ROLE OF MICRORNAS IN TUMORIGENESIS AND THEIR MODULATION BY VERSICAN 3’ UNTRANSLATED REGION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Laboratory Medicine and Pathobiology
University of Toronto

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2010

Abstract

MicroRNA is a single-stranded RNA of about 22 nucleotides in length and is expressed endogenously. It functions as a gene regulator by pairing imperfectly with 3’ untranslated region (3’UTR) of target mRNAs, leading to translational inhibition. MicroRNA is implicated in many regulatory pathways and hence affects various cellular activities. In the development of cancer, genetic alterations occurred at miRNA locus and its expression level is dysregulated in various cancers versus normal tissue counterparts. It is thus important to find the targets of dysregulated microRNAs contributing to progression of cancer. To facilitate long term functional studies, a microRNA expression construct with unique futures was generated. Stable expression of miR-378 enhanced cell survival, reduced caspase-3 activity, and promoted tumor growth and angiogenesis. By algorithmic predictions and proteomic analysis, two tumor suppressors, SuFu and Fus-1, were found to be translationally regulated by miR-378. Target validation was confirmed by co-transfection experiments and luciferase activity assays, reassuring its oncogenic role by regulating two tumor suppressor genes simultaneously. Conversely, microRNA can also function as a tumor suppressor by modulating expression of Versican, an extracellular matrix protein known to facilitate tumorigenesis and angiogenesis. By a novel PCR method, more than one microRNA were found to bind to Versican 3’UTR.
Among these microRNAs, targeting of Versican and Fibronectin by miR199a-3p was validated. Expression of a fragment of Versican 3’UTR was expected to antagonize the function of miR-199a-3p. Stable expression of Versican 3’UTR resulted change in cell morphology and increased cell-cell adhesion. Analysis of primary tissues from transgenic mice expressing versican 3’UTR showed an increase expression of Versican and Fibronectin, and organ adhesion was found between liver and its surrounding tissues. In addition, 3’UTR also modulated the level of miR-199a-3p and miR-136, alleviating translation of negative cell cycle regulators, PTEN and Rb1. This resulted in reduced cell proliferation and hence diminished tumor growth. These findings suggest a role of microRNA in tumor growth, providing a valuable target for therapeutic intervention.
Acknowledgments

In blink of an eye, my life as a graduate student is truly coming to an end. In these five years, people have walked in and out of my life, but their warm friendship remains at the bottom of my heart.

First and foremost, I must thank my supervisor, Dr. Burton B. Yang, for always being there for me. You have given me opportunities as well as challenges by providing me various interesting projects to work with. Your encouragement and guidance have always been greatly appreciated, and my research career would have never started with such an enriching experience without you. I thank my advisory committee members, Dr. Heyu Ni and Dr. Arun Seth, for your keen advice and supportive feedback. You have encouraged me to think broader and more critically- a skill I would definitely need in my future career.

Thanks to all the helping hands that have been there with me during my stay in Sunnybrook. To Samantha, Deng, Titan, Xiong, and Shireen, my collaborators; Tatiana, Carol, Zina, William, Jennifer, and Minhui, thank you for your help and assistance whenever I needed; Dave, Amy, Bing, Lillian, Vinaya, and Casey, who have taught me fundamentals and given me your care; Rachel, Linda, JJ, JI, Eric, Jana, Judy, Les, Nick, Yuqin, Cynthia, Jeya, Lena, Stephen, Fred, Melinda, and all my friends in Sunnybrook. My pursuit of this degree would have been mostly frustration and work spotted by moments of excitement if your friendship was not present. I thank you for your scientific contribution and unwavering support. Your presence has made my journey here filled with laughs and sweats. I will never forget these joyful moments. To David and Samuel, my best friends, I thank you for just listening to me in countless occasions.

Lastly, I must thank my parents for your continued support and patience, and my brother, Franck, for believing in me while I am going through these five years of training. You all have always been my motivation in difficult times. Without you, I would not have transformed into the person I am today. For this, I am sincerely and humbly grateful.

Daniel Y. Lee

April 22, 2010
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ABBREVIATIONS

3’UTR = 3’ Untranslated Region
5’UTR = 3’ Untranslated Region
EGFR = Epidermal Growth Factor Receptor
FACS = Flow Assisted Cell Sorting
FBS = Fetal Bovine Serum
FN = Fibronectin
GFP = Green Fluorescent Protein
miRNA = microRNA
PcG = Polycomb Group proteins
PCR = Polymerase Chain Reaction
PI3K = Phosphatidylinositol-3 Kinase
Piwi = P-element induced wimpy testis
PRC2 = Polycomb Repressive Complex 2
Pri-miRNA = primary microRNA transcript
pre-miRNA = microRNA precursor
PTEN = Phosphotase and Tensin Homolog
Rb1 = Retinoblastoma 1
RNase P = Ribonuclease P
RTK = Receptor Tyrosine Kinase
SuFu = Suppressor of Fused
VCAN = Versican
VEGF = Vascular Endothelial Growth Factor
Xist = X-inactive-specific-transcript
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Chapter 1

Literature Review
1.1 Overview

The human genome contains approximately 250,000 protein-coding genes, comprising merely 1.5% of our genome [1]. Expression pattern of these genes is unique in different tissues, and has to be carefully coordinated to achieve homeostasis and proper functionality in response to external stimuli. Biological functions are mostly mediated by proteins, but they often require activation and modification to maintain proper conformations before exerting their roles. Besides physical and chemical modification of proteins, there are also multiple regulatory barriers to the transcription and translation of genes. To undergo transcription, protein transcription factors play key roles in initiating transcription through their DNA-binding domains. Transcription factors interact with cis-elements present in the promoter regions of a gene for transcription activation. In a mRNA, 5’ untranslated region (5’UTR) contain regulatory elements that are important for translation initiation and RNA stability. Regulatory elements present in the 3’ untranslated region (3’UTR) also govern RNA stability. RNA decay can be preceded by endonuclease activity activated through the Iron-Responsive Elements (IREs), or by deadenylation, which is regulated by AU-rich elements (ARE). Interestingly, the 3’UTR of mRNA has evolved to play a regulatory role by enabling additional translational control. Translational efficiency of mRNAs has been found to be determined by the abundance and the types of miRNAs that are present in the cell.

In 1993, the first miRNA from Caenorhabditis elegans was discovered by Ambros and his coworkers [2]. Mutations in two genes, named lin-4 and lin-14, resulted in failure of worms to mature and differentiate properly during development. Further characterization of the genes was surprising because lin-4 only transcribed a 22-nucleotide RNA that encoded no protein. The short RNA sequence complemented to
multiple sites in the 3’ untranslated region (3’UTR) of lin-4 mRNA, suggesting a potential RNA interaction between individual RNA strands. And hence it was proposed that lin-4 is an endogenous regulator that inhibited translation of lin-14 mRNA by binding to its 3’UTR. It was not until 2001 when the word of miRNA was coined to recognize this broad class of small RNA regulators found in both plants and animals. miRNAs are defined as non-coding RNA sequences about 22 nucleotides in length, and they regulate gene expression in a sequence-specific manner. miRNAs are evolutionarily conserved across species [3]. Depending on sequence familiarity, miRNAs are grouped into families. Family members usually share high similarity in their seed sequence (nucleotide 2 to 7), and are thought to target the same genes with similar seed sequence. Up to January 2010, 721 miRNAs were found and validated in the human genome [4]. According to studies from a few years ago, the number of miRNAs was expected to be at least a thousand predicted by computer models, which is about 3% of the total number of genes [5]. These miRNAs are expected to regulate one third of human genes [6], but a new prediction model has shown that more than 60% of human protein-coding genes are conserved for miRNA regulation [7]. Profiling of miRNA expression in different tissues yields the tissue-specific expression of miRNA, and this specificity offers suggestions as to their ability in determining cell fate [8, 9]. Expression level of miRNAs is also stage-specific during tissue development, which further implies their importance in developmental control [10]. And hence, our knowledge in miRNA functions expands as more and more findings link miRNA’s function with its targets.

Although miRNA processing has been better elucidated, there is still very little known about how miRNA expression is regulated. Microarray technology development and miRNA-specific advancement has allowed a broader inspection of overall transcription of miRNAs. Large scale investigations using clinical samples has led to the
conclusion that a group of miRNAs are differentially expressed in malignant tumors compared to normal tissues [11, 12]. In order to understand the origin of miRNA transcription, the location of miRNA genes were mapped, and many of them were found to be situated in the reported genomic fragile sites or regions associated with cancer [13]. Growing evidences, including previous experiences with protein-coding genes have shown that genomic alterations, such as chromosomal translocation, amplification, and deletion, are accounted for differential miRNA expressional in cancer [14]. Subsequently, miRNA expression profile in many different cancer types was used as a signature for diagnostic and prognostic purposes. However, miRNAs can be more than just biomarkers that allow us to assess genetic and cellular characteristics of the cells. The ability of miRNA to potentially target multiple genes enables its direct and far-reach effect on promoting cancer. Up-regulation of oncogenic miRNAs lowers expression of tumor suppressor genes, and down-regulation of certain tumor suppressor-like miRNAs allows elevated translation of oncogenes. Understanding the function of these miRNAs and their targets will be an important step in dissecting miRNAs’ effects, reflected by its deregulated status in cancer. These works will not only improve our understanding in the physiological role of miRNA, but also aid in developing a counter strategy against uncontrolled cellular behavior due to dysregulated miRNAs.

1.2 Biogenesis of microRNAs

1.2.1 Transcription of microRNA genes

In order to identify the mode of transcription of miRNAs, their genomic locations are first annotated. According to genomic screening of over two hundred miRNAs, more
than half of these miRNAs are transcriptionally linked to other genes, which codes for proteins or non-coding RNAs (ncRNA) [15]. More specifically, 40% of analyzed miRNAs are located in the intron of protein-coding genes, while 10% of them are transcribed in the intron of ncRNA transcripts. In addition, 10% of miRNAs overlaps with exons of ncRNA, suggesting further complexity in the maturation of miRNAs. In general, two third of miRNAs actually share transcriptional machinery with a protein coding gene or ncRNA.

Besides intronic and exonic miRNA, other miRNAs are located between genes. Many intergenic miRNAs are clustered in the genome. Multiple miRNAs are separated by up to 10 kilobases are transcribed together in one transcript [16]. For the intergenic miRNAs, there has been reports of TATA box binding motifs upstream of miRNA genes [17]. Having their own transcription elements means that miRNAs can be independently transcribed as well, but transcription factors need to be recruited for initiating the transcription of intergenic miRNAs. Recent studies have reported the involvement of several transcription factor, such as MYC and P53, in regulating expression of miRNAs (Table 1.1) [18].

P53 is a well known transcription factor controlling genes involved in a variety of biological activities such as cell cycle and apoptosis. Its expression is usually quite low due to constant degradation by ubiquitination, but its expression stabilizes through transactivation in response to stress signals [19]. Many genes that are activated by p53 are often involved in pro-apoptosis, senescence-induction, and cell-cycle inhibition. Upon p53 activation, several independent studies all identified miR-34a being strongly up-regulated by p53 in many cultured cell lines and also in primary mouse embryonic fibroblast [20, 21]. More interestingly, expression of two other members of the miR-34 family, miR-34b and miR-34c, are also induced by p53. These two family members are
Table 1.1 Transcription factors and specifically regulated miRNAs

<table>
<thead>
<tr>
<th>TF</th>
<th>Regulated miRNA</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>↑ miR-34 family</td>
<td>Suppression of tumorigenesis</td>
</tr>
<tr>
<td>AR</td>
<td>↑ miR-125b-2</td>
<td>Androgen independent growth</td>
</tr>
<tr>
<td>Myc</td>
<td>↑ miR-17-92</td>
<td>Stimulate tumor growth</td>
</tr>
<tr>
<td></td>
<td>↓ miR-15a, miR-34a</td>
<td>Tumor suppression</td>
</tr>
<tr>
<td>E2F family</td>
<td>↑ miR-17-92</td>
<td>Stimulate tumor growth</td>
</tr>
<tr>
<td>MyoD</td>
<td>↑ miR-1, miR-133</td>
<td>Regulation of muscle-specific proteins</td>
</tr>
<tr>
<td>Twist</td>
<td>↑ miR-10b16</td>
<td>breast cancer invasion &amp; metastasis</td>
</tr>
</tbody>
</table>


**Table 1.1 Transcription factors and specifically regulated miRNAs**

This is a list containing some transcription factors that were reported to regulate the miRNA expression. Most of these transcription factors bind to specific regulatory binding sites upstream of miRNAs and recruit co-activators and basic transcriptional machineries.
transcribed from a different chromosome from miR-34a (chromosomes 11 and 1, respectively). In addition, miR-34a and miR-34c have identical seed regions and are thus predicted to target the same mRNAs. Further functional analysis showed that both miR-34b and miR-34c work co-operately with p53 in suppressing cell proliferation [22], while miR-34a induces growth arrest through regulating E2F pathway [23]. This is an example of a transcription factor activating miRNAs transcription in order to function synergistically.

