015). Mir-122 stability in the liver is established via the selective addition of single adenosine to 3' by poly(A) polymerase GLD2 (Katoh et al., 2009). Multiple different uridyl transferases (TUT1, TUT2, TUT3, ZCCHC6, and ZCCHC11) also participate in miRNA 3' tailing. However, this time activity of miRNA is affected, not stability. Both in human(A549) and mouse (MLE-15) cell lines, mir-26 uridylation by ZCCHC11 results in decreased mir-26 activity and enhanced target gene (IL6) expression (Jones et al., 2009). Some miRNAs possess intrinsic sequences, like AU/UA, for their regulation. High density of these dinucleotides on miRNAs directly correlates with short miRNA half-life in human neuronal cells (Sethi & Lukiw, 2009). There are also ribonucleases which specifically degrade certain miRNAs. For instance, XRN1 affects the stability of let-7, miR-57, miR-59, miR-235, miR-241 in C.elegan (Bossé et al., 2013) and mir-382 in human HEK293 cells (Bail et al., 2010), directly.

1.3.4.3. Regulation by RNA-binding Proteins

Besides the RNA-binding proteins (RBPs) in core miRNA processing machinery (DROSHA, DGCR8, DICER and AGO) there are other RBPs that can regulate the

stability of miRNAs. GW182(Glycine-Tryptophan Protein Of 182 KDa) and AGO2(Argonaute RISC Catalytic Component 2) has found to protect miRNA from degradation in mouse embryonic fibroblast (Diederichs & Haber, 2007; Winter & Diederichs, 2011; Yao, La, Chen, Chang, & Chan, 2012).This increased stability is most likely because 5' and 3' ends of miRNAs are protected from nucleases due to GW182 and AGO2 interaction with miRNAs. In addition, Translin, another RBP, is shown to bind mir-122a and increases miRNA stability in mouse testis (Yu & Hecht, 2008).

There are some RBPs, which can bind to the terminal loop or stem of miRNAs progenitors and affect the fate of the miRNA positively or negatively. This binding can affect the pri-miRNA processing in nucleus or pre-miRNA processing in the cytoplasm. LIN28B, for example, interact with primary and precursor let7 family of miRNAs and recruits TUT4 and TUT7 (uridyltransferases), causing degradation of miRNAs (Heo et al., 2009).

Nuclear factor (NF) 90/45 can bind to stem-loop of pri-let7a-1, pri-mir-21, and pri-mir-15a and inhibits Drosha processing (Sakamoto et al., 2009). Another regulation over mir-21 is established by SMAD where pri-mir-21 processing is advanced with the association of SMAD with Drosha, p53 and pri-mir-21 simultaneously (Davis, Hilyard, Lagna, & Hata, 2008).

HNRNPA1 and KSRP regulate let-7a processing. While KSRP promotes let-7a biogenesis, binding of HNRNPA1 to the conserved terminal loop of pri-let-7a-1 prevents miRNA processing by Drosha and leads to decreased mature let-7a levels (Michlewski & Cáceres, 2010). HNRNPA1 also binds to mir-18a terminal loop and change the stem-loop conformation, facilitating better cleavage by Drosha (Michlewski et al., 2010).

There is also indirect regulation of miRNA processing over miRNA biogenesis machinery. Impaired pre-miRNA export to the cytoplasm, post-translational regulation of AGO proteins, degenerate Drosha or Dicer activity and many other

similar events lead to a change in mature miRNA output in cells (Behm-Ansmant et al., 2006; Kawahara, 2014). As a hormonal regulation, Estrogen is the most studied for its relationship with miRNA processing. In the presence of E2(ligand), ER-alpha can inhibit miRNA processing. This regulation is manifested through direct binding of ER-alpha(N-terminus) to Drosha(C-terminus) and followed by dissociation of Drosha from pri-miRNAs (Yamagata et al., 2009). In contrast to ER-alpha, TGF-β and BMP signaling pathways facilitate Drosha-mediated miRNA processing in human muscle cells (Davis et al., 2008).

1.4. miR-21

Hsa-mir-21 is one of the first identified miRNAs in humans (Lagos-Quintana et al., 2002) and it is discernibly conserved across mammalian species (Figure 1.4). It is also the most commonly expressed miRNA in many different cancer types (Iorio Marilena V. et al., 2007). MIR21 is located on the long arm of chromosome 17 that is amplified in many cancers (Feng & Tsao, 2016). Deregulation of mir-21 is associated with various cardiovascular, inflammatory, and developmental diseases and of course with different cancers (Feng & Tsao, 2016). It is classified as an oncogenic miRNA (oncomiR) (Folini et al., 2010). That is why understanding the regulation of mir-21 and its relationship with its host gene (VMP1) is crucial.

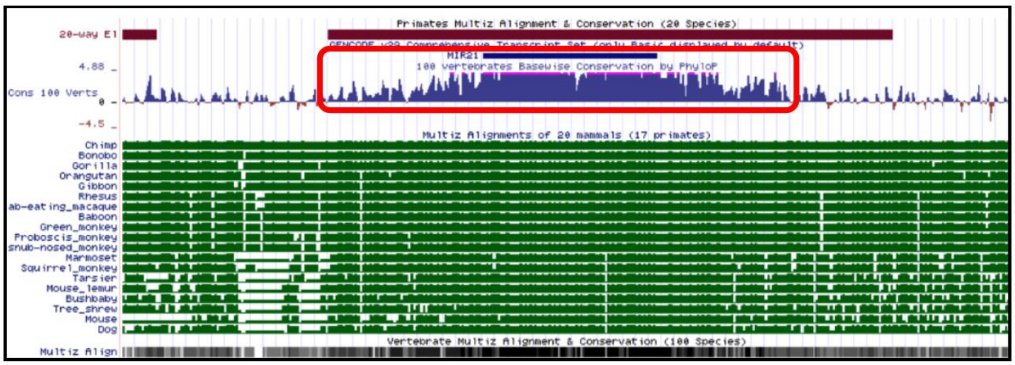

Figure 1.4. Conservation of mir-21 across mammalian species.

Continuous green lines blue peaks indicate higher similarity. Red region shows the highly conserved mir-21 region. In the green area, conservation among 20 different vertebrates is listed. On the bottom, conservation across 100 different species is indicated with shades or gray. Darker shades show high similarity. The image is taken from UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly.

There are two sources for mir-21 expression:

1) At first glance, it is logical to assume that mir-21 expression depends on the promoter of its host gene, VMP1. However, VMP1 gene is usually polyadenylated up to 194bp upstream of mir-21 hairpin (red star in figure 1.5); meaning that canonical VMP1 transcripts do not include mir-21 hairpin. Transcriptional regulation of pri-mir-21 transcript is predominantly independent of VMP1 (Z. Wang, 2012).

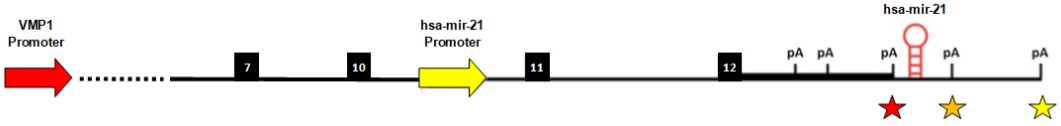

Figure 1.5. Overview of VMP1 and mir-21 locus with polyadenylation signals.

VMP1 promoter (red arrow) and mir-21 promoter (yellow arrow) is shown, Black boxes indicate the exons and their numbers. VMP1 transcript is able to utilize the third poly(A) site from left (red star). VMP-mir-21 transcript exclusively uses second poly(A) from left (orange star). mir-21 transcript exclusively uses the last poly(A) site (yellow star).

However, there is an alternative polyadenylation variant of VMP1 that extends beyond mir-21 hairpin. This less frequent source of mir-21 is called VMP1-mir-21 transcript (Z. Wang, 2012). Unlike the non-spliced pri-mir-21 transcript, VMP1-mir-21 transcript utilizes the proximal poly(A) site (4th from left, right after mir-21 hairpin,orange star in figure 1.5) (Z. Wang, 2012). Moreover, how the first three poly(A) sites that are readily utilized by VMP1 is skipped to produce non-spliced mir-21 transcript is still a matter of debate.