RNA polymerase II is the first enzyme that is thought to be responsible in transcribing the primary miRNA (pri-miRNA) (Fig. 1.1) [24]. Some miRNAs, which are surrounded by Alu repeats area of chromosomes, are found to be transcribed by RNA transcriptase III instead [25]. These transcripts containing pri-miRNA can be several kilobases long, and also have a 5’ 7-methylguanosine gap and a 3’ polyadenylated tail similar to a conventional messenger RNA (mRNA) [26].

1.2.2 microRNA processing in nucleus

To become a mature miRNA from a pri-mRNA transcript that is kilobases long, cleaving and splicing are definitely required during miRNA maturation processes. Long pri-miRNA is first processed into a precursor miRNA of about 70 nucleotides long. It has a hairpin stem of about 33 base-pairs, a terminal loop and two single-stranded flanking regions upstream and downstream of the hairpin.

The cleavage of pri-miRNA proceeds concurrently along with the transcription of the gene or ncRNA [27]. In the nucleus, primary miRNA is first endonucleolytically cleaved by a miRNA microprocessor composed of Drosha and DGCR8 (Pasha). Drosha is a RNase III endonuclease that performs the cleavage through its RNase III domain (Fig.
Fig. 1.1 Canonical pathway of miRNA biogenesis

Primary miRNA is cleaved by the microprocessor in the nucleus. Resulting precursor miRNA is exported from nucleus by Exportin-5. In the cytoplasm, Dicer removes the loop from precursor miRNA. Guide strand of the mature miRNA is loaded with Argonaute (Ago2) into the RISC complex, where it guides RISC to silence target mRNAs. The complementary strand is subsequently degraded.
Fig. 1.2 Diagrams of proteins related to miRNA processing

RNase III domain makes single cut on a RNA strand. Double-stranded RNA-binding domain is expected to bind dsRNA. WW domains binds to proline rich domain at low affinity. CRM1 domain is involved in nuclear/intracellular trafficking and secretion. Helicase domain inhibits RNase III activity. PAZ domain prefers binding to a 3' nucleotide overhang of ssRNA. DUF283 domain may serve a structural role. Function of other domain still remains unknown.
1.2) [28]. It cleaves precisely 11 base-pairs away from the bottom of the hairpin stem where the 5' and 3' flanking regions separate [29]. In order for the microprocessor to recognize pri-miRNA, conservation of the pri-miRNA stem structure is essential. Single Nucleotide Polymorphism (SNP) in the stem has been reported to block proper processing by Drosha [30]. The flanking single-stranded RNA below the stem loop is also mandatory for Drosha cleavage, but the sequence of terminal loop is not important in most cases [31]. Therefore, these fundamental yet conserved requirements allow processing of pri-miRNA to occur even during transcription of the entire transcript.

While Drosha acts as the catalytic subunit of the microprocessor, DGCR8 recognizes the pri-miRNA through its double-stranded RNA binding domain, and also stabilizes its interaction with Drosha through its conserved C-terminal domain [32]. DGCR8 functions as a molecular ruler that allows Drosha to cut at an accurate position. The precursor miRNA (pre-miRNA) that is cleaved by Drosha always has a 2 nucleotide overhang as a characteristic product of RNase III [33]. The 2 nucleotide overhang later becomes an important property for recognition by other proteins in downstream processes.

The expression level of the microprocessor complex and its activity is tightly maintained. The working Drosha-DGCR8 complex also cleaves stem loops present in the 5’UTR and the coding region of DGCR8 mRNA, resulting in its instability [34]. This acts as a feedback mechanism to ensure no additional complex will be assembled. The microprocessor complex can also be customized to enhance processing of a specific miRNA. There has been report of a heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) participating as a subunit in the Drosha-DGCR8 complex. This RNA-binding protein binds specifically to pri-miR-18a but not the other miRNAs in the same cluster, and the knock-down of hnRNP A1 greatly reduces expression of pre-miR-18a [35]. In this case,
hnRNP A1 acts as a trans-acting factor by binding to the conserved loop of pri-miR-18a. This binding changes the conformation of pri-miR-18a and favors its processing by the miRNA microprocessor [36]. However, this mechanism is not applicable to all the miRNAs. miRNA comparisons across species shows that approximately 14% of miRNAs have conserved loop sequences.

After nuclear processing, pre-miRNA is transported by Exportin-5 to the cytoplasm in a Ran guanosine triphosphate-dependent manner [37]. Exportin-5 recognizes pre-miRNA not by its sequence but by its characteristic hairpin stem and 3’ protruding overhang [38]. These recognition requirements allow the transport of correctly processed pre-miRNAs only. High affinity binding of Exportin-5 to pre-miRNA protects it as soon as it is processed by Drosha [39].

1.2.3 microRNA processing in cytoplasm

Once inside the cytoplasm, pre-miRNA is further processed into a shorter double stranded RNA duplex, composing of a mature strand and a complementary strand. This processing is carried out by another RNase III endonuclease, Dicer [40]. Dicer has a PAZ (Piwi-Argonaute-Zwille) domain (Fig. 1.2), which preferentially binds to the 3’ overhang of single stranded RNA [41]. The distance between its PAZ domain and two RNase III domains is 65 angstroms, which is about 25 nucleotides in length according to crystal structure analysis. Therefore, after excision of pre-miRNA terminal loop, double stranded RNA is about 22-25 nucleotide in length with 2 nucleotides overhang on each 3’ side [41, 42]. Knocking down the expression of Dicer greatly reduces miRNA maturation [43], and deletion of Dicer in mice leads to lethality in the early development [44]. This result has pointed out the importance of miRNAs in maintaining embryonic
stem cell population, and has led to other studies on miRNA's function in embryonic cells [9].

Additional mechanisms that regulate Dicer processing have been reported in the literature. Dicer is shown to be negative regulated by its product, let-7 miRNA, by targeting the coding region of Dicer mRNA [45]. Targeting of Dicer mRNA leads to the negative feedback of Dicer expression and thus its activity. In one study, pre-miR-138 was observed in abundance in the cytoplasm of many cell types, but mature miR-138 was found to only in certain cell types. This observation points out the possibility that miRNA expression can be regulated post-transcriptionally and that miRNA expression could be restricted in distinct cell types [46]. Although the specific mechanism is not found, but it is possible that a tissue-specific RNA binding protein is required to recruit Dicer in the processing of pre-miR-138. A ternary complex has been reported where TAR (Human Immunodeficiency Virus Trans-Activating Response) RNA Binding Protein (TRBP) and Protein Activator of PKR (PACT) assembles with Dicer. Dicer alone is sufficient for pre-miRNA processing. Like DGCR8 to Drosha, TRBP acts to stabilize Dicer [47]. Both TRBP and PACT were shown to enhance binding of RNA to Dicer and greatly facilitates the processing efficiency of Dicer [48]. By default, the helicase domain of Dicer inhibits its own cleaving activity [49]. TRBP interacts directly to the helicase domain of Dicer and changes it conformation to improve its processing efficiency. In addition, TRBP also functions by recruiting the excised RNA duplex to its final stop, the Argonaute proteins [50].

1.2.4 Guide strand loading into RISC

Following excision by Dicer, short miRNA duplex is formed. Dicer and its
cleavage-associated proteins TRBP or PACT dissociate from the miRNA duplex. However, only one strand from the miRNA duplex will be the guide strand, which in terms means guiding the functional unit. The other strand of the duplex is referred as the complementary strand and is degraded subsequently. The mechanism for this asymmetry in strand selection still remains unclear [51], but it is suspected that strand selection is dependent on subunits of a ribonucleoprotein complex called the RNA-induced Silencing Complex (RISC). RISC is composed of Dicer, TRBP for RNA binding, and Argonaute2 (Ago2) for catalytic activity [52]. Ago2 seems to pertain the ability to remove complementary strand by initiating an endonucleolytic cut at the passenger strand [53]. Degradation of the passenger strand facilitates the loading of the guide strand and activation of RISC. In vitro reconstitution experiments has shown that no chaperons or co-factors other than the basic machineries are required for RISC formation [54]. Dicer, TRBP, and Ago2 are the basic components needed for miRNA processing and functioning. However, particular helicases may be involved in facilitating unwinding of specific miRNAs. For example, P68 RNA helicase has been shown to facilitate selection of let-7 by RISC, and knock-down of P68 RNA helicase has been shown to inhibit let-7 functioning [55].

Theoretically, either strand of the miRNA duplex can be the guide strand. A study has attempted to predict strand selection by comparing outcomes of selection in validated precursor miRNA pairs with random RNA duplexes [56]. Random RNA duplexes do not form good base-pairing stems. It was found that miRNA pairs generally exhibit lower free energy than random duplexes. Moreover, the most profound effect on stability was on the first nucleotide of 5' anti-sense termini. Base-pairing including U nucleotide, such as A:U, U:A, G:U, are particularly not as stable compared with C:G pairs, which allows its incorporation into RISC. The ease in unwinding the RNA duplex
might explain in part why many miRNAs that start with a U nucleotide are selected.

1.2.5 miRNA editing

There has also been many reports of miRNA modifications. MiRNA modifications affect base pairing and stability within the miRNA duplex, and thus most likely will influence strand selection. miRNA editing occurs after transcription by replacing adenosine (A) with Inosine (I). Inosine differs from guanosine only in the lack of an exocyclic amine group, and preferentially pairs with cytosine. Adenosine Deaminases acting on RNA (ADAR) is involved in recoding RNA sequences [57]. ADAR is known to function sometimes in genes but mostly in non-coding repetitive RNA sequences [58]. In an attempt to investigate the frequency of miRNA editing and its potential editing sites, Blow and coworkers sequenced 99 miRNAs and found that there was A-to-I editing in at least 6% of miRNAs [59]. The presence of edited nucleotides in the pri-miRNAs indicates that RNA editing occurs early in miRNA biogenesis. This means that nuclear processing by Drosha, exporting by Exportin-5, cytoplasmic processing by Dicer, loading into RISC, and complementarity to target sites can be potentially affected. As an example, a recent study has shown that ADAR edits miR-22 precursor, potentially affecting miRNA maturation, strand selection, and thus site targeting [60]. Another study looked into the RNA editing of miR-151 primary transcript, which resulted in complete blockage of its cleavage by Dicer, and thus edited pre-miR-151 RNAs accumulated in the cytoplasm [61]. By comparing the original and edited miRNAs in each stage of processing, the effect of RNA editing on miRNA biogenesis was more thoroughly investigated. Most edited primary miRNAs analyzed were found to be suppressed in their processing by Drosha although in some cases, deamination at a particular position
appeared to enhance Drosha processing [62]. Wildtype or edited RNA was then subjected to *in vitro* processing assays using primary or precursor miRNA processing machineries. Interestingly, deamination at different positions had different effects on Drosha and Dicer processing. RNA editing affects the processing of miRNAs, indicating that the major function of primary miRNA editing is the modulation of miRNA biogenesis and miRNA expression levels, rather than generation of new mature miRNAs and redirection of target gene selection. Therefore, RNA editing provides a source of control for miRNA-mediated gene regulation.

1.2.6 Importing mature microRNA back to nucleus

Mature miRNAs are excised to about 22 nucleotides in the cytoplasm, and is subsequently incorporated into RISC to guide gene silencing. Despite the short size of mature miRNA, miR-29b was found to contain a nuclear localization element (AGNGUN, where N is any nucleotide) besides a conserved seed sequence for gene targeting [63]. Since the seed sequence is located at the 5’ end, the nuclear localization element is located at the 3’ end of miR-29b. The other members of miR-29 do not have the same hexanucleotide sequence although they are transcribed together in a polycystronic transcript. This study not only suggests that family members are functionally redundant, but it also introduces studying miRNA functionality in the nucleus.