2) Mir-21 has its own promoter that is in the 10th intron of Vacuole Membrane Protein 1(VMP1) (Fujita et al., 2008). This mechanism results in the primary mir-21 transcript that is not spliced and extends through rightmost poly(A) signal (yellow star in figure 1.5). This is the main source of mir-21 expression.

Given its independent nature from VMP1, there are so far 3 different promoters identified for pri-mir-21 transcript. The first one that is called miPPR-21 is the most characterized promoter ((Fujita et al., 2008). Other two promoters are located downstream of the miPPR-21. They are overlapping and found to be induced in a cell line-specific manner ((Kumarswamy, Volkmann, & Thum, 2011). miPPR-21 promoter produces 4.3kb gene product starting within 10th intron of VMP1 and it the most abundant pri-mir-21 transcript (Kumarswamy, Volkmann, & Thum, 2011).

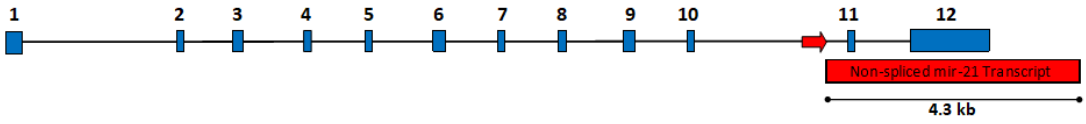

Figure 1.6. VMP1 and non-spliced mir-21 transcript.

Blue boxes indicate the exons and their numbers are located above of each exon. mir-21 promoter is shown as red arrow. Non-spliced mir-21 transcript (4.3kb) is shown with red rectangle. Size of each box reflects their relative size to each other.

1.4.1. Transcriptional Regulation of mir-21

Regulation of mir-21 transcript is achieved by different means. The miPPR-21(Fujita et al., 2008), includes binding sites for NFI, STAT3, p53, SRF, AP-1, and C/EBPα (Kumarswamy et al., 2011) From this list, NFI and C/EBPα are proven to repress the mir-21 transcription. On the other hand, direct AP-1 (Fujita et al., 2008), and AR (Ribas et al., 2009) binding induce mir-21 promoter activity. Additionally, Ras, EGFR, and ERK1/2 trigger promoter activity (Huang et al., 2008; Seike et al., 2009; Talotta et al., 2009). In addition to those, STAT3 and Estrogen Receptor exhibit their effect on the mir-21 promoter in cell type and condition-dependent manner, respectively (Ribas & Lupold, 2010).

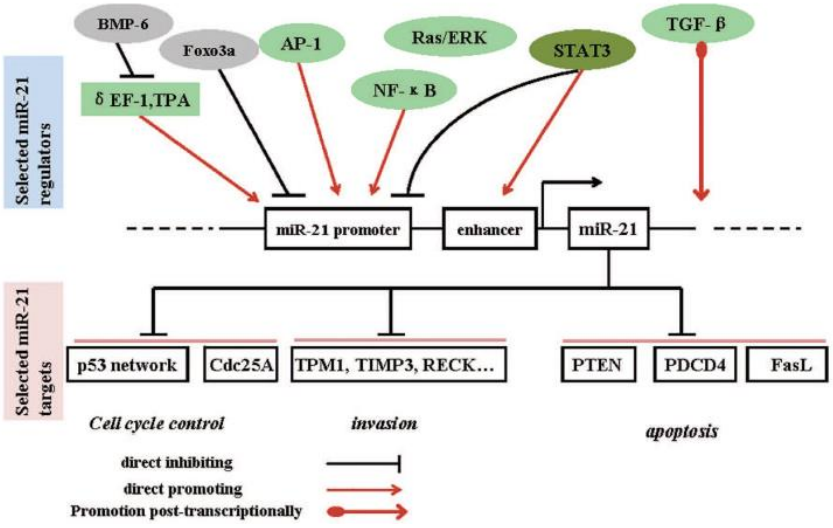

Figure 1.7. The upstream regulation of miR-21.

Top section: Ras/ER K, AP-1, NFκB signaling pathways could induce miR-21 transcription, while BMP-6 and Foxo3a inhibit the transcription of miR-21. STAT3 regulates miR-21 transcription in the opposite direction depending on the cell and tissue type. Bottom section: validated mir-21 targets in cell cycle, cell invasion and apoptosis. Figure is taken from (Pan, Wang, & Wang, 2010).

In summary, mir-21 is subjected to unique and complex (transcriptional) regulation through various proteins and pathways acting on mir-21 promoter/s, and through alternative polyadenylation of VMP1/mir-21 locus.

1.4.2. Relationship with Breast Cancer

MIR21 is the only common miRNA that is dysregulated in 540 tumor samples from six different tumors (Iorio Marilena V. et al., 2007). Expression levels of mir-21 are also induced by different cancer-related pathways including Activator protein 1, Androgen receptor, STAT3 and Estradiol pathways. It is a well-known oncomiR targeting various genes that are related to tumor cell proliferation, invasion, and apoptosis (Feng & Tsao, 2016). Mir-21 targets well-characterized tumor suppressors like p53, RECK, PDCD4, cdc25a, FasL, and PTEN (Feng & Tsao, 2016). High expression of mir-21 correlates with decreased adhesion, increased migration, high-proliferation rate and suppression of apoptosis; all favoring tumor growth and migration (Kumarswamy, Volkmann, & Thum, 2011).

1.5. Aim of the Study

Deregulation of miRNAs in (breast)cancer is quite common. Therefore, unraveling the mechanism of miRNA regulation is essential. HNRNPA1 as an RBP has been shown to regulate as-of-yet only a few miRNA genes. Considering all the different RNA-binding properties of HNRNPA1, we hypothesized that HNRNPA1 may regulate other miRNAs directly or indirectly. We utilized bioinformatics and biochemical assays in breast cancer cell line MCF7 to test this hypothesis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Databases

DIANA mirPath v.3 is used to analyze the miRNA and the pathways they are involved with (available at http://snf-515788.vm.okeanos.grnet.gr/).

G4 Hunter (available at https://bioinformatics.cruk.cam.ac.uk/G4Hunter/) and QGRS Mapper (available at http://bioinformatics.ramapo.edu/QGRS/index.php) are used to predict G-quadruplex structures.

GTEx Portal (The Genotype-Tissue Expression) is used for non-spliced mir-21 transcript image.

Immunohistochemistry (IHC) results are taken from The Human Protein Atlas (available at https://www.proteinatlas.org/).

Kaplan-Meier Plotter is used to plot KM-plots (available at https://kmplot.com/analysis/).

RBPmap (Mapping Binding Site of RNA Binding Proteins) (available at http://rbpmap.technion.ac.il/) and RBPDB (The Database of RNA-binding Protein Specificities) (available at http://rbpdb.ccbr.utoronto.ca/) are used to predict potential HNRNPA1 binding to pri-mir-21 sequence and stem-loop.

UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly (available at https://genome.ucsc.edu/) is used to locate the VMP1 and mir-21 locus, and to get the conservation data for mir-21.

2.2. High-throughput miRNA profiling with nCounter, NanoString

Total RNA from MCF7 cells (pSuper EV, pSuper NT, pSuper HNRNPA1-sh, MCF7-crispr EV, MCF7-A1 CRISPR colonies 1 and 10) was isolated using Phenol-Chloroform Extraction with Trizol Reagent (Invitrogen, CAT# 15596-018) as indicated by the manufacturer's protocol. RNA purity was surveyed by a Nanodrop spectrophotometric measurement (Thermo Scientific) of the OD260/280 proportion with satisfactory qualities falling somewhere in the range of 1.9 and 2.1. RNA was diluted to 50 mg using 1 ml RNase-free water and 3 ml was used for miRNA profiling on the multiplexed nCounter Human v2 miRNA stage (NanoString Technologies, Seattle, WA, USA). RNA samples were set up by ligating a specific DNA tag (miR-tag) onto the 3' end of each mature miRNA according to the manufacturer's protocol. Excess tags were expelled by restriction digestion at 37 C. Hybridizations were done by combining 5 ml of each miRNA-Tag samples with 20 ml of nCounter Reporter probes in hybridization buffer and 5 ml of nCounter Capture tests at 65 C for 16–20 h. Excess probes were evacuated utilizing two-step magnetic bead-based purification on the nCounter Prep Station. Abundance of specific target molecules was measured utilizing the nCounter Digital Analyzer by counting the individual fluorescent barcodes and evaluating target molecules. The data were gathered utilizing the nCounter Digital Analyzer and were examined utilizing the nCounter Analysis System as per the manufacturer's instruction.