Further studies proceed by searching for transporters that carry miRNA to the nucleus. A specific member of the Argonaute family, Nuclear RNAi Defective-3 (NRDE-3), is responsible for transporting siRNAs but not long dsRNA from the cytoplasm to the nucleus [64]. NRDE-3 normally resides in the cytoplasm. Upon binding to RNA, it then redistributes to the nucleus. Since this is a study performed in *C. elegans*,
the short RNA transported in human may be involved with gene regulation rather than simply gene silencing. Besides NRDE-3, another member of the Argonaute family, Ago2, is also found inside the nucleus. This nuclear subpopulation of Ago2 is transported by Importin-8, and knock-down of Importin-8 reduces the nuclear presence of Ago2 [65]. Importin-8 normally resides in processing-bodies (P bodies), and is recruited to Ago2-associated protein complex for nuclear localization and potentially for miRNAmediate gene regulation.

The presence of Argonaute proteins and mature miRNAs in the nucleus suggests their regulatory role in transcription. A conserved miRNA, miR-320, is encoded within the promoter region of a cell cycle gene, POLR3D, in an antisense orientation. It is found to provide negative feedback to POLR3D transcription by base-pairing with sense strand in perfect complementarity [66]. Since transcription is affected instead of translation, a different protein complex composed of Argonaute-1 (AGO1), Polycomb Group (PcG) component EZH2, and tri-methyl histone H3 lysine 27 (H3K27me3), is found associated with miR-320. This miRNA-associated protein complex is referred as the Transcription Silencing Complex. In another study, mature miR-206 was found to accumulate with 28S rRNA in both cytoplasm and nucleolus, while the miR-206 precursor was found in the cytoplasm [67]. Precursor and mature forms of other miRNAs have also been found clustered in the nucleolus [68]. It is not clear why miR-206 is associated with rRNA, but further modification of miRNAs or targeting of mRNAs that transit to the nucleolus are suspected. Therefore, besides the known role of miRNA-mediated post-transcriptional gene regulation in the cytoplasm, recent evidence indicates that some miRNAs may also have nuclear roles in regulating transcription.
1.2.7 microRNA degradation

Compared to the extensive study on miRNA biogenesis, relatively less is known about miRNA degradation. In Arabidopsis, Small RNA Degrading Nuclease 1 (SDN1) is the 3’ to 5’ exonuclease that is proven to degrade mature miRNAs specifically [69]. Knockdown of SDN genes in vivo results in elevated miRNA levels and developmental defects. The other exonuclease, XRN2, degrades in 5’ to 3’ fashion [70]. The same genes are present in eukaryotes and are expected to exert similar functions.

Although degradation pathways are not fully understood, observations of modified RNAs are reported to affect miRNA degradation. Adenylation of miRNAs is found to prevent them from degrading in plants, specifically P. trichocarpa [71]. In Arabidopsis, HEN1 was found to be a miRNA methyltransferase which methylates the 3’ end of miRNAs [72]. In HEN1 mutants, miRNAs are not methylated. As a result, miRNAs decrease in abundance but increase in length by addition of one to five uridines. The function of uridylation on miRNA is currently unknown, but uridylation has been associated with miRNA-mediated mRNA degradation [73]. Past studies have shown that the 3’ end of miRNA-mediated cleavage products are tagged with one to twenty-four uridines, and they are associated with shortening of 5’ end by decapping. Further evidence is still required to determine the fate of uridynated miRNAs in mammals.

RNA editing has also been shown to affect the fate of transcribed miRNAs. Hyper editing occurs when up to 50% of adenosines are converted to inosine in dsRNA. In a study following the flow of hyper-edited RNA duplex, the RNA duplex that contains multiple and alternating U:I and I:U pairs are degraded by a cytoplasmic ribonuclease, Tudor Staphylococcal Nuclease (Tudor-SN) [74]. Tudor-SN has been found to be a subunit of RISC, and preferentially cleaves dsRNA containing Inosine–Uracil pairs [75].
An artificial RNA duplex was used to illustrate the ability of Tudor-SN in degrading hyper-edited RNAs. In a later study, pre-miR-142 is shown to be edited in hematopoietic tissues by ADAR [57]. As a result, its processing by Drosha is suppressed and the expression level of mature miR-142 is low as expected. Conversely, mature miRNA-142 level increased substantially in ADAR null mice. Therefore, RNA editing is a potential pathway that miRNAs can be degraded post-transcriptionally in the cytoplasm. However, more work is currently needed in describing when and how miRNAs are degraded after their transcription.

1.3 Post-transcriptional regulation by microRNA

1.3.1 Translational inhibition

Mature miRNAs are eventually transferred to Argonaute proteins, and guides the RISC in RNA silencing. To regulate translation, miRNA base-pairs with its target sites in 3’UTR of mRNA [76]. Translational repression has also been reported when target sites are located in 5’UTR or coding regions [77, 78]. However, the mechanistic details of miRNA functioning in translational repression are still poorly understood. Results from different studies are sometimes contradictory because they are conducted in different systems and species. Therefore, potential outcomes of miRNA-mediate translational repression using results from some current studies will be briefly described in the following sections (Fig. 1.3).

1.3.1.1 Repression at translational initiation

Translational initiation starts with the recognition of mRNA 5’ cap structure (Fig. 1.4),
**Fig. 1.3 Proposed mechanism of miRNA-mediated translational inhibition**

RISC incorporated with miRNA exerts translational inhibition through multiple mechanisms. By interacting with other proteins, it blocks translation elongation by promoting ribosome drop-off a), degrading the nascent translated polypeptide b). Other mechanisms such as blocking translational initiation c) and promoting mRNA degradation d) will eventually transport mRNA to processing bodies.
Fig. 1.4 Diagram of translation initiation complex including eIF4E

Eukaryotic initiation factor 4 (eIF4) has been known to participate in recruitment of mRNA to the ribosome. Cartoon representation of its associated proteins during translation initiation is shown.
m\textsuperscript{7}GpppN (7-methylguanosine, where N is any nucleotide), by eIF4E, which is a subunit of the eukaryotic translation initiation factor eIF4 [79]. eIF4G, another subunit of translational initiation complex, interacts with eIF3, which recruits 40S ribosomes, and also Polyadenylate-binding Protein 1 (PABP1), which binds to the poly(A) tail of the interacting mRNA. Therefore, the translational initiation complex allows circularization of mRNA by interacting with its 5' cap through eIF4E and its 3' poly(A) tail through eIF4G:PABP1 interaction. This has been suggested to enhance translation efficiency by ribosome recycling [80]. An alternative pathway to initiate translation is through recruiting 40S ribosomes from Internal Ribosome Entry Site (IRES), which is usually located at 5’ UTR [81]. The exact detail of miRNA-mediated regulation is still under debate.

A study carried out in human cells shows that mRNAs with 5’ cap and 3’ tail are susceptible to miRNA-mediated translational inhibition but not mRNAs with IRES or unmethylated cap [82]. The importance of methylated cap for translational repression is further corroborated by experiments using bi-cistronic mRNAs which contain a methylated cap in 5’ end of first Open Reading Frame (ORF) and IRES at 5’ end of second ORF [83]. These data suggest translational repression is due to recognition of eIF4E by 5’ cap or recruitment of eIF4G by eIF4E (Fig. 1.3C). It is also possible that interaction between PABP1 and eIF4G is blocked. One study supports the notion that recognition of 5’ cap by eIF4E is interrupted. This study argues that the similarity of two conserved aromatic rings in both Ago2 and eIF4E are important for cap structure recognition [84]. Single amino acid mutation in the aromatic rings from phenylalanine to tryptophan affects translational repression but not Ago2’s loading of miRNA nor its catalytic activity. This data indicates that Ago2 competes for binding with 5’ cap and thus prevents translational initiation. In consistency with this finding, the addition of purified
initiation factor eIF4F rescues mRNA from miRNA-mediated inhibition [85].

Another notion regarding miRNA-mediated translational inhibition is that there may be a lack of ribosome assembly during translation initiation. In a study, eIF6 and 60S ribosomal subunit proteins were co-immunoprecipitated with RISC in human cells [86]. eIF6 is known for its role in ribosome assembly by binding to 60S ribosome, preventing its interaction with 40S ribosomal subunit [87]. Knock-down of eIF6 by siRNAs markedly relieved miRNA-mediated repression on expression of reporter construct, whereas the mutant construct was unaffected. Further investigation is needed in helping to explain how eIF6 is recruited to RISC. It is also unclear why mRNA with IRES is translated without perturbation in this case.

1.3.1.2 Repression during translational elongation

Results from several studies have proposed that premature ribosomes drop-off during translation (Fig. 1.3A). In yeast, a transcription factor Hac1p is found only translated when an intron at the 3′ end of its mRNA is removed by splicing [88]. Unspliced HAC1 mRNA is deposited in poly-ribosomes but its translation is stalled. Base-pairing of this intron sequence with 5'UTR diminishes ribosome loading, while mutation of either intron or a 20 base-pair sequence in the 5'UTR restores Hac1p mRNA translation. Another study describes the association of Oskar mRNA with poly-ribosomes without translation in Drosophila. Nascent polypeptide-associated Complex (NAC) is found to be essential for Oskar mRNA localization and hence its translation during development [89]. Later, Bruno is found to bind to Bruno Response Element at 3'UTR of Oskar mRNA and also interacts with EIF4E binding protein Cup [90]. Therefore, it is possible that miRNA inhibits translation using a similar mechanism.
More evidence will be required to demonstrate the involvement of miRNAs in stalling translational elongation by interrupting ribosomes.

1.3.1.3 Repression through nascent peptide degradation

The finding of translationally repressed mRNAs associated with polysomes has led to speculations that proteins are continuously synthesized from these mRNAs. Proteins are not detected due to rapid degradation of newly synthesized polypeptides by proteases recruited by miRNA-incorporated RISC (Fig. 1.3B). In a study carried out in *C. elegans*, lin-4 is found to target 3'UTR of lin-14 mRNA and repress its translation [91]. The transcription process of lin-14 mRNA, its state of polyadenylation, and association with polysomes are not affected, and its translational initiation is normal under various tests and assays. The reason may be that translational elongation was blocked or synthesized polypeptide was released prematurely. In a later study, partially complementary siRNAs are used to investigate miRNAs-mediated translational repression in human cells [92]. Contradictory to other findings, IRES-initiated translation is repressed by short RNAs. In a bicistronic reporter system, partially complementary siRNA reduces translational efficiency by 2-fold, but the expression of suppressor tRNA overcomes this inhibition due to ribosome pausing at stop codon. In addition, the repressed mRNAs associated with polysomes are detected with peptidyl transferase activity. Pulse-chase experiments by radioactively labeled methionine showed presence of smears which represent short-lived nascent polypeptide chains. These evidences suggest that translational repression do happen after initiation of translation. However, further efforts are need to specify the associated proteases.
1.3.1.4 Repression by mRNA degradation

Messenger RNA degradation can be initiated terminally by either decapping or deadenylation [93], although sequence-specific endonucleolytic cleavage by polysomal ribonuclease 1 (PMR-1) may also occur (Fig. 1.5) [94]. In general, mRNA degradation usually begins with deadenylation from 3’ end of mRNA by deadenylases. Following deadenylation, decapping enzyme, such as DCP1 and DCP2, removes 5’ cap of mRNA, exposing mRNA for exonucleolytic decay from 5’-to-3’ by enzyme XRN1. Alternatively, deadenylated mRNAs can be degraded in 3’-to-5’ direction by cytoplasmic exonucleases.

In a study investigating miRNA-mediated translational repression, some miRNA-targeted mRNAs are found destabilized (Fig. 1.3D) [95]. This study is carried out in *D. melanogaster*. Argonaute protein, Ago1, is found to interact with GW182, a marker for processing bodies where mRNAs are degraded or stored [96]. The same interaction is also found in human cells [97]. GW182 is shown to function downstream of Argonaute proteins by silencing mRNA expression and promoting mRNA degradation. For GW182-mediated mRNA decay, CCR4:NOT is recruited for deadenylation, and DCP1:DCP2 decapping complex is also required. Results from another study also supports the importance of decapping in miRNA-mediated mRNA decay [98]. Decapping activators are depleted in the experiment, which leads to the accumulation of deadenylated mRNAs while suppressing miRNA-mediated gene silencing. However, these studies also show that translational repression and mRNA degradation can occur as a combination. The subunits that are recruited during target site recognition by miRNA-incorporated RISC may determine the degree of translation inhibition.
Degradation of mRNAs by deadenylation-dependent decapping is the major mechanism by which mRNAs are degraded. After deadenylation or decapping, 3'-to-5' or 5'-to-3' exonucleolytic decay is followed respectively.
1.3.2 Target switching by RNA editing

Most edited primary miRNA are less frequently processed by the miRNA biogenesis machineries and hence edited miRNA is unlikely to exert its function. However, there are still cases reporting that edited miRNAs provide an alternative function. It was found that deamination in the seed region of miR-376 is a tissue-specific modification [99]. For example, editing frequency of pri–miR-376a is about 98% in the human medulla. Consequently, only the edited version of mature miR-376a2-5p is detected in this region of the brain. Moreover, editing in the seed sequence of miR-376 resulted in alteration of its targets. After miRNA editing, Phosphoribosyl Pyrophosphate Synthetase 1 (PRPS1) became its new target in vivo. PRPS1 is involved in uric acid synthesis pathway and thus edited miR-376 became an example of tissue-specific regulation in uric acid level by miRNA.

A-to-I editing by ADAR has also been reported in 3’UTR. In the three thousand deaminated sites assessed, potential miRNA target sites are generated due to how edited 3’UTR complements a subset of miRNAs [100]. This result reveals the creation of novel miRNA target site by ADAR’s RNA editing activity.

1.3.3 Translational Activation

Translational repression mediated by miRNAs is a well accepted mechanism for post-transcriptional gene regulation. However, few exceptions to this concept have been discovered. AU-Rich Element (ARE) is a cis-element that affects mRNA stability and translation in response to stimuli [101]. Upon serum starvation, ARE in the 3’UTR of Tumor Necrosis Factor-alpha (TNF-α) transforms into an activation signal during cell cycle arrest [102]. Immunoprecipitation reveals that Fragile-X-mental-retardation-related
protein 1 (FXR1) and Ago2 are associated with ARE diffusely in the cytoplasm during translational activation. Further analysis showed that miR369-3 recruits the association of these proteins with the AREs to activate translation by base-pairing with 2 target sites within the ARE region of TNF-α 3’UTR [103]. This study showed that switching between repression and activation in coordination with cell cycle phases. During cell proliferation, miRNAs repress translation. While cells arrests or differentiate, miRNAs may activate translation instead.

Besides translational activation, a case of miRNA-mediate transcriptional activation has also been reported. By in silico scanning, miR-373 was found to target promoter of E-Cadherin and cold-shock domain-containing protein C2 (CSDC2), and promoter targeting induced transcriptional activation [104]. Mismatch mutations of miR-373 indicates that gene induction was specific to the miR-373. Although the proteins associated with miR-373 are not specified in this study, this finding revealed a new mode for miRNA functioning.

1.4 microRNAs contribute to progression of cancer

Malignant transformation is a multi-step process of normal cells acquire genetic and epigenetic alterations [105]. During this process, cells may gain changes that enhance growth and survival (oncogenic) and ones that impair cancer progression (tumor suppressor-like). At the end of the transformation processes, transformed cells are growth independent, apoptosis resistant, tissue invasive and metastatic. Oncogenes and tumor suppressor genes are particularly powerful in transforming cells because they often strongly affect proliferation, apoptosis, or even both. For example, p53 is a well-known tumor suppressor that monitors cell cycle and induces apoptosis, but is
missing or mutated in 50% of all human cancers [106].

1.4.1 miRNAs in cancer diagnosis

Today, miRNAs are gaining increasing importance as gene expression regulators. Its ability to affect cellular activities by regulating gene expression makes it potentially powerful in contributing to malignant transformations. Analysis of a number of human tissue and cell lines shows that miRNA precursor processing is reduced in cancer cell lines [107]. Many of these precursor miRNAs are retained in the nucleus, and thus they are not processed into mature miRNAs. This result is in consistence with an observation of global reduction of mature miRNAs in tumors compared with normal tissues [108]. Loss of Dicer expression has been reported in tumors as well [109]. In addition, knockdown of other major miRNA biogenesis machineries- Drosha, DCG8, and Dicer, substantially decreases production of miRNAs and promotes a more transformed phenotype of cells tested [110]. All of these data suggest that miRNA are expressed to maintain proper cell functioning.

Expression of miRNAs is different in tumors compared with levels in normal tissues. For example, mir-125b, mir-145, mir-21, and mir-155 expression are clearly deregulated in breast cancer, as confirmed by microarray and northern blot [111]. Deregulation of miRNA expression may be a result of genetic alteration. In a study, 53% of 200 surveyed miRNAs genes are found located at fragile sites of chromosomes, where miRNA genes are much more likely to be susceptible to amplification, deletion, or breakpoint [13]. This finding, however, suggests that miRNAs may have a crucial role in cancer development. Over-expression of oncogenic miRNAs can result in down-regulation of tumor suppressor genes, whereas under-expression of tumor suppressor-like miRNAs will
lead to up-regulation of oncogenes. Therefore, studies linking the targets of these miRNAs with their functions will unveil the underlying role of miRNAs in cancer (Table 1.2).

1.4.1.1 miRNAs as oncogenes

1.4.1.1.1 miR-17-92-1 cluster

The miR-17-92-1 cluster is located in a non-protein-coding gene C13orf25 at 13q31 [112], and it transcribes a polycystronic transcript encoding a number of miRNAs- miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-20a, miR-19b-1, and miR-92-1. This chromosomal region was found to be amplified in diffuse large B-cell lymphoma and a number of other lymphomas, leading to over-expression of these miRNAs. In fact, expression of these miRNAs were also found in other solid tumors [12]. Over-expression of these miRNA promotes proliferation and inhibits apoptosis through working synergistically with c-myc to accelerate tumor development [113]. Dysregulated expression or functioning of c-myc is one of the most frequently cause of malignancies. It was later demonstrated that c-my activates transcription of the miR-17-92-1 cluster, as well as the transcription factor, E2F1, which regulates cell cycle progression (Fig. 1.6A) [114]. Interestingly, E2F1 transcription factor is the best-characterized target of the miR-17-92-1 cluster through miR-17–5p and miR-20a. This negative feedback provides moderate control over the proliferation signal by E2F1. Further studies then showed that the core promoter region of miR-17-92-1 cluster contains transcription factor binding sites for E2F1, E2F2, and E2F3 [115]. Therefore, the miR-17-92-1 cluster has a role in fine tuning E2F signaling pathway (Fig. 1.6B).
<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Dysregulation</th>
<th>Function</th>
<th>Validated targets</th>
<th>Oncogene (ONC) or tumour suppressor (TS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15a and miR-16-1</td>
<td>Loss in CLL, prostate cancer and multiple myeloma</td>
<td>Induces apoptosis and inhibits tumorigenesis</td>
<td>BCL2, WT1 RAB9B and MAGE83</td>
<td>TS</td>
</tr>
<tr>
<td>let-7 (a, b, c, d, e, f, g and i)</td>
<td>Loss in lung and breast cancer and in various solid and haematopoietic malignancies</td>
<td>Induces apoptosis and inhibits tumorigenesis</td>
<td>RAS, MYC and HMGA2</td>
<td>TS</td>
</tr>
<tr>
<td>miR-29 (a, b and c)</td>
<td>Loss in aggressive CLL, AML (11q23), MDS lung and breast cancers and cholangiocarcinoma</td>
<td>Induces apoptosis and inhibits tumorigenicity. Reactivates silenced tumour suppressor genes</td>
<td>TCL1, MCL1 and DNMTs</td>
<td>TS</td>
</tr>
<tr>
<td>miR-34</td>
<td>Loss in pancreatic, colon, breast and liver cancers</td>
<td>Induces apoptosis</td>
<td>CDK4, CDK6, cyclin E2, EZF3 and MET</td>
<td>TS</td>
</tr>
<tr>
<td>miR-145</td>
<td>Loss in breast cancer</td>
<td>Inhibits proliferation and induces apoptosis of breast cancer cells</td>
<td>ERG</td>
<td>TS</td>
</tr>
<tr>
<td>miR-221 and miR-222</td>
<td>Loss in erythroblastic leukaemia</td>
<td>Inhibits proliferation in erythroblasts</td>
<td>KIT</td>
<td>TS</td>
</tr>
<tr>
<td>miR-221 and miR-222</td>
<td>Overexpression in aggressive CLL, thyroid carcinoma and hepatocellular carcinoma</td>
<td>Promotes cell proliferation and inhibits apoptosis in various solid malignancies</td>
<td>p27, p57, PTEN and TIMP3</td>
<td>ONC</td>
</tr>
<tr>
<td>miR-155</td>
<td>Upregulated in aggressive CLL, Burkitt's lymphoma and lung, breast and colon cancers</td>
<td>Induces cell proliferation and leukaemia or lymphoma in mice</td>
<td>MAF and SHIP1</td>
<td>ONC</td>
</tr>
<tr>
<td>miR-17–92 cluster</td>
<td>Upregulated in lymphomas and in breast, lung, colon, stomach and pancreatic cancers</td>
<td>Induces proliferation</td>
<td>E2F1, BIM and PTEN</td>
<td>ONC</td>
</tr>
<tr>
<td>miR-21</td>
<td>Upregulated in glioblastomas, AML (11q23), aggressive CLL and breast, colon, pancreatic, lung, prostate, liver and stomach cancers</td>
<td>Inhibits apoptosis and increases tumorigenicity</td>
<td>PTEN, PDCD4, TPM1 and TIMP3</td>
<td>ONC</td>
</tr>
<tr>
<td>miR-372 and miR-373</td>
<td>Upregulated in testicular tumours</td>
<td>Promotes tumorigenicity in cooperation with RAS</td>
<td>LATS2</td>
<td>ONC</td>
</tr>
</tbody>
</table>

**Table 1.2 miRNAs function as a oncogene or tumor suppressor gene**

Several of the miRNAs that have been described as oncogenes or tumor suppressors and their targets are listed.

AML, acute myeloid leukaemia; BCL2, B cell leukaemia/lymphoma 2; BIM, Bcl2-interacting mediator of cell death; CLL, chronic lymphocytic leukaemia; DNMT, DNA methyltransferase; HMGA2, high mobility group AT-hook 2; LATS2, large tumour suppressor homologue 2; MCL1, myeloid cell leukaemia sequence 1; MDS, myelodysplastic syndrome; PDCD4, programmed cell death 4; PTEN, phosphatase and tensin homologue; SHIP1, SH2 domain-containing inositol-5'-phosphatase 1; TCL1, T cell lymphoma breakpoint 1; TIMP3, tissue inhibitor of metalloproteinases 3; TPM1, tropomyosin 1; WT1, Wilms tumour 1.

(Croce CM, Nat Rev Genet., 2009)
Fig. 1.6 Involvement of miR-17-92-1 cluster in the E2F signaling pathway

(A) E2F1–3 are involved in a positive auto-regulatory loop. Activation of the mir-17–92 cluster by E2F1–3 would balance the positive auto-regulatory loop of E2F1–3 by a negative feedback loop.

(B) Double feed-forward loop occurs between E2F1–3 and Myc, and the miR-20a. Since E2F1–3 activate the transcription of MYC, and vice versa.

1.4.1.1.2 miR-155

mRNA-155 is coded in the non-coding gene B-cell integration cluster (BIC), and is often accumulated in several B cell lymphomas [116]. Over-expression of miR-155 has also been reported in several solid tumors [12]. The progression of malignancy is provided by the development of transgenic mice over-expressing miR-155 in B cells [117]. When miR-155 is expressed under control of VH promoter-Ig heavy chain Eμ, pre-leukemic pre-B cell proliferation is observed in spleen and bone marrow, which eventually lead to B-cell malignancy. Another miR-155 target, the Tumor Protein 53-induced Nuclear Protein 1 (TP53INP1), is a proapoptotic stress-induced p53 target gene that reduces pancreatic tumor growth [118]. Finding of this target helps to explain the oncogenic role of miR-155 gene.