2.3. Cell Culture

MCF7 cells (Michigan Cancer Foundation-7) grown in Dulbecco's Modified Eagle's Medium with High Glucose containing 4500 mg/L Glucose ,1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 10 % Fetal Bovine Serum (FBS) and 1 % penicillin-streptomycin. Plasmocin treatment (Invitrogen, CAT# ant-mmp) is done to eliminate mycoplasma contamination All cell culture supplements were obtained from Biowest (Riverside, USA). Cell lines were cultured as monolayers and incubated at 37 ºC with 5% CO2 and 95% humidified air. Cells were frozen in

liquid nitrogen at 70-80% confluency with 5% DMSO (dimethyl sulfoxide) (Sigma, Cat# 154938) in order to store cells for long term. Cell pellets were obtained with by 300-500 g centrifugation for 5 minutes. Cell thawing was done at 37°C.

2.3.1. Transfection

pSpCas9(BB)-2A-Puro (PX459)-HNRNPA1-CRISPR vector (designed and cloned by Murat Erdem, Erson Lab) was used to generate HNRNPA1-deleted MCF7 cells. Stable cell lines were created with 0.45 ug/ml Puromycin selection. They are maintained with 0.225 ug/ml Puromycin (Cat# 108321-42-2; Roche). pSuper EV and pSuper A1sh vectors were designed and cloned by Murat Erdem. G418-Geneticin (Roche, CAT# 4727878001) is used for selection (800 ug/ml) and maintenance (400 ug/ml) of MCF7-sh cells. Transfections were performed with Turbofect Transfection Reagent (Thermo Fisher Scientific, CAT#R0531) according to manufacturer's manual.

2.4. RNA isolation and Real Time PCR

Total cellular RNA was isolated with Roche High Pure RNA Isolation Kit (CAT#11828665001) or Phenol-Chloroform Extraction with Trizol Reagent (Invitrogen, CAT# 15596-018) and further processed with an overnight DNAse I enzyme treatment (Thermo Fisher Scientific, CAT#EN0521) according to the manufacturer's manual (Table 2.1). DNA contamination was checked by PCR with GAPDH primers (F: 5'GGGAGCCAAAAGGGTCATCA-3', R: 5'-TTTCTAGACGGCAGGTCAGGT-3'). 1-5 µg RNA was reverse transcribed by RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, CAT# EP0441) using oligo-dT primers and stored at -20 ºC (Table 2.2). Synthesis of cDNA is also confirmed with PCR using GAPDH primers. Quantitative Real-Time PCR (RT-qPCR) reaction was performed using BioRAD SYBR Green Supermix (CAT#172-5270) with 0.5 µM forward and reverse primers and 1 µl cDNA. RT-qPCR Machine BioRAD CFX-Connect was used. Ct values were calculated using relative standard curve method and the fold change was calculated by ΔΔCq method. B-actin and

RPLP0 mRNA expression values are used as house-keeping controls (reference genes). The primers used in the study are given in Table 2.3. RNAs were quantified via BioDrop Duo (Isogen Life Science). RNA sample purity was, A260/A280 ratio was between 1.8 and 2.0 and A260/A230 ratio was above 2.0.

Table 2.1. *DNase Treatment*

Total RNA	10 µl
10 X Reaction Buffer	10 µl
DNase I (Roche)	6 µl
MG water	variable
TOTAL	100 µl
Incubate tubes at 37°C for 2 hours, Continue with rest of the protocol.	

Table 2.2. *cDNA Synthesis*

RNA	1000-5000 ng
Primer (oligodT or random hexamer)	1 µl
MG water	variable
TOTAL	12 µl
Briefly centrifuged and incubated for 5 min. at 70 °C. Put on ice for 1 min.	
5X Reaction Buffer	4 µl
Ribolock RNAse inhibitor	1 µl
dNTP mix	2 µl
RevertAid RT enzyme	1 µl
TOTAL	20 µl
Incubate the tubes for 60 minutes at 42°C; stop the reaction by heating at 70°C for 5 minutes.	

Table 2.3. *List of Primers*

Primer Name	**Primer Sequence** up: Forward Primer down: Reverse Primer	**Primer Length (bp)**	**Annealing Temperature (Celsius)**
hsa-mir-21-5p	TCGTGACATCTCCATGGCTG CCAGACAGAAGGACCAGAGT	20 20	59
hsa-mir-98a	TGCCTGCTGCCCTTATTAGA GATGGAGGCAACACTGCTAAG	20 21	62
BCL-2	CTTTGAGTTCGGTGGGGTCA GGGCCGTACAGTTCCACAAA	20 20	64
PDCD4	ACCCTGCAGATCCTGATAACT CGCCTTTTTGCCTTGGCATT	21 20	64
hs STAT3	TTCTGGGCACAAACACAAAAG TCAGTCACAATCAGGGAAGC	21 20	61
hs VMP1 exon7	ATCGGTACAGCAATCGGAGA TCTGCATGTTCCAGCATCTCT	20 21	59
hs VMP1 exon12	TGGTCGTTGTCATGGTGTGT GTTCAACCGCTGCTGGATTC	20 20	59
VMP1 i10	TACAGCTCTTGGTTTCTCCCA AAGGCAGGGATTGGATAGGAAG	21 22	60
VMP1 i11	GTTTCTTCTCCCCTCTGGGAA CACTGGCAAACCAACTTCACT	21 21	60
HNRNPA1 111bp	GCTCACGGACTGTGTGGTAA GGCCTTGCATTCATAGCTGC	20 20	62
GAPDH	GGGAGCCAAAAGGGTCATCA TTTCTAGACGGCAGGTCAGGT	20 21	56
RPLP0	GGAGAAACTGCTGCCTCATA GGAAAAAGGAGGTCTTCTCG	20 20	59
ACTB qPCR	TGACGTGGACATCCGCAAAG CTGGAAGGTGGACAGCGAGG	20 20	61

2.5. Protein Isolation and Western Blot

Total cellular proteins were isolated with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, #78501) containing phosSTOP (Roche-CAT#04906837001) and protease inhibitor cocktail (Roche-CAT#11873580001). Protein concentrations were measured with the BCA kit assay. Total protein extracts were denaturated with 6X Loading dye at 100°C for 10 minutes. SDS-PAGE is performed using 5% stacking and 8-12% separating gels and blotting was done onto PVDF membrane (Roche). 5% bovine serum albumin (BSA) in TBS-T (Tris Buffer

Saline- Tween) were used as blocking reagent at room temperature for 1 hour. Blocking was followed by overnight incubation with the primary antibodies: B-actin (1:2000) (Santa-Cruz, CAT# sc-47778), HNRNPA1 (1:2000) (4B10, Santa-Cruz, CAT# sc-32301), HNRNPA1 (1:2000) (9H10, Santa-cruz, CAT# sc-56700) with a subsequent 1-hour incubation of secondary antibody (Santa Cruz, anti-mouse CAT#sc-2005). Membranes were visualized by BioRAD Clarity™ Western ECL Blotting Substrates (CAT#1705060) according to the manufacturer's instructions.

2.6. Statistical Analysis

Student's t-test was performed to examine the difference between the control and treatment groups. ANOVA was performed to measure the mean difference of more than 2 samples. A P value less than 0.05 was deemed statistically significant. All graphs and the statistical analysis were created/calculated via GraphPad Software (La Jolla California, USA). Statistical significance was assumed at $p < 0.05$, (*) while $p < 0.01$ is denoted "**", $p < 0.001$ is denoted as "***", and $p < 0.0001$ is denoted as "****".

CHAPTER 3

RESULTS

3.1. Effect of HNRNPA1 Downregulation on miRNAs in MCF7 cells

To investigate the effect of HNRNPA1 on miRNAs, we silenced HNRNPA1 transcript in breast cancer cell line MCF7 using short hairpin RNA-mediated gene silencing. The confirmation of silencing was done by RT-qPCR (Figure 3.1).