1.4.1.2 miRNAs as tumor suppressors

1.4.1.2.1 miRNA Let-7 family

Let-7 has many closely related and highly conserved family members [119]. It is lost in many human malignancies [13], and is also the earliest to be proven to regulate several oncogenes, including RAS, HMGA2, and MYC [120-122]. Mutations in the RAS family of proto-oncogenes (comprising of H-RAS, N-RAS and K-RAS) are found in 20% to 30% of all human tumors, and activating RAS gene results in cellular transformation [123]. Interestingly, the 3'UTR of human RAS genes contains multiple target sites for members of Let-7 family. Introducing let-7 into lung cancer cells reduces cell growth in colony formation assay [124]. In a later study, over-expression of let-7 in several human lung cancer cell lines resulted in decreased levels of Ras, and the depletion of let-7 led
to a marked increase in Ras protein expression. Therefore, Let-7 miRNAs function as tumor suppressors by maintaining expression level of oncogenic RAS proteins.

Expression of Let-7 is greatly reduced in breast Tumor-initiating Cells compared with non-Breast Tumor-initiating Cells [125]. Targeting of many different genes by Let-7 miRNAs gives rise to characteristics of Tumor-initiating Cells. In addition, increased expression of let-7 in self-renewing tumor-initiating cells then decreased their proliferation and metastatic capacity.

1.4.1.2.2 miR-15a and miR-16-1

The tumor suppressor-like properties of miR-15a and miR-16-1 are well-studied. Chronic Lymphocytic Leukemia (CLL) is the most common type of leukemia and is characterized by a deletion at chromosome 13q14 [126]. Frequent deletions and loss of heterozygosity in chromosome 13q14 have suggested inactivation of tumor suppressor genes at this location [127]. However, no protein coding genes are found at the time. Deletion analysis finally narrowed down to a fragment containing three non-protein coding genes. One of them is named Leukemia-associated Gene 2 (LEU2), which is flanked by Alu sequences and long interspersed element (LINE) [128]. Later miR-15a and miR-16-1 were found located in the first intron of LEU2, and reduction of both miRNAs is found in 68% of CLL cases. Further study then show that anti-apoptotic B Cell Lymphoma 2 (BCL2) is targeted by miR-15a and miR16-1 [129]. Deletion or inactivity of miR-15a and miR-16-1 relieves translational repression on BCL2. In addition, introducing miR-15a and miR-16-1 are shown to induce apoptosis in a leukemia cell line model.
1.4.2 microRNAs in cancer prognosis

The earliest finding of miR-15a and miR-16-1 playing a causal role in inducing CLL links miRNAs with cancer [130]. Mapping of miRNAs reveals that their genomic location is situated in fragile regions of the genome [13], and hence can substantially contribute to their deregulated expression in tumor tissues. Expression profiling of miRNA has been used extensively in defining the miRNA signature of different cancers, and the classification accuracy is almost 100% [131]. Differential miRNA expression is a feature that can be particularly useful in diagnosis, and it has been identified in lung cancer [132], breast cancer [111], liver cancer [133], pancreatic cancer [134], and more.

For example, miR-15a and miR16-1 have been shown to target BCL2, causing CLL [129]. Therefore, expression of miR15a and miR16-1 contributes to pathogenesis of CLL. In addition, a unique miRNA signature of 13 miRNAs (out of 190 miRNAs analysed) differentiate CLL cases from other leukemias, in accordance with current biomarkers-IGHV mutation and ZAP-70 expression [135]. This miRNA signature is also associated with disease progression.

miRNA expression pattern is also associated with stages and progression of cancer. For example, miRNA profile can reflect tissue of origin and also predicts survival with lung adenocarcinoma [132]. In a recent study, different miRNA signatures was found to be associated with different stages of neoplastic progression and their hallmark capabilities [136]. Pancreatic neuroendocrine tumors of RIP-Tag2 mice expressing SV40 T-antigen in pancreatic β cells were collected in different stages of cancer. miRNA expression was found to be altered in an instructive way during different stages (Fig. 1.7). The differential miRNA expression in different stages of cancer progression is thought to have its functional role that is yet to be elucidated.
Fig. 1.7 Differential miRNA expression associated with cancer progression

Progression from normality to metastasis of the pancreatic islets is marked by distinctive miRNA signatures at different neoplastic stages. Gray arrowhead from Metastasis signature to Met-like tumor indicates the majority of the metastasis signature is present in met-like primary tumors.

(Olson P et al. Genes Dev., 2009)
Our understanding of the roles of miRNAs in cancer is still incomplete and only a handful of tumor-associated miRNAs are shown to act as oncogenes or tumor suppressors. More studies are required to move from miRNA expression profiling to their biological activities in order to understand their involvement in the etiology of malignant transformation.

1.5 Noncoding RNA affects gene expression

Over the last decade, many thousands of noncoding RNAs have been found through microarray and shotgun sequencing [137]. It has become clear that most of our genome is transcribed and a substantial portion of these transcripts do not code for proteins [138]. Sizes of these noncoding transcripts vary from about 22 nucleotides for microRNA to thousands of nucleotides such as Xist RNA (X-inactive-specific-transcript). Characterization of these noncoding RNA (ncRNA) indicates that their promoters are quite conserved in order to ensure its expression [139]. These ncRNAs are distributed in both cytoplasm and nucleus, and their diverse impacts in gene expression have been reported in various studies. For example, microRNAs have been known to modulate mRNA translation, and Xist RNA plays essential roles in chromosome X inactivation. The specific expression profiles of noncoding RNAs within different brain regions and cell populations suggest their intrinsic functions, which are still largely unknown [140].
The ncRNAs have been studied in mammals, plants, fungi and various other organisms and their diverse impacts on both transcription and translation are gradually recognized.

Traditional roles of ncRNAs are exemplified by ribosomal RNA (rRNA) and transfer RNA (tRNA). Both of them function as RNA molecules and are irreplaceable components in the process of translation. tRNA base-pairs with a specific codon located on the messenger RNA (mRNA) and bring along a corresponding amino acid. rRNA provides peptidyl transferase activity by binding amino acids collected from tRNAs during translational elongation. However, many more ncRNAs are discovered and their roles in the cell have been subsequently uncovered. Telomerase RNA has a structural role by being the core of a telomerase, and serves as the template while the ends of replicated chromosomes are sealed [141]. ncRNAs also have catalytic functions by processing RNAs. For example, Ribonuclease P (RNase P) is an endoribonuclease that generates the mature 5’-end of a precursor-tRNA [142]. Small RNAs such as small nucleolar RNA (snoRNA) modifies rRNA and tRNA by methylation [143] and pseudo-uridylation [144]. Modifications on RNAs affect their stability. Piwi (P-element induced wimpy testis)-interacting RNA (piRNA) is slightly longer than microRNA and is an epigenetic regulator [145]. It associates with Piwi protein and Polycomb Group proteins (PcG) in order to repress gene expression [146]. It has been first known to prevent retrotransposon transposition by silencing its expression in germ cells [147].
Recently, several thousand noncoding RNAs are identified, and their genomic location, sequence, and promoter region are found highly conserved [148]. Expression pattern of this group of intergenic noncoding RNA is distinct across different cell types, and this pattern correlates with expression of protein-coding genes that are important during development. These long ncRNAs are also found to interact and exert transcription regulatory role with chromatin-modifying complexes such as Polycomb Repressive Complex 2 (PRC2) [149]. Shorter ncRNAs have also been reported to affect transcription at a local level by targeting transcriptional activators or repressors. For instance, the steroid receptor RNA activator (SRA) functions as a transcriptional co-activator for several nuclear hormone receptors [150]. It operates in a ribonucleoprotein complex that is required for hormone receptors to co-activate transcription. ncRNAs have also been reported to act as a transcriptional repressor by directly targeting RNA polymerase II. B2 RNA is a 178-nucleotide mouse ncRNA that binds to the core of RNA Polymerase II after heat shock, and therefore represses transcription of specific mRNA genes [151, 152].

Although many ncRNAs that regulate gene expression are being revealed, these examples are a small portion of regulatory network by RNAs. The detail mechanisms of their action remain to be elucidated.
1.6 Rationale and general hypothesis

Cancer is an accumulation of uncontrolled proliferation and inappropriate cell survival. During the process of tumor formation, damage to oncogenes or tumor suppressor genes in a cell facilitates its cancerous progression. In addition, altering expression of extracellular matrix proteins will change the composition of stromal microenvironment. Altered extracellular cellular molecules influence cellular activities and also cells’ response to soluble proteins such as cytokines and growth factors. More importantly, this external oncogenic signal feedbacks to both the cancerous cell and its neighboring cells. Recently, growing evidence indicates that miRNAs function as gene regulators, and impairment of its expression is implicated with tumorigenesis. Therefore, investigation of miRNA function may reveal unexplored mechanism contributing to tumorigenesis and will be useful in the diagnosis and treatment of cancer.

miRNAs consist of a very small portion of the RNA pool within cells, but modulates translational efficiency in cells of different origin. Tissue-specific gene expression can be suppressed by 60% as miRNA partially base-pair with its target mRNA sequences [7]. It has been estimated that expression of at least one-third of genes are regulated by miRNAs [153]. Currently, seven hundred miRNAs have been discovered and validated in human, but only a slight fraction of these miRNAs are studied. miRNA genomic sites are often located in fragile sites, and their expression pattern are different between cancerous and healthy tissues. Based on these findings, I hypothesize that one miRNA can regulate a biological event, such as tumorigenesis, by modulating expression of several genes that is associating with this biological event.

This hypothesis was tested using several approaches: i) characterizing oncogenic functions of miR-378 by regulating multiple tumor suppressors, ii) approaching study
from 3'UTR’s perspective by screening for miRNAs that modulate genes involved in tumor formation such as VCAN, and lastly, iii) inhibiting or antagonizing miRNA with 3'UTR harbouring the miRNA binding sites.

i) Characterizing oncogenic function of miR-378

In this novel study, we investigated the oncogenic potential of miR-378. miR-378 is located at human chromosome 5q33, a frequently reported genomic weak point. Computational algorithms, Pictar [153], generated several tumor suppressor genes as its potential targets. Interestingly, in another study investigating the 3'UTR of Vascular Endothelial Growth Factor (VEGF), miR-378 was found to surprisingly up-regulate VEGF expression \textit{in silico} [154]. Our initial investigation also revealed that expression of miR-378 was greatly elevated in tumor tissues compared to its neighbour cells. All of these evidences suggested that miR-378 may possess oncogenic functions. Therefore, this study initiated by investigating the contribution of miR-378 and its targets in tumor formation. Level of miR-378 was quantitated in various cell lines and two cell lines of different origin were used in the \textit{in vitro} cell model. These two cell lines were chosen because they both express intermediate level of miR-378. If the effect of miR-378 can be observed in both cell lines, the observed phenomenon is likely to be universal.

In order to determine the function of miR-378, its expression must be stable for observation and cell function analysis. Customized miR-378 expression construct was engineered. The effect of miR-378 on human astrocytoma cells U87 and breast cancer cells MT1 were monitored in cell survival assay and tumor formation assay. There are several tumor suppressor genes that are potential targets of miR-378. Predicted targets of miR-378 were confirmed by luciferase reporter assay and co-transfection experiments. Direct effect of miR-378 was further studied by introducing miR-378
ii) Screening for miRNAs that target tumor growth-promoting gene, VCAN

Versican is a hyaluronan-binding protein that belongs to the family of large aggregating chondroitin sulfate proteoglycans located primarily in the extracellular matrix. An increase in intensity of stromal staining with increasing tumor grade suggested that versican deposition might be associated with histological grade or degree of differentiation in breast cancer. Patients with tumors containing versican-rich peritumoral stroma experienced shorter relapse-free intervals than patients with versican-poor tumors [155]. Furthermore, Versican also serves as an early diagnostic marker and functions as an active modulator of metastasis in other types of cancer [156, 157]. Intensity and distribution of VCAN is also different in tumors derived from miR-378-transfected cells compared to control tumors. It is likely that VCAN expression is affected by miRNAs during progression of cancer. In this part of my study, the miRNAs that regulate VCAN expression are screened and identified. Identification of the regulatory miRNAs expands the understanding of miRNA functioning, and also reveals targets for future gene therapy for cancer.

In order to prove VCAN is regulated by miRNAs, its 3'UTR must confer translational influences. This was carried out by tailing VCAN 3'UTR to VCAN C-terminal G3 domain or a GFP coding region. Expression of G3 or GFP was determined by western blot or FACS respectively. PCR of miRNA:UTR was developed to predict miRNA interaction \textit{in silico}. Through this experiment, multiple miRNAs were found to bind to VCAN 3'UTR at physiological temperatures. Among them, miR-199a-3p was investigated for its regulatory role on Versican and Fibronectin expression. Targeting of VCAN and FN were confirmed by luciferase reporter assay. Transgenic mice expressing VCAN 3'UTR were
then generated. Antagonizing miR-199a-3p was expected to relieve VCAN and FN mRNAs from translational repression. Expression of VCAN and FN in vivo was then investigated by western blot and immunohistochemistry.

iii) Antagonizing miRNAs function by expressing 3'UTR

In previous study, miR-199a-3p was expected to modulate VCAN and FN expression. Antagonizing miR-199a-3p functions by expressing VCAN 3'UTR was expected to relieve VCAN and FN mRNAs from miR-199a-3p-mediated translational inhibition. In order to monitor the function of VCAN 3'UTR, it was expressed stably in a human astrocytoma cell line, U343. Since FN and VCAN were both extracellular matrix molecules, they were expected to affect cell adhesion. Change in cell morphology was observed during this process. In the 3'UTR transgenic mice, internal organs were found to adhere to each other as well.

The effect of VCAN 3'UTR on tumor formation and miRNA activity were also investigated. Mouse breast cancer cell line, 4T1, was stably transfected with VCAN 3'UTR, and was analyzed for its tumorigenic potential by tumor formation assays. 4T1-induced tumor growth and metastatic capacity closely mimicked human breast cancer, and was an appropriate animal model for stage IV human breast cancer. From previous data, a number of miRNAs bound to VCAN and target negative cell cycle regulators, Rb1 and PTEN. Antagonizing these miRNAs resulted in increased expression of Rb1 and PTEN, which was confirmed by expression analysis of tumors by western blot and immunohistochemistry. Reduction in cell proliferation was observed in cell proliferation assays, and decelerated cell cycle progression as measured by FACS.
Confirmation of Rb1 and PTEN targeting was carried out by luciferase reporter assays. VCAN 3’UTR was expected to bind to miRNAs. To further explore the effect of VCAN 3’UTR, steady state expressions of miRNAs were specifically analyzed by RT-qPCR in primary tissues and cancer cells.

1.7 Thesis objectives and organization

There are a total of five chapters in this thesis. The first chapter provides a current but general background of miRNA, from its biogenesis to degradation. The known mechanisms of miRNA-mediated gene regulation are also discussed. Since miRNAs regulate cell activities in various ways, specific attention on its involvement with promoting tumor growth will be addressed. Background on how miRNAs are currently being used or proposed for prognostic and diagnostic purposes is briefly introduced.

The following three chapters are the core of this thesis, and present original experimental data. Each chapter starts with an abstract describing the content of the chapter in short. They are then followed by an introduction, which provides specific background to the study that is beyond the scope of literature review in Chapter 1. It also outlines the specific goal of the study. Materials and method section describes the methods used in the experiments. Results section presents figures obtained from the experiments and includes discussion on the obtained results and implication.

Chapter 2 starts off this study by describing the oncogenic property of miR-378. The study was based on findings from literature and computational predictions made using public miRNA databases. In the early stages of my study on miRNA, a lot of miRNA-specific tools were not available. miRNAs were too short for conventional RNA
isolation methods, PCR, and RT-qPCR. Therefore, a lot of efforts were made to develop tools in order to study miRNAs, and these designs are described in detail. The ability of miR-378 to enhance cell survival and thus promote tumor growth was completely, or at least partially, due to down-regulation of two tumor suppressor genes - SuFu and Fus-1. This result places miR-378 upstream of the regulatory network and is thus one of the causes of tumor formation. Few studies in the past have linked miRNA to oncogenes or tumor suppressor genes. This is one of the first study to show that a single miRNA can regulate more than one gene simultaneously, causing tumorigenesis.

Experimental finding in Chapter 3 reinforces the general hypothesis and the results from Chapter 1. miR-199a-3p was found to regulate both VCAN and FN expression at the same time, in order to regulate cell adhesion. Both VCAN and FN are extracellular matrix proteins, but VCAN was shown to promote tumor growth by autocrine and paracrine mechanisms. A different experimental approach was taken in this study. The 3'UTR of VCAN were screened for interacting miRNAs, and a miRNA:UTR identification PCR was developed. This work suggests that miRNA studies do not always have to initiate from miRNAs. Our current knowledge on oncogenes and tumor suppressors can also be utilized in miRNA research. In addition, 3'UTR harbouring target sites of miRNAs is currently being used to inhibit miRNA function. The experimental approach was novel and successful, and its ability to inhibit miRNA function was proven both in vitro and in vivo.

Chapter 4 demonstrates the same antagonizing approach of using 3’UTR to inhibit miRNA function. As a result, the functions of a number of miRNAs, miR-199a-3p, miR-144, miR-136, were inhibited. Normally, these miRNAs synergistically target two negative cell cycle regulators- Rb1 and PTEN, but instead bound to 3’UTR, which is expected to act as a decoy. This results in reduced cell proliferation and thus tumor
growth. Moreover, 3'UTR not only antagonizes miRNAs but also enhances instability of specific miRNAs by forming a RNA duplex, but this may not occur in a different tissue.

Finally, Chapter 5 discusses the biological significance of these findings. Since miRNA research just emerged within the last decade, many fundamental questions still remain. While these findings improve our knowledge of miRNA's involvement in cancer, recommendations on potential future studies are proposed in this chapter.
Chapter 2

Oncogenic role of miR-378 by promoting cell survival, tumor growth, and angiogenesis

A version of this chapter is published in Proceedings of National Academic of Science of United States of America (Lee DY, Deng Z, Wang CH, and Yang BB. 104(51):20350-5.)
2.1 Abstract

MicroRNAs are endogenously transcribed non-coding RNA that post-transcriptionally regulates translation. Gene expression involved in proliferation, differentiation, and apoptosis are frequently altered during tumorigenesis, and thus miRNAs play important roles in cancer development. In this study, we show that expression of miR-378 enhances cell survival, reduces caspase-3 activity, and promotes tumor growth and angiogenesis. Proteomic analysis indicates that Suppressor of Fused (SuFu) is a potential target of miR-378, which was confirmed in vitro and in vivo. In a luciferase activity assay, expression of a luciferase reporter construct containing the target site in SuFu was repressed when it was co-transfected with miR-378. Conversely, transfection of SuFu reversed the effect of miR-378 in cell survival, suggesting an important role for miR-378 in tumor cell survival and thus growth. We also discovered that miR-378 targets another tumor suppressor gene, Fus-1. Expression of luciferase constructs harboring the target sites in Fus-1 was also repressed by miR-378. Fus-1 constructs with or without its 3'UTR were also generated. Co-transfection experiments showed that the presence of miR-378 repressed Fus-1 expression post-transcriptionally. In cell survival assay, suppression of Fus-1 expression by siRNA against Fus-1 enhanced cell survival, and the transfection of the Fus-1 construct reversed the function of miR-378 in cell survival. Our results suggest that expression of miR-378 enhanced
cell survival, tumor growth, and angiogenesis through repression of two tumor suppressors, SuFu and Fus-1 simultaneously.

2.2 Introduction

Genetic alterations happen during somatic events. These changes by themselves are rarely sufficient enough to cause cancer, but a combination of these events over a prolonged period of time do. It has long been believed that genetic alterations of protein-coding genes, such as oncogenes and tumor suppressors, are the major contributors to the development of cancer. An example of these is the case of Chronic Myeloid Leukemia (CML). Chromosomal translocation at t(9:22) generated a fusion protein composed of BCR and ABL1 gene[158]. Gleevac, or Imatinib, was developed specifically to inhibit the unregulated activity of ABL1 tyrosine kinase [159]. Recently, miRNAs have emerged as a new class of gene expression regulator. Its dysregulation may have oncogenic or tumor suppressors properties, and thus has became a potential contributor in initiation and progression of cancer. For example, miR-15 and miR-16 are frequently deleted or down-regulated in approximately 68% of Chronic Lymphocytic Leukemia (CLL) cases [130]. These miRNAs were later found to function as tumor suppressors by inducing apoptosis through regulating expression of Bcl2 [129]. On the other hand, miR-155 is often accumulated in various lymphomas and thus is thought to be oncogenic [117]. It is until recently that its proliferative function was found to be attributed by targeting SHIP1 [160].

In the initial study, we investigated the oncogenic potential of miR-378. miR-378 is located at human chromosome 5q32, a frequently reported genomic weak point, building its genetic basis causing various types of cancers. Genomic instability in this
particular loci has been reported to cause unregulated transcriptional activity of local genes [161]. Computational algorithms predicted several tumor suppressors as its potential targets. Interestingly, in another study investigating the 3'UTR of Vascular Endothelial Growth Factor (VEGF), miR-378 was found to up-regulate VEGF expression in silico [154]. All of the evidence obtained suggested that miR-378 may exert oncogenic function. Therefore, we investigated the function of miR-378 and its targets that mediate the observed phenomenon.

2.3 Materials and Methods

Construct Generation

All of our constructs were confirmed by sequencing, and the primers used in the following are listed in Table 1. A microRNA construct expressing miR-378 was designed by our lab and the DNA was synthesized by a biotech company (Top Gene Technologies, Montreal). Briefly, the pre-miRNA-378 was ligated into the mammalian expression vector BluGFP that contains a Bluescript backbone, a CMV promoter driving green fluorescent protein (GFP) expression, and a promoter driving miR-378. This plasmid was developed in our lab and is expected to simultaneously express a piece of RNA and produce GFP (Fig 2.1A). This means that every fluorescent cell also expresses mature miR-378. Based on this plasmid, another plasmid named miR-378-1 was generated to express one copy of miR-378, made using two primers miR-378-1copy and miR-378C. The sequence of the construct is illustrated in Fig 2.1B.

Using a similar approach, a construct transcribing miR-378 antisense was generated. Briefly, the primer anti-miR-378 was designed to incorporate an anti-miR-378 sequence into the expression vector by PCR. PCR products were digested by restriction
Fig. 2.1 Generation of miR-378 expression construct

(A) Diagram of a construct expressing GFP, a neomycin-resistance gene, and two hairpin structures of pre-miR-378 that give rise to two copies of miR-378. Underlined nucleotides are restriction sites, capitalized alphabets are miRNA precursor sequences, and alphabets in lower case are artificial sequence for PCR purpose.

(B) Diagram of a similar construct, mir-378-1, expressing one copy of miR-378 and its detail sequence arrangement.
digestion and ligated as described above to produce the anti-miR-378 construct.

A luciferase reporter vector (pMir-Report; Ambion, Austin, TX) was used to generate luciferase reporter constructs (Figure 2.2). Two primers (huSufuN3’-SacI and huSufuC3’-MluI) were used to clone a fragment of the SuFu 3’-UTR by RT-PCR. PCR product was digested with SacI and MluI, followed by insertion into a SacI- and MluI-open pMir-Report vector. To generate a mutant containing a mutation in the miR-378 target sequence, a primer (huSufuC3’mu-MluI) harboring this mutation was utilized in combination with previously used forward primer huSufuN3’-SacI in a PCR reaction. The PCR product was digested with SacI and MluI and inserted into a SacI- and MluI-open pMir-Report vector. To serve as a negative control, a non-related sequence was amplified from the coding sequence of the chicken versican G3 domain using two primers chver10051-Spel and chver10350-Sacl. It is expected that no endogenous microRNA would target this fragment as it is located in the coding sequence. The amplified PCR product was digested with SpeI and SacI, followed by insertion into a SpeI- and SacI-open pMir-Report vector.

To study the function and regulation of SuFu and Fus-1 in miR-378-regulated cell activities, we needed to transcribe SuFu and Fus-1 with or without their 3’-UTR. The Sufu construct was a kind gift of Dr. James Rutka from the University of Toronto. To enhance expression of SuFu, a Kozak sequence was engineered into the Sufu construct using two primers, Kozak-MycHindIII and husufuCXbaI (Fig. 2.3A). A fragment of the Sufu 3’-UTR cloned was linked to the Sufu construct using the primers Kozak-MycHindIII and husufuCSacl, producing the SufuUTR construct (Fig 2.3C). Expression of Sufu with a Kozak sequence was shown to increase the expression level as compared to that without a Kozak sequence (Fig 2.3B).

Fus-1 was cloned with two primers, FusKozak-BamHI and FusMyc-Xhol, by
A fragment of Sufu 3'UTR was inserted into the luciferase report vector pMir-Report producing a construct named Luc-Sufu. The potential mir-378 target sequence was labelled in blue. Mutations labelled in red color were generated in the mir-378 target sequence producing a mutant construct named Luc-Sufu-mu. A control sequence (Ctrl) was obtained from the G3 domain of chicken versican.
Fig. 2.3 Diagrams of SuFu expression constructs

(A) A SuFu expression construct was generated starting with a restriction site HindIII (underlined) followed by a typical Kozak sequence (gccgccacc), a myc epitope, the coding sequence of SuFu, and a restriction site Xbal.