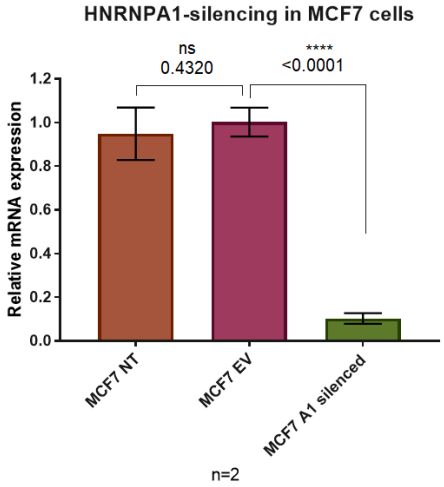

Figure 3.1. Establishing an HNRNPA1 silencing model.

Real Time PCR results for HNRNPA1 silencing. RNAi model is designed by my fellow co-worker Murat Erdem. Around 10-fold decrease in HNRNPA1 mRNA levels is observed in MCF7 A1-silenced cells compared to controls. Significance levels and p-values are located on the corresponding bars. The PCR result is the combination of 2 biological replicates, each with 3 technical replicates.

Next, to reveal whether miRNA levels were modulated by A1 levels, we used the NanoString nCounter miRNA panel using A1 shRNA and control cells (NT = non-targeting shRNA). Out of 798 miRNAs in the NanoString panel, there were 62

differentially expressed miRNAs exhibiting > 1.5-fold or < 0.67-fold changes in MCF7shRNA cells. Remaining microRNAs were either not altered or their expression levels were low.

Among those differentially regulated miRNAs, 19 were upregulated and 43 microRNAs were downregulated in HNRNPA1-silenced cells (Table 3.1).

Table 3.1. *List of selected upregulated and downregulated miRNAs.*

The intensity of each miRNA in both cell types and their ratio (intensity in A1sh cells/intensity in pSuper EV cells) are written on the same row.

Mature miRNA	***pSuper EV***	***pSuper A1sh***	***A1sh/EV***
hsa-miR-135a-5p	389.59	22.14	0.057
hsa-miR-3065-5p	51.85	5.82	0.112
hsa-miR-193a-5p+hsa-miR-193b-5p	41.48	6.63	0.160
hsa-miR-615-5p	33.32	7.96	0.239
hsa-miR-320e	50.6	13.4	0.265
hsa-miR-1290	26.13	7.32	0.280
hsa-miR-92a-3p	26.13	8.26	0.316
hsa-miR-625-5p	40.94	13.21	0.323
hsa-miR-582-5p	53.4	18.73	0.351
hsa-miR-7-5p	2399.18	901.57	0.376
hsa-miR-4454+hsa-miR-7975	12931.75	5495.69	0.425
hsa-miR-106a-5p+hsa-miR-17-5p	413.38	182.05	0.440
hsa-let-7f-5p	898.44	397.12	0.442
hsa-miR-30c-5p	70.85	31.73	0.448
hsa-miR-423-5p	179.02	80.9	0.452
hsa-miR-21-5p	**4863.32**	**2202.9**	**0.453**
hsa-miR-30a-3p	67.83	33.22	0.490
hsa-miR-193b-3p	40.84	20.62	0.505
hsa-let-7a-5p	13573.9	7029.74	0.518
hsa-miR-3065-3p	21.57	11.21	0.520
hsa-miR-140-5p	52.61	27.4	0.521
hsa-miR-30a-5p	153.49	80.31	0.523
hsa-let-7g-5p	2026.79	1080.78	0.533
hsa-miR-6724-5p	56.86	30.41	0.535
hsa-miR-10a-5p	30.37	16.3	0.537
hsa-miR-30b-5p	106.5	58.01	0.545
hsa-miR-126-3p	316.24	180.73	0.571
hsa-miR-365a-3p+hsa-miR-365b-3p	640.02	378.73	0.592

Table 3.1 Cont'd

hsa-miR-181b-5p+hsa-miR-181d-5p	166.02	99.7	0.601
hsa-miR-378i	482.07	295.79	0.614
hsa-miR-378g	152.44	93.87	0.616
hsa-let-7e-5p	1785.62	1108.66	0.621
hsa-miR-20a-5p+hsa-miR-20b-5p	640.14	406.35	0.635
hsa-miR-362-3p	63	40.38	0.641
hsa-miR-148a-3p	1015.82	656.56	0.646
hsa-let-7b-5p	5244.58	3409.24	0.650
hsa-miR-26b-5p	433.72	285.51	0.658
hsa-miR-1180-3p	295.83	457.17	1.545
hsa-miR-497-5p	55	85.16	1.548
hsa-miR-148b-3p	502.14	809.84	1.613
hsa-miR-1260a	97	158.3	1.632
hsa-miR-324-5p	105.96	174.33	1.645
hsa-miR-149-5p	66.51	110.35	1.659
hsa-miR-548y	13.58	23.11	1.702
hsa-miR-28-5p	197.98	343.62	1.736
hsa-miR-424-5p	202.77	354.77	1.750
hsa-miR-210-3p	11.64	21.31	1.831
hsa-miR-612	41.23	82.15	1.992
hsa-miR-873-3p	54.21	125.92	2.323
hsa-miR-598-3p	22.94	58.57	2.553
hsa-miR-6721-5p	25.21	65.21	2.587
hsa-miR-495-3p	5.59	29.09	5.204
hsa-miR-1183	9.07	43.82	4.831
hsa-miR-455-5p	17.19	84.56	4.919
hsa-miR-127-3p	2.5	22.96	9.184
hsa-miR-135b-5p	1.36	232.21	170.743

Majority of these miRNAs are predicted to take part in essential cellular pathways like; TGF-beta, RAS, Hippo, PI3K-Akt, Estradiol, cAMP, and Wnt signaling pathways (Table 3.2). Considering these pathways are all cancer-related, the potential role of HNRNPA1 in cancer progression can be partly due to miRNAs.

Table 3.2. *List of Pathways related with miRNAs from NanoString results*

p-values, number of genes affected in the pathway and number of miRNAs involved are listed on the same row with each pathway.

KEGG Pathways	***p-value***	***#genes***	***#miRNAs***
Pathways in cancer	6.44E-07	280	56
PI3K-Akt signaling pathway	7.87E-05	230	57
MAPK signaling pathway	0.000544	171	56
Ras signaling pathway	1.61E-05	159	53
Rap1 signaling pathway	1.93E-05	152	52
Endocytosis	0.000126	148	56
Regulation of actin cytoskeleton	0.004393	145	54
Focal adhesion	0.000408	144	55
Proteoglycans in cancer	9.13E-07	139	55
cAMP signaling pathway	0.007072	134	53
Viral carcinogenesis	0.003512	118	54
Protein processing in endoplasmic reticulum	0.003117	113	54
Hippo signaling pathway	6.44E-07	109	52
Adrenergic signaling in cardiomyocytes	0.000266	100	53
Ubiquitin mediated proteolysis	0.000126	99	53
FoxO signaling pathway	0.000155	99	53
Signaling pathways regulating pluripotency of stem cells	0.000544	98	52
Axon guidance	3.97E-06	97	54
Hepatitis B	0.005249	95	54
AMPK signaling pathway	7.23E-05	94	56
Wnt signaling pathway	0.013393	93	53
…	…	…	…
Full list is in appendices			

To select the most relevant miRNA, we narrowed down the differentially regulated miRNAs using fold change and high expression criteria in MCF7 cells. The heatmaps show the set of such candidate miRNAs (Figure 3.2).

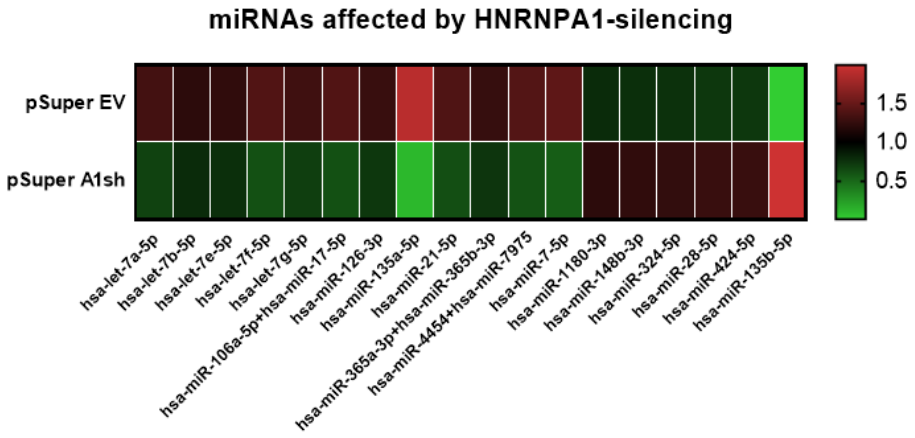

Figure 3.2. Heatmap results of selected miRNAs.

A combination of most significant miRNAs with high expression levels in either pSuper EV or pSuper A1sh cells are shown. While red color indicated low, green color indicated high expression. For each miRNA, normalization against their initial intensities is performed. Change in the intensities are color-coded with the bar located on the right of the figure.

One of the most interesting miRNAs was miR-21. Mature mir-21 level was decreased 55% in HNRNPA1-silenced cells compared to the control (Figure 3.3). It is associated with many different pathways in breast cancer (see mir-21 chapter in the introduction).

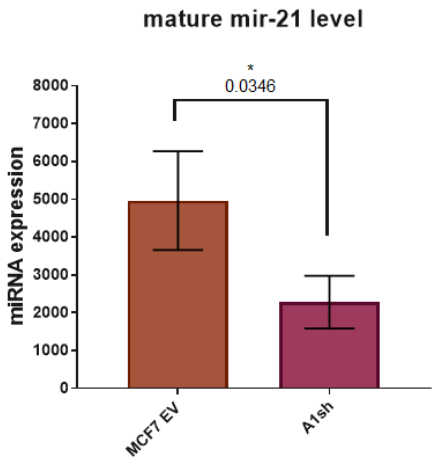

Figure 3.3. Read count of mature mir-21 in the silencing model.

NanoString intensity results are shown here. Student's t-test is performed and significance is indicated above bars. There is a clear decrease in mature mir-21 levels in HNRNPA1-silenced cells. The intensity of mature mir-21 was dropped from 4863.32 to 2202.9 in A1sh cells, compared to the control (EV). The average intensity of selected list of miRNAs was 915.5. It means, initial mir-21 levels were very high and it decreased 55% in response to HNRNPA1 silencing.

3.2. Pri-mir-21 levels decreased in HNRNPA1-silenced cells

To begin understanding how A1 might regulate miR-21 which is a known oncogenic miRNA in ER+ breast cancer cells (Blenkiron et al., 2007; Petrović et al., 2014), we wanted to confirm the NanoString results.

First, we used the shRNA transfected MCF7 cells to detect pri-mir-21 levels. RT-qPCR revealed around 6-fold decrease in pri-miRNA-21 levels in MCF7-shRNA cells compared with control MCF7 cells (Figure 3.4).

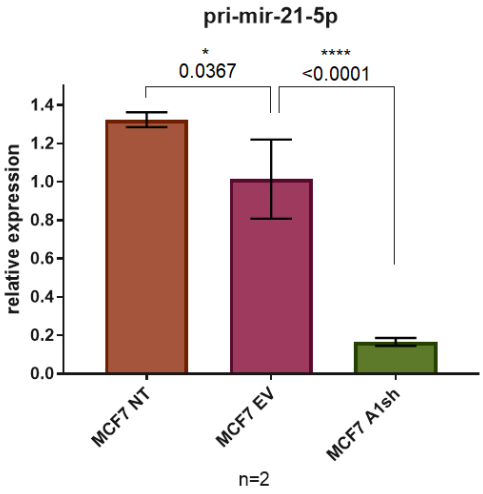

Figure 3.4. Real-Time PCR results for pri-mir-21-5p in HNRNPA1-silenced MCF7.

The result is the combination of 2 biological replicates, each with 3 technical replicates. Against the controls (MCF7 NT and MCF7 EV) MCF7 A1sh shows around 10-fold decrease in HNRNPA1 mRNA expression. The significance levels and p-values are indicated above of each corresponding bar. Ordinary one-way ANOVA is performed against MCF7 EV.

3.3. CRISPR/Cas9 deletion of HNRNPA1

Next, we performed CRISPR/Cas9 targeted genome editing of HNRNPA1 gene using PX459 vectors (see material and methods). We confirmed the deletion with Western Blot (figure 3.5. A). In this CRISPR set, we have obtained several different monoclonal HNRNPA1-deleted cells. However, with time HNRNPA1 protein levels have gradually increased in CRISPR-cells (Figure 3.5. B). We performed the second CRISPR with the same construct. However, once again HNRNPA1 protein levels increased in time (Figure 3.5 C and D). Possible reasons are duplication on the target gene locus (resulting in only heterozygous deletion) and ineffective puromycin selection with poor Cas9 efficiency. HNRNPA1 gene is located on chr12 q13.13 which is duplicated in some MCF7 subclones (Nugoli et al., 2003). This may explain why we get this phenotype. The further experiments were done with cell pellets collected from different periods of time to see the effect of this phenomenon on mir-21 and its downstream pathways.

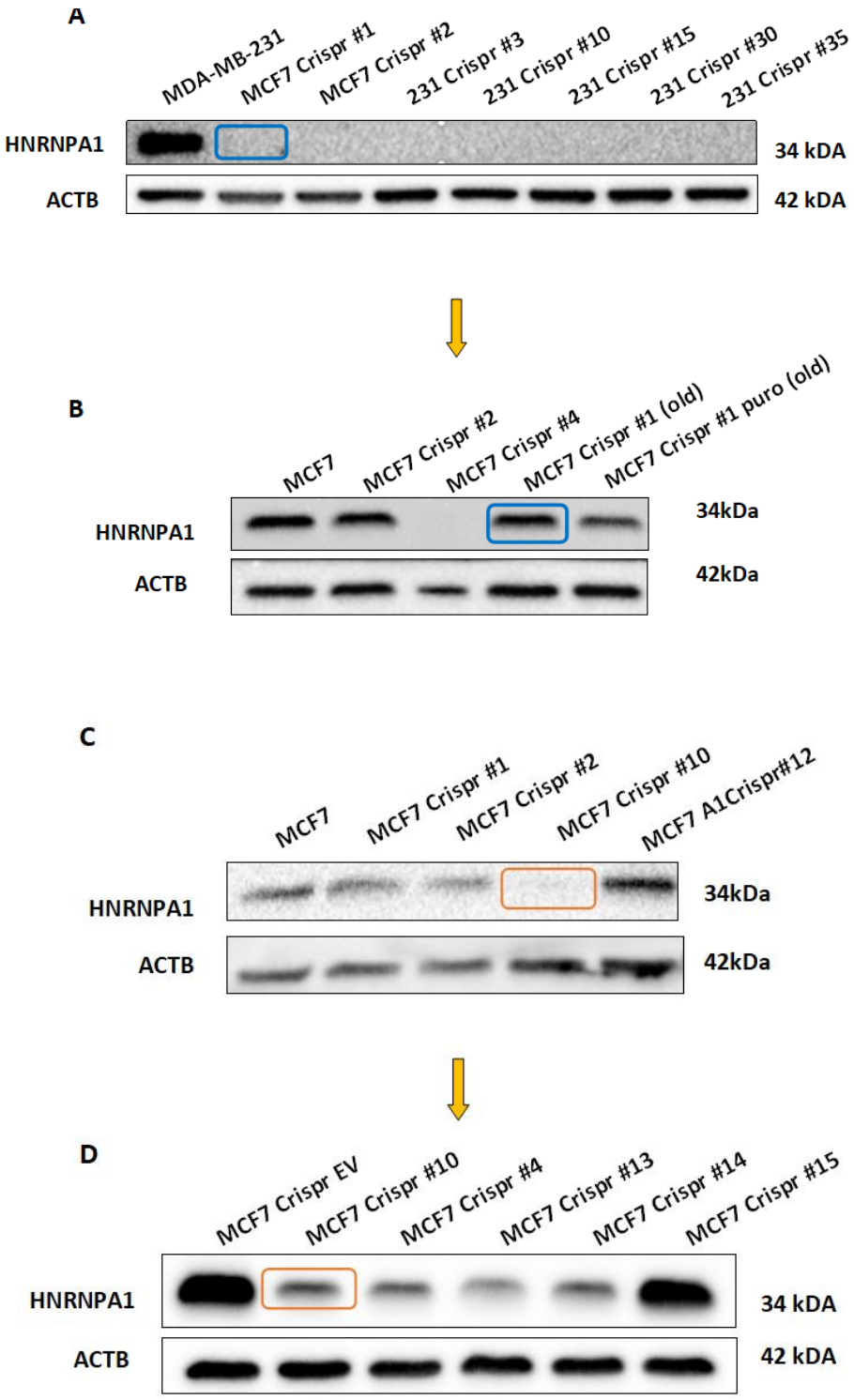
A
MDA-MB-231
MCF7 Crispr #1
MCF7 Crispr #2
231 Crispr #3
231 Crispr #10
231 Crispr #15
231 Crispr #30
231 Crispr #35
HNRNPA1
ACTB
34 kDA
42 kDA
B
MCF7
MCF7 Crispr #2
MCF7 Crispr #4
MCF7 Crispr #1 (old)
MCF7 Crispr #1 puro (old)
HNRNPA1
ACTB
34kDa
42kDa
C
MCF7
MCF7 Crispr #1
MCF7 Crispr #2
MCF7 Crispr #10
MCF7 A1Crispr#12
HNRNPA1
ACTB
34kDa
42kDa
D
MCF7 Crispr EV
MCF7 Crispr #10
MCF7 Crispr #4
MCF7 Crispr #13
MCF7 Crispr #14
MCF7 Crispr #15
HNRNPA1
ACTB
34 kDA
42 kDA