(B) Expression of SuFu construct with or without a Kozak sequence.

(C) The coding sequence of SuFu and a fragment of SuFu 3’UTR were linked as shown producing a construct SuFu-3’UTR.
RT-PCR. PCR product was then digested with BamHI and XhoI and inserted into a BamHI- and XhoI-opened pcDNA3 vector (Fig. 2.4A). The 3’-UTR of Fus-1 cloned above was amplified through PCR using two primers, FusN3’-XhoI and FusC3’-ApaI. The PCR product was digested with XhoI and ApaI and inserted into the Fus-1 construct digested with XhoI and ApaI, producing the FusUTR construct (Fig. 2.4B).

To generate a siRNA against Fus-1, the primers huFus-si323P1 and huFus-si382P2 were used in PCR. PCR product was digested with BglII and HindIII and inserted into a BglII- and HindIII-digested BluGFP vector, generating a construct expressing siRNA against Fus-1 (Fig. 2.5).

The Fus-1 3’-UTR contains a potential target sequence for miR-378 located at nucleotides 748-770. A luciferase reporter construct was generated using the primers huFusN3’-SacI and huFus780R-MluI by RT-PCR. The PCR product, after restriction enzyme digestion by SacI and MluI, was inserted into a SacI- and MluI-open pMir-Report vector, producing Luc-Fus (Fig. 2.6A). A mutant construct was generated using two primers, huFusN3’-SacI and huFus780Rmu-MluI. Using the same procedure as above, this PCR product was used to produce the Luc-Fus-mu construct (Fig. 2.6B).

**RT-PCR and RNA analysis**

Cells (2.5 × 10^6) were harvested, and total RNA was extracted with the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instructions. RT-PCR assays were performed as previously described. Briefly, 2 µg of total RNA was used to synthesize cDNA by reverse transcription, a portion of which (equal to 0.2 µg of RNA) was used in a PCR with two appropriate primers. PCR products were visualized through agarose gel electrophoresis using ethidium bromide staining. For RT-PCR of mature microRNAs, specific primers were designed, and the procedure for reactions is given on
Fig. 2.4 Diagrams of Fus-1 expression constructs

(A) A Fus-1 expression construct was generated starting with a restriction site BamHI (underlined) followed by a typical Kozak sequence (gccgccacc), the coding sequence of Fus-1, a myc epitope, and a restriction site XbaI.

(B) The coding sequence of Fus-1 and a fragment of Fus-1 3’UTR were linked as shown producing a construct Fus-1-3’UTR.
Fig. 2.5 Diagram of siRNA construct against Fus-1

(A) Map of Fus-1 siRNA construct.

(B) siRNA-mediated silencing of Fus-1 was examined by RT-PCR.

(C) Little Fus-1 protein was detected in siRNA-transfected cells compared to the vector control.
**Fig. 2.6 Luciferase report constructs for Fus-1.**

Fus-1 contains a potential target sequence for mir-378-3p. Luciferase report constructs containing Fus-1 3'-UTR were generated. The potential mir-378 target sequence was labelled in blue. Mutations labelled in red color were generated in the mir-378 target sequence producing mutant constructs Luc-Fus-mu.
Figure S2.1.

**Western Blotting**

Cells were seeded in a 6-well plate at $2 \times 10^5$ cells/well and lysed after 48 hours after in 100 µl of lysis buffer containing protease inhibitors (150 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.5 M EDTA, 20% Triton X-100, 8 M Urea, and 1x protease inhibitor cocktail). After addition of lysis buffer, cells were collected and sonicated for 15 seconds on ice. All samples were subjected to SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes followed by immunostaining with a primary monoclonal antibody against the myc tag (Abcam, clone 9E10, 1:1500) or Sufu (Novus Biologicals, 1:500) overnight at 4°C. The secondary antibody used was goat anti-mouse IgG (1:2000) for 1 hour at room temperature. After detection of the protein bands with ECL kit (Millipore) by autoradiography, the blot was stripped and re-probed with the primary antibody against β-actin (Sigma-Aldrich, 1:5000) to confirm equal loading of samples.

**Luciferase Activity Assay**

Luciferase activity assays were performed using the Promega Luciferase Assay System (Madison, WI). In brief, COS-7 cells ($10^5$ cells/well) were seeded onto 12-well tissue culture plates overnight. The cultures were changed for serum-free medium prior to transfection with different combinations of DNA (e.g. 10 ng luciferase reporter construct mixed with 10 ng β-Gal plasmid and 300 ng miR-378 construct). Twenty hours after transfection, the transfection reagent was replaced with serum-free medium, and the cultures were maintained at 37°C for 24 hours. Cells were then harvested using trypsin/EDTA and lysed with 150 ml of 1x lysis buffer diluted from luciferase 5x lysis buffer on ice for 30 min. After centrifugation at 14000 rpm for 15 seconds, the
supernatant was used to measure β-Gal and luciferase activity in 96-well plates in triplicate by TopCount NXT (a microplate scintillation and luminescence counter produced by Packard Instrument Company, Meriden, CT). For the luciferase activity assay, 10 μl of sample was mixed with 70 μl of Luciferase Assay Substrate A. For β-Gal activity assay, 50 μl of each sample was mixed with 90 μl of Substrate B (o-nitrophenyl-β-D-galactopyranoside or OPNG) followed by incubation at 37ºC for 1 hour before reading. Luciferase activities were normalized against β-Gal activity.

**Cell survival assay**

Cells (1.5 x 10^5 cells/well or 2 x 10^5 cells/well) were seeded on 35 mm Petri dishes in DMEM containing 0 to 10% FBS, and incubated for different time periods. Cell numbers were counted using trypan blue staining.

**Immunofluorescence**

Cells cultured on chambered slides were fixed in 3.7% paraformaldehyde at 37ºC for 30 min, permeabilized by incubation with 0.1% Triton X-100 for 15 min, and blocked with 1% BSA in PBS. They were then immunostained with primary antibody diluted in blocking solution at room temperature for 1 hour. Cells were rinsed three times with PBS and incubated with secondary antibody coupled to Texas red or FITC for 45 min. Slides were then rinsed three times with PBS before mounting, and visualized using a Leica confocal microscope.

**Tumorigenicity assays in nude mice**

Six-week-old nude mice strain Balb/c were injected with miR-378- or GFP-transfected U87 cell lines (5 x 10^6 cells in 100 PBS). Tumors were measured
weekly thereafter. Tumor volume $V$ was measured using a caliper by determining the length $L$ and width $W$, where $V = (L \times W^2)/2$. Four weeks after injection, tumors were fixed in 10% buffered formalin, processed, and embedded in paraffin. Immunohistochemistry was performed on 5 μm paraffin sections mounted on charged slides. Sections were stained with Hematoxylin and Eosin (H&E) or immunostained with either anti-Sufu antibody, anti-CD34 monoclonal antibody, or with secondary antibody as a control using the ABC and DAB Kits from Vector Laboratories Inc (Burlingame, CA).

**Statistical Analysis**

The results (mean values ± SD) of all the experiments were subjected to statistical analysis by t-test unless otherwise mentioned. The level of significance was set at $p < 0.05$.

2.4 Results and Discussion

2.4.1 Generation of miRNA expression construct

Although miRNAs have emerged as key regulators of gene expression, our understanding of their specific functions has been limited by several obstacles. First, regulation of miRNA expression is not well known. Second, tracking the functions of a particular miRNA is difficult. Third, synthetic miRNAs are readily degraded by enzymes in a few days, making it impossible to monitor miRNA function for long-term studies *in vitro* or *in vivo*. As a result, there are very few miRNAs whose functions have been reported in the early studies of miRNA. In order to investigate miRNA function in long-term, we have developed an expression construct to transcribe precursor of miR-378 and GFP, with an antibiotic selection marker (Fig. 2.1). The advantage of this
construct is the stability of microRNA expression after stable transfection. Furthermore, transfection of this construct allows selection with neomycin, as well as rescue of positive clones by monitoring GFP or by cell sorting pooled cells. Since the stable cells were obtained by cell sorting, they were cultured continuously through several passages. The cells continued to show expression of GFP three months after sorting. In this miRNA expression model, two cell lines were used- U87, a human astrocytoma cell line, and MT1, a human breast carcinoma cell line. We have detected the expression of pre-miR-378 by RT-PCR using RNA prepared from U87 cells stably transfected with miR-378 (Fig. 2.7A). The pre-miR-378 was then successfully processed to mature miR-378, as detected by RT-PCR with primers designed by us (Fig. 2.7B). Because a viral promoter was used to transcribe miR-378, knowing that a suitable level of mature miR-378 was being expressed is essential. RT-qPCR amplifying miR-378 specifically was carried out, and there is about 2.3-fold of miR-378 being expressed in the stable pooled cells (Fig 2.7C). This part of the study enabled an appropriate, effective, and stable expression of mature miR-378 in our model.

2.4.2 Expression of miR-378 promotes tumorigenesis

We monitored the effect of miR-378 expression on cell activity. U87 cells transfected with miR-378 or control vector expressing GFP alone were maintained in varied serum-containing culture conditions. The cells were allowed to overgrow, resulting in extensive cell death, and cell survival was monitored with a fluorescent microscope. Transfection with miR-378 enhanced cell survival as compared with control in all serum conditions by microscopic examination (Fig. 2.8) and cell counting (Fig. 2.9A). In order to determine that increased cell death resulted to decreased cell number, the detached and adherent cells were harvested separately and counted with Trypan
Fig. 2.7 Expression mature and precursor miR-378 in stable cells

(A) RT-PCR of pre-miR-37 using RNA prepared from U87 cells stably transfected with miR-378 or control vector, confirming transcription of pre-miR-378. Gapdh was also tested for control purpose.

(B) RT-PCR of mature miR-378 using the same RNA, confirming proper processing of pre-miR-378.

(C) RT-qPCR was carried to quantified level of mature miR-378. U6 RNA was also quantified to normalized the samples.
Fig. 2.8 Examination of stably transfected U87 cells in cell survival assay

U87 cells transfected with miR-378 or control vector were maintained in various serum-containing conditions, but the cells were allowed to overgrow. Cell survival was monitored with a fluorescent microscope. Transfection with miR-378 enhanced cell survival, as compared with the control cells.
(A) The number of survived cells were harvested and counted for statistical analysis. Significant differences are indicated by asterisks. **, p < 0.01. Error bars, SEM (n=4).

(B) The numbers of dead cells and surviving cells were counted for statistical analysis. Significant differences are indicated by asterisks. * p < 0.05; **, p < 0.01. Error bars, SEM (n=4).

Fig. 2.9 Quantification of dead and survived cells in cell survival assay
Fig. 2.10 Comparison of caspase 3 activity in stably transfected cells

Caspase 3 activity was determined in cells transfected with miR-378 or a control vector in serum-free or in 10% serum-containing conditions. Significant differences are indicated by asterisks. **, p < 0.01. Error bars, SEM (n=6).
Fig. 2.11 Effect of anti-miR-378 in cell survival assay

(A) U87 cells transfected with miR-378, construct expressing antisense of miR-378, or control vector were maintained were subjected to cell survival assay. Representative microscopic pictures were shown. Transfection with anti-miR-378 reverted the function of miR-378 in cell survival.

(B) The numbers of survived cells were counted for statistical analysis. Significant differences are indicated by asterisks. * p < 0.05; * , p < 0.05. Error bars, SD (n=3).
blue staining (Fig. 2.9B). The results revealed that transfection with miR-378 reduced cell death and increased cell survival. Similar experiments were carried out using MT-1 cell line, after stable transfection and fluorescent sorting. The miR-378 stably transfected MT-1 cells also demonstrated increased cell survival and reduced cell death, compared to MT-1 cells stably transfected with control vector (Fig. S2.2), suggesting a conservation of miR-378 functioning in different cell types.

To determine whether the cells death was due to apoptosis, caspase 3 activity assays were performed. Stably transfected U87 cells were cultured in 1% or 10% serum-containing conditions and their caspase 3 activities were measured. Results showed that caspase 3 activity dropped significantly in the miR-378-transfected cells compared to the vector-transfected cells (Fig. 2.10). Since caspase 3 activity is essential for apoptosis, decreased caspase 3 activity explains the decrease in apoptotic cells transfected with miR-378.