Figure 3.5. Western Blots for HNRNPA1-CRISPR deletions.

The CRISPR construct was designed by Murat Erdem.A) Confirmation of the first HNRNPA1 CRISPR-set. This experiment is a joint work with Murat Erdem. HNRNPA1 deletion in different MCF7 and MDA-MB-231 colonies is clearly observed. Wild-type MDA-MB-231 is used as control. B) Western Blot result of old and new CRISPR-colonies. CRISPR colony #1 from the first set shows HNRNPA1 protein expression in time. C) Western Blot results of the second HNRNPA1 CRISPR-set. CRISPR colony #10 is showing no- HNRNPA1 protein levels. MCF7 is used as control D) Western blot results of second set CRISPR colonies. It was performed later than Western Blot in (C) section. CRISPR #10 demonstrated HNRNPA1 levels, too. For all four experiment, B-actin levels are used as loading controls. Size of each protein is indicated on right side of each graph. HNRNPA1(Santa-cruz 4B10) antibody is used in 1:2000 dilution.

3.4. Pri-mir-21 level decreases upon HNRNPA1 deletion via CRISPR/Cas9

After the 6-fold change in pri-mir-21 expression in the A1sh cells, we measured the pri-mir-21 levels in different CRISPR colonies. In colony #1.1 and #1.2 we observed 5.9 and 3.6-fold decrease in pri-mir-21 levels, respectively (Figure 3.6 A). In the CRISPR colony #10, we observed 1.5-fold decrease in HNRNPA1 mRNA expression compared to the control in early times (down to 65% expression compared to the control). However, pri-mir-21 expression showed an increase in these cell lines over time (Figure 3.6 B).

Interestingly, we detected a continuous trend where complete depletion of A1 resulted in the most downregulation of pri-mir21. With high HNRNPA1 protein level, high pri-mir-21 levels were observed among different colonies. These results confirmed the initial analysis and suggested that A1 might directly or indirectly regulate miR-21 levels in mcf7 cells.

To conclude, we observed a decrease in the primary mir-21 levels in three different silencing constructs (one shRNA-silencing and two different CRISPR sets), in which HNRNPA1 protein levels correlated with pri-mir-21 levels.

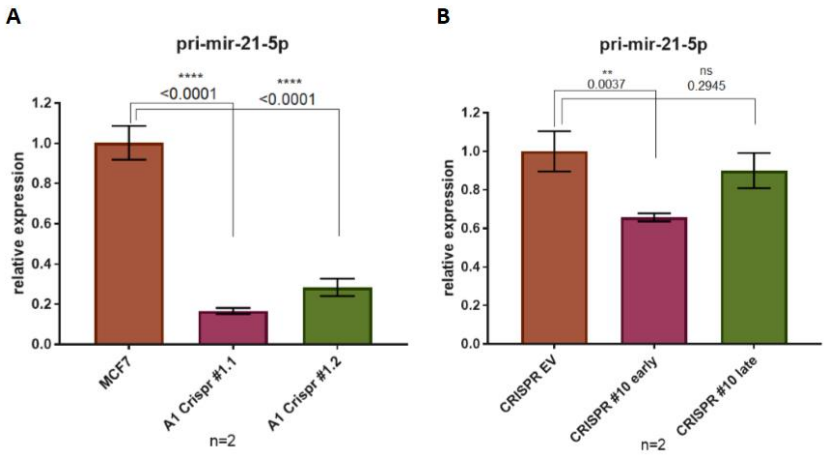

Figure 3.6. Real Time PCR results of pri-mir-21 in CRISPR colonies.

A) Decrease in pri-mir-21-5p levels in CRISPR colony #1 cells. #1.1 and #1.2 originated from same cells and grown separately. Both showed significant decrease in pri-mir-21-5p levels compared to control. B) pri-mir-21-5p levels in CRISPR colony #10 in early and late time. In early time, pri-mir-21-5p level is significantly (**) decreased compared to control, but it goes back to same level as control in late time. Ordinary one-way ANOVA is performed against controls. Significance levels are indicated above each corresponding bar. Both experiments were performed in 2 biological replicates, each with 3 technical replicates.

Following the confirmation of the significant decrease of pri-mir21 levels in A1 silenced models, we sought to understand how pri-mir21 levels might be regulated by A1.

We checked whether A1 might directly bind to pri-miR21 using both RBPmap (Motifs Analysis and Prediction of RNA Binding Proteins) and RBPDB (RNA-binding Protein Database), and found no potential binding motif. It is also worth mentioning that HNRNPA1 does not bind to the loop of mir-21 (Michlewski & Cáceres, 2010). Hence, the next possibility was that transcription of mir21 was regulated by A1.

3.5. Effect of HNRNPA1 loss on pri-mir-21 and its host gene (VMP1)

Mir21 locus maps to chr17:59,841,266-59,841,337 (Kent et al., 2002). Interestingly mir21 gene overlaps with a host gene called VMP1 (Vacuole membrane protein 1). Because of this overlap, we tested whether VMP1 expression was also altered in HNRNPA1 silenced cells (Figure 3.7). To do that, we designed two different primer sets for exons (exon #7 and exon #12) and two for introns of VMP1(intron #10, intron #11). The primer locations are marked as arrows in Figure 3.7.

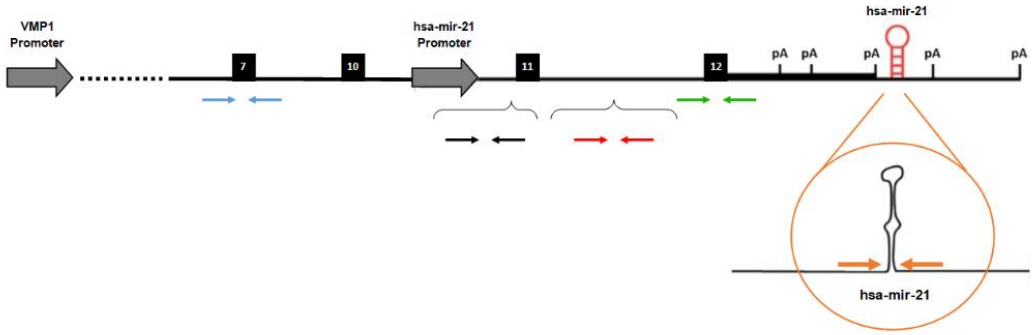

Figure 3.7. Overview of VMP1 and mir-21 locus.

Thicker grey arrows indicate VMP1 and mir-21 canonical promoter. Black boxes are exons of the gene and inside their numbers located. Exon 11 and Exon 12 is shared between both promoters. pA indicates the polyadenylation sites. Red hairpin is the has-mir-21 hairpin structure. Thinner arrows are primers. They are named as followed: blue: exon 7 primers, black: intron 10 primers, red: intron 11 primers, green: exon 12 primers and orange: pri-mir-21 primers.

A 5' primer set mapping to Exon 7 of VMP1 gene amplified the VMP1 transcript comparable to control MCF7 cells, supporting that pri-21 is independently expressed/processed from VMP1 (Figure 3.8). Another primer set from Exon 12 showed a 2-fold decrease compared to control (Figure 3.8). While the only source for exon #7 is VMP1 transcript, exon #12 is shared by both VMP1 and mir-21 transcript; supporting the idea that HNRNPA1 might regulate mir-21 independent of its host gene.

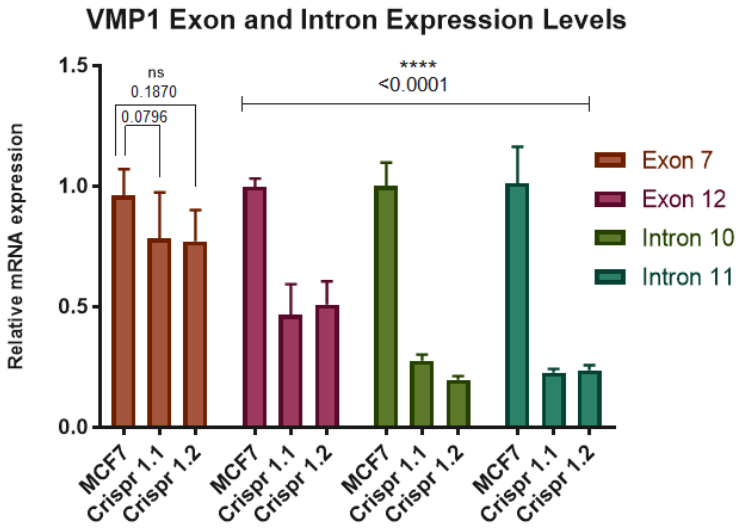

Figure 3.8. Real Time PCR results of VMP1 intron and exons in CRISPR colonies.

Exon 12 showed more decrease in HNRNPA1-silenced cells than Exon 7 does. Both intron 10 and 11 expressions are quite low in HNRNPA1-silenced cells. Two-way ANOVA is performed against each control. Significance levels are indicated above each corresponding bar. The experiments are performed in 2 biological replicates, each with 3 technical replicates.

Next, we designed primers from the Intron 10 and 11 of VMP1. This region overlaps with the pri-mir-21 transcript. Actually, the promoter region of mir-21 is located in VMP1 intron #10 and it produces 4.3 kb non-spliced pri-mir-21 transcript (Fujita et al., 2008) (Figure 3.7 and 3.9). Intron 10 primers are designed for the sequences right before exon 11 starts, to ensure that it is located at downstream of the mir-21 promoter. Because pri-mir-21 transcript is not spliced, we deduced that the expression values of these introns indicate the mir-21 transcription. RT-qPCR results showed expression from both introns decreased 5-fold compared to control Figure 3.8). These results suggested that the effect of HNRNPA1 on mir-21 regulation might be through transcriptional regulation.

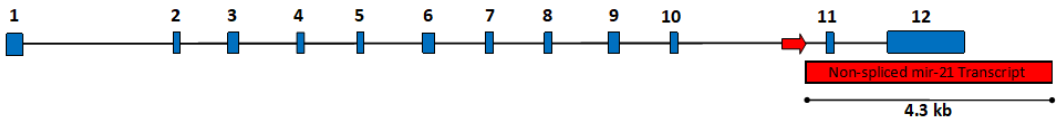

Figure 3.9. Overview of VMP1 and mir-21 transcript.

Blue boxes are exons of VMP1 with numbers indicating the exon numbers. Red arrow is mir-21 canonical promoter that is located in intron #10. Red box is showing the non-spliced 4.3 kb mir-21 transcript. The figure is based on GTEX isoform expression results.

3.6. Effect of HNRNPA1 loss on mir-21 target mRNAs

Next, while the mature mir-21 levels still need to be confirmed, we sought to test known mir21 mRNA targets. One of the known targets is PDCD4 (Programmed cell death 4) which is associated with apoptosis in cancer cells. Pdcd4 mRNA is known to be degraded upon mir-21 binding (Z. Sun, Li, Kaufmann, & Albers, 2014).

Using CRISPR models for HNRNPA1, we performed RT-qPCR and results suggested almost a 2-fold upregulation of PDCD4 in HNRNPA1-CRISPR #1.1 cells compared with control MCF7 cells (Figure 3.10).

Another target is STAT3 (Signal transducer and activator of transcription 3) implicated intensely in cancer. RT-qPCR resulted in 1.8-fold upregulation of STAT3 in HNRNPA1-CRISPR #1.1 cells compared with the control Figure 3.10).

Lastly, expression of a third mir-21 target, BCL-2 (B-cell lymphoma 2), was measured by RT-qPCR. 1.5-fold upregulation of BCL-2 in HNRNPA1-CRISPR #1.1 cells was observed (Figure 3.10).

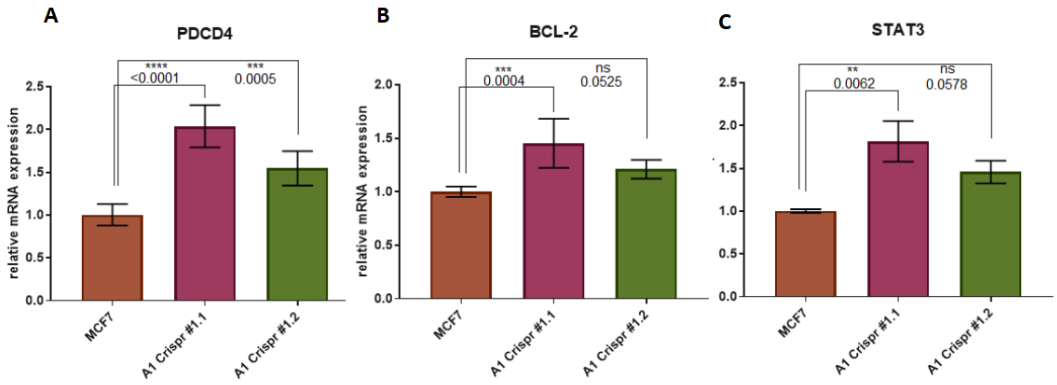

Figure 3.10. Expression of different targets of mir-21.

A) PDCD4 mRNA level. Both Crispr cells shows increased PDCD4 expression. The Real Time PCR experiment is repeated 2 times each with 3 technical replicates. B) BCL-2 mRNA level. Only Crispr #1.1 cells showed significant increase in BCL-2 levels. The Real Time PCR experiment is repeated 2 times each with 3 technical replicates. C) STAT3 mRNA level. Real Time PCR experiment is repeated once with 3 technical replicates.

All three targets showed same upregulation pattern in response to HNRNPA1 loss in the other CRISPR-colony (#1.2). However, the change in the upregulation of each target mRNA was lower in CRISPR #1.2 cells compared to CRISPR #1.1 cells (PDCD4=1.55, STAT3=1.46, BCL-2=1.21) (figure 3.10). If we correlate the primary mir-21 levels with mature mir-21 levels, we can speculate that this may be due to the high expression of pri-mir-21 in CRISPR#1.2 cells compared to CRISPR#1.1.

In summary, expression of three different mir-21 target mRNAs increase in the HNRNPA1-silenced cells and their expression levels are negatively proportional with primary mir-21 levels (see Crispr 1.1 and 1.2 results in Figure 3.6A and Figure 3.10).

CHAPTER 4

CONCLUSION

In conclusion, HNRNPA1 silencing led to a change in the expression of 62 different miRNAs in MCF7 cells. Among those, we chose mir-21 to investigate the effect of HNRNPA1 on miRNAs. In different silencing set-ups (sh-RNA and CRISPR colonies), we observed primary mir-21 levels were decreased in a way that is proportional to the level of HNRNPA1 loss. Mir-21 transcript was affected by the loss of HNRNPA1 more than the host (VMP1) transcript does. In addition, introns found in mir-21 transcript showed decreased expression, suggesting that HNRNPA1 may regulate pri-mir-21 transcriptionally.

Next, to confirm the mature mir-21 decrease observed in NanoString experiment, we measured the mRNA expression levels of three different mir-21 targets PDCD4, BCL-2, and STAT3. Expression level of all three has increased in response to HNRNPA1 loss, again proportional to extent of HNRNPA1 loss.

For most of the deregulated miRNAs in cancer, change in mature miRNA levels do not correlate with primary miRNA level. Most of the time, the regulation occurs post-transcriptionally. However, for the mir-21, that is not the case. For example, during mammalian development, there is a good correlation between primary and mature levels of mir-21 (Thomson et al., 2006). This suggest the idea that mir-21 transcription and processing is tightly coupled. This idea reinforces the importance of transcriptional regulation of mir-21, potentially via an HNRNPA1 regulated transcription factor.

We observed that extent of HNRNPA1 protein loss correlated with pri-mir-21 levels. On top of that, pri-mir-21 levels negatively correlated with mir-21 target genes. If we combine this with the tightly coupled transcription and processing of mir-21, we may

suggest that HNRNPA1 may regulate mir-21 transcriptionally and this is directly reflected on mature mir-21 levels, and consequently on mir-21 targets.

The effect of HNRNPA1 loss on VMP1 transcript was not significant, however, there was a clear decrease in pri-mir-21 transcript. This may indicate a (in)direct regulation mir-21 promoter region by HNRNPA1. Hence, it will be important to unravel how mir-21 promoter might be regulated (in)directly by A1.

Interestingly, the ability of HNRNPA1 to unwind the G-quadruplex(G4) structures on promoters is already known to facilitate the transcription. Transcription of KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog) (Cogoi, Rapozzi, Cauci, & Xodo, 2017) and splicing factor TRA2B (Transformer 2 Beta Homolog) (Nishikawa, Kuwano, Takahara, Nishida, & Rokutan, 2019) mRNAs are two known examples regulated by HNRNPA1 binding to G4 on their promoters. We have found two potential G4 structures on the canonical mir-21 promoter using QGRS (Quadruplex forming G-Rich Sequences Mapper) (Kikin, D'Antonio, & Bagga, 2006) and G4 Hunter (Bedrat, Lacroix, & Mergny, 2016) (see Appendix). This finding can also be investigated for possible regulation of mir-21 promoter by HNRNPA1.

In this work, we investigated the role of HNRNPA1 on miRNAs and found that mir-21 expression is potentially regulated by HNRNPA1. Ongoing experiments will help us understand how.

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APPENDICES

A. Buffers used in Western Blot Experiments

Tris Buffered Saline (10X)

- 12.1 grams Tris (20mM)
- 40 grams NaCl2 (137mM)
- Complete to 500mL by adding dH2O
- Adjust pH to 7,6 by adding 37% HCl
- STORE AT ROOM TEMPERATURE

(1X TBST)

- Mix 10X TBS (5mL) with 45 ml dH20
- Add 50 uL Tween 20 (If 0.1% TBST is needed)
- Put on shaker for 10 mins
- STORE AT ROOM TEMPERATURE

6X SDS Loading Buffer (store at -20C)

- 5.25 mL Tris-HCl (pH 6.8- 1M)
- 1.54 grams SDS
- 5.4 mL Glycerol
- 750 mL B-mercaptoethanol
- 2 mg Bromophenol blue
- 15 mL dH2O
- Warm a while and shake until dissolved

10% SDS solution

- 10 grams SDS is dissolved in 100 mL dH2O. Store at Room Temperature.

1M Tris-HCl

- 12 grams Tris Base is dissolved in 60 mL dH2O
- Adjust pH to 6.8 and complete the volume to 100 mL..
- Store at 4C.

1.5M Tris-HCl

- 18 grams Tris Base is dissolved in 60 mL dH2O
- Adjust pH to 8.8 and complete the volume to 100 mL..
- Store at 4C.

10 % APS

- 1gram Ammonium Persulfate in 10 mL dH2O
- Filter sterilization with 0.2 um filter and store at -20C.

Running Buffer (10X)

- 30 grams Tris (250mM)
- 180 grams Glycine (2500mM)
- 10 grams SDS (1%)
- Complete the volume to 1liter with dH2O and store at 25C
- Dilute to 1X with only dH2O to use it.

Transfer Buffer (10X)

- 30.3 grams Tris
- 144 grams Glycine

- Complete the volume to 1liter with dH2O and store at 25C or 4C

Transfer Buffer (1X)

- 10X Transfer Buffer 100 mL
- dH2O 700 mL
- Methanol 200 mL
- Always mix in this order!
- Store at 4 C.

Stripping Buffer (Mild)

- 15 grams Glycine
- 15 grams SDS
- 10 mL Twee 20
- Complete the volume to 1liter with dH2O and adjust the volume to pH 2.2
- Take 50 mL aliquots and heat up to 65 C prior to use.

Blocking Buffer

- 2 grams Skim Milk or BSA in 4 mL TBST or PBST
- Put on shaker to dissolve for 10 mins
- Use fresh each time
- Antibody is also dissolved in blocking buffer

B. DIANA mirPath v3 Results

Table B.1. *Full List of Pathways related with miRNAs from NanoString results*

p-values, number of genes affected in the pathway and number of miRNAs involved are listed on the same row with each pathway.

KEGG Pathways	*p-value*	*#genes*	*#miRNAs*
Pathways in cancer	6.44E-07	280	56
PI3K-Akt signaling pathway	7.87E-05	230	57
MAPK signaling pathway	0.000544196	171	56
Ras signaling pathway	1.61E-05	159	53
Rap1 signaling pathway	1.93E-05	152	52
Endocytosis	0.000126026	148	56
Regulation of actin cytoskeleton	0.004393158	145	54
Focal adhesion	0.000407679	144	55
Proteoglycans in cancer	9.13E-07	139	55
cAMP signaling pathway	0.007071952	134	53
Viral carcinogenesis	0.003511985	118	54
Protein processing in endoplasmic reticulum	0.003117466	113	54
Hippo signaling pathway	6.44E-07	109	52
Adrenergic signaling in cardiomyocytes	0.000266239	100	53
Ubiquitin mediated proteolysis	0.000126026	99	53
FoxO signaling pathway	0.000155175	99	53
Signaling pathways regulating pluripotency of stem cells	0.000544196	98	52
Axon guidance	3.97E-06	97	54
Hepatitis B	0.005248841	95	54
AMPK signaling pathway	7.23E-05	94	56
Wnt signaling pathway	0.013393463	93	53

Table B.1 Cont'd

Neurotrophin signaling pathway	0.000407679	89	50
Dopaminergic synapse	0.026216439	89	54
Platelet activation	0.018386531	86	53
Sphingolipid signaling pathway	0.002497197	82	52
Cholinergic synapse	0.000912029	79	54
Thyroid hormone signaling pathway	0.01354329	79	54
Glutamatergic synapse	0.000826009	78	51
Choline metabolism in cancer	0.00447336	75	51
HIF-1 signaling pathway	0.026216439	73	50
Estrogen signaling pathway	0.000155175	69	51
Circadian entrainment	0.000407679	69	51
Retrograde endocannabinoid signaling	0.01255374	69	54
Morphine addiction	1.06E-06	68	54
Prostate cancer	0.000325191	68	50
Melanogenesis	0.026216439	68	49
ErbB signaling pathway	0.000149584	67	53
GABAergic synapse	0.000149584	63	54
Gap junction	0.000912029	63	49
Glycerophospholipid metabolism	0.045715783	62	48
TGF-beta signaling pathway	2.60E-06	61	49
Adherens junction	4.25E-05	59	49
Small cell lung cancer	0.048306418	59	50
Melanoma	0.000750905	56	50
Renal cell carcinoma	2.60E-06	55	51
Chronic myeloid leukemia	0.001209651	55	50

Table B.1 Cont'd

Pancreatic cancer	6.99E-05	53	48
Gastric acid secretion	0.036086119	53	50
p53 signaling pathway	0.007338061	51	47
Central carbon metabolism in cancer	0.002716563	50	49
Colorectal cancer	0.002333958	48	47
Prolactin signaling pathway	0.039288332	48	47
Glioma	0.001387402	47	51
mTOR signaling pathway	0.004243804	47	48
Amphetamine addiction	0.036086119	46	42
Endometrial cancer	0.010071225	39	47
Lysine degradation	0.003997008	36	46
Cocaine addiction	0.01535183	34	41
Endocrine and other factor-regulated calcium reabsorption	0.032092955	31	47
Circadian rhythm	0.021206741	25	43
Prion diseases	0.004555208	18	31
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	0.035850118	18	32
Fatty acid biosynthesis	2.34E-05	9	23