To confirm the function of miR-378 in cell survival, U87 cells were transfected with a construct expressing an antisense sequence against miR-378 (anti-miR-378) followed by cell survival assay. Again, expression of miR-378 prolonged cell survival. However, expression of miR-378 antisense abolishes the function of miR-378 and cell survival significantly was significantly reduced (Fig. 2.11).

When the miR-378-transfected U87 cells were maintained in serum-free conditions in Petri dishes, we observed a dramatic change in cell morphology (Fig. S2.3). The miR-378-transfected cells rounded up, while the control cells were still spreading on the dishes. The ability of these cells to survive without attachment, along with its increased survival and decreased apoptosis, are all characteristics that initiate tumor formation. We also found that the level of miR-378 is higher in human papillary thyroid tumor compared to its adjacent tissues (Fig. 2.12). Consequently, a tumor formation assay was
Fig. 2.12 Quantification of miR-378 levels in primary human tumor
Expression of miR-378 was analyzed by RT-qPCR with RNA isolated from papillary thyroid tumor and adjacent normal tissue. *, P=0.05. Error bars indicate SD (n =3).
carried to investigate the oncogenic potential of miR-378. Both groups of transfected cells were injected subcutaneously into nude mice. Two weeks after the injection, we could already detect a clear difference in tumor size. By four weeks, mice injected with the miR-378-transfected cells had much bigger tumors than mice injected with the GFP-transfected cells (Fig 2.13A). The difference in tumor size was very significant two weeks after cell injection (Fig. 2.13B). Dramatic difference in tumor growth indicates that expression of miR-378 greatly promoted the process of tumor formation, because wild-type U87 cells normally take 4 weeks to form visible tumors. In an independent experiment using a different batch of miR-378-transfected cells, similar results were also obtained (Fig S2.4). The tumors were collected and sectioned for immunocytochemistry using anti-CD34 antibody. The tumors formed by miR-378-transfected cells contained larger blood vessels than those formed by the vector-transfected cells (Fig. 2.14), implying that miR-378 played a role in promoting blood vessel formation through the expansion of small, existing vessels into large, so-called "mother vessels" [162]. Although the number of blood vessels per unit field did not seem to increase, it is probable that the total number of blood vessels in the larger tumors had to be higher to allow expansion of the tumor. The formation of the mother vessels may be important in facilitating such expansion.

2.4.3 Repression of Suppressor of Fused (SuFu) by miR-378

We then examined differential protein expression affected by miR-378 transfection. U87 cells stably transfected with miR-378 or control vector were harvested and subjected to proteomic analysis performed by WEMB Biochem Inc (Richmond Hill, ON). A large number of proteins were down regulated due to the presence of miR-378. This analysis was repeated independently using a different pool of miR-378- or
Fig. 2.13 Tumor growth curve of miR-378 and control-transfected U87 cells

(A) U87 cells transfected with miR-378 or vector were injected subcutaneously into nude mice. Four weeks after injection, mice were photographed and sacrificed.

(B) Tumor sizes were measured and tumor growth curve was obtained (n=3; **, p<0.01).
Fig. 2.14 Staining of tumor blood vessels with CD34 antibody
Tumors formed by cells transfected with miR-378 or control were subjected to immunohistochemistry with anti-CD34 antibody. Expression of the miR-378 construct promoted levels of tumor-associated vascularization.
vector-transfected cells. In both experiments, SuFu (Suppressor of Fused) was consistently down regulated greatly (Fig. 2.15). SuFu is reported to function as a tumor suppressor [163], because loss of its function causes a failure to regulate both the Sonic Hedgehog and the WNT signaling pathways, leading to excessive tumor cell proliferation [164]. Repression of Sufu expression may be an essential component of miR-378's functionality to enhance cell survival and tumor growth. Other proteins which are the potential targets of miR-378 were also detected to be down regulated in U87 cells transfected with miR-378, compared to cells transfected with the GFP control vector.

We analyzed the sequence of SuFu 3'UTR and found a potential target site (nucleotides 4645-4676 of SuFu, GeneBank access number NM_016169) complementing imperfectly to miR-378 (Fig. 2.16, upper panel). This sequence is conserved in SuFu mRNA of human, chimpanzee, mouse, rat, and canine genomes (Fig. S2.5), suggesting an importance for miR-378 regulation. RNA and protein lysates were collected from various human cell lines. Expression of miR-378 and SuFu were correlated, corroborating their potential miRNA-target relationship. Repression of SuFu expression found in proteomic analysis was confirmed by Western blot analysis of U87 cells stably transfected with miR-378 or the GFP vector probed with anti-Sufu monoclonal antibody. We detected a clear reduction of SuFu expression in cells transfected with miR-378, compared to cells transfected with control vector (Fig. 2.17A). Conversely, transfection with miR-378 antisense reverted the translational repression exerted on SuFu mRNAs by miR-378 (Fig. 2.17C). RNA was also prepared from same cells and was subjected to RT-PCR. Little difference was detected (Fig. 2.17B). This indicates that miR-378 repressed SuFu expression at the translational level. The cells transfected with miR-378 and vector were also subjected to immunostaining using
**Fig. 2.15 Proteomic analysis of stably transfected cells**

U87 cells transfected with miR-378 or a control vector were harvested and subjected to proteomic analysis. Repression of SuFu expression was found in two independent experiments.

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Fig. 2.16 Imperfect matching of miR-378 and its target site in SuFu 3’UTR

Computational algorithms were used to predict miR-378 binding site in the 3’UTR of SuFu. miR-378 was found to have the strongest binding from nucleotides 4645-4677 (upper panel). Mutations were introduced to interrupt this interaction (lower panel, red color).
Fig. 2.17 Western blot of miR-378 lowers SuFu expression in cells

(A) Cell lysate from miR-378- or vector-transfected U87 cells was subjected to electrophoresis on a 12% SDS-polyacrylamide gel and probed for SuFu and actin protein expression.

(B) RNA prepared from the same cells was subjected to RT-PCR. SuFu and GAPDH transcription levels were compared.

(C) Cells transfected with miR-378 antisense reverts translational repression on SuFu mRNA.
anti-SuFu antibody. These experiments further confirmed the repressed SuFu expression in cells transfected with miR-378 (Fig. 2.18).

We further looked for evidences of repressed Sufu expression by miR-378 in vivo. Previously obtained tumors from mice injected with miR-378- or GFP-transfected cells were sectioned and stained with H&E, secondary antibody (serving as a negative control), and anti-SuFu antibody. In the tumor sections derived from cells transfected with the GFP control, we observed cells with pink cytoplasm with condensed blue nuclei. This is characteristic of apoptotic cells after H&E staining [165]. However, no such cells were found in the tumors from cells transfected with miR-378 (Fig. 2.19). Instead, SuFu expression was greatly repressed as seen in the tumor sections. This result confirmed that the strong support of miR-378 in tumor growth and angiogenesis was mediated by the SuFu-associated pathway.

To directly demonstrate the repression of SuFu by miR-378, we integrated a fragment of the SuFu 3'UTR containing the miR-378 target sequence into a luciferase report vector. Luciferase activity was significantly repressed in the construct harboring the miR-378 target sequence (Fig. 2.20), compared with the control vector harboring a non-related fragment (Luc-Ctrl) or the SuFu 3'-UTR fragment containing a mutation in the miR-378 target site (Fig. 2.16). To test the possibility of an additive effect between the target sequence and the miR-378 hairpin structure, we generated a miR-378 expression construct containing one copy of the miR-378 hairpin structure. Luciferase activity assays indicated that the miR-378 construct harboring two miR-378 hairpin structures produced greater repressive strength on luciferase activity than the one containing one miR-378 hairpin structure (Fig. S2.7).

To reinforce the targeting of miR-378 on SuFu, co-transfection experiments were carried out. SuFu constructs with (SufuUTR) or without (Sufu) SuFu 3'UTR were
Fig. 2.18 Immunostaining stably transfected cells with anti-SuFu antibody

Immunocytometry staining was performed in U87 cells stably transfected with miR-378 or GFP, followed by confocal microscopic examination of GFP and SuFu expression. Transfection with miR-378 repressed SuFu expression levels.
Fig. 2.19 Immunostaining tumor sections with anti-SuFu antibody
Tumors formed by cells transfected with miR-378 or control vector were sectioned and stained with H&E, secondary antibody (negative control or Neg), and anti-SuFu antibody to examine SuFu expression.
Fig. 2.20 SuFu is targeted by miR-378 in luciferase activity assay
COS-7 cells were co-transfected with the miR-378 and luciferase reporter construct, which has been engineered with a fragment of the Sufu 3’-UTR harbouring the target sequence of miR-378 (Luc-Sufu) or a mutant (Luc-Sufu-mu). As a negative control, the luciferase reporter construct was engineered with a non-related fragment of cDNA (Ctrl). Significant differences are indicated by asterisks. **, p < 0.01. Error bars, SEM (n=3).
generated (Fig. 2.3). These constructs were then transiently co-transfected with miR-378 or control vector. Repression of SuFu expression by miR-378 was confirmed by Western blot probing with anti-SuFu antibody (Fig. 2.21A). RT-PCR analysis of mRNA from these cells produced similar levels of PCR product (Fig. 2.21B). These results indicate that miR-378 only affected SuFu translation but not transcription.

To finalize the function of miR-378 in cell survival by targeting SuFu, rescue experiments were carried out. U87 cells stably transfected with miR-378 or GFP vector were transiently transfected with Sufu or SufuUTR, and were subject to cell survival assay. Survived cells were examined under a light microscope (Fig. 2.22A) and were counted with trypan blue staining (Fig. 2.22B). Results from this experiment indicated that the introducing SuFu into miR-378-expressing cells reversed the effect of miR-378 on cell survival. The number of survived cells was similar between miR-378- or vector-transfected cells after SuFu was being expressed in these cells. In the presence of the 3'-UTR by transfecting with SufuUTR construct, SuFu expression was repressed by in the presence of miR-378. High level of survived cells reinforces our finding that SuFu 3'UTR contains a target site for miR-378. Nevertheless, without additional expression of miR-378 by transfecting with GFP controls, SufuUTR appeared to enhance cell survival compared to Sufu transfection alone. This might be due to repression of SufuUTR by endogenous miRNAs.

2.4.4 Targeting of Fus-1 by miR-378

While searching for potential targets of miR-378, we also identified Tumor Suppressor Candidate 2 (TUSC2, or Fus-1, GeneBank Access number NM_007275) as one of the potential targets [166]. Fus-1 appeared to harbour a standard target sequence for miR-378 at nucleotides 748-770 (Fig. 2.23, upper panel). Sequence
Fig. 2.21 Western blot and RT-PCR analysis of SuFu and miR-378 co-transfection

(A) Cell lysate was prepared from U87 cells co-transfected with miR-378 and SuFuUTR or GFP vector, followed by Western blot probing with the anti-SuFu antibody, or anti-actin antibody.

(B) Level of SuFu and GAPDH mRNA was also examined through RT-PCR.
Fig. 2.22 Comparing cell survivals in co-transfected stable cells

(A) U87 cells stably transfected with miR-378 or GFP vector were transiently transfected with Sufu or SufuUTR, and were subjected to cell survival assay. Cells were examined under microscope.

(B) Survived cells were counted by tryphan blue staining. Significant differences are indicated by asterisks. **, p < 0.01. Error bars, SEM (n=5).
**Fig. 2.23 Imperfect matching of miR-378 and its target site in Fus-1 3’UTR**

Computational algorithms were used to predict miR-378-3p binding site in the 3’UTR of Fus-1 (upper panel). Mutations were introduced to interrupt this interaction (lower panel, red color).
analysis indicated that this target sequence was conserved across different species (Fig. S2.8). To investigate whether Fus-1 supports in miR-378’s function in cell survival, Fus-1 construct was generated. We cloned Fus-1 cDNA by RT-PCR and linked it to a myc tag in pcDNA3.1 expression construct. Myc tag was included in the transcription because no antibody recognizes Fus-1 (Fig. 2.4A). In order to determine its regulation by miR-378, this construct was expressed in GFP- or miR-378-transfected U87 cells. Cell lysate was prepared from these cells and subjected to Western blot analysis by probing with the anti-myc antibody 9E10. A band of the expected size was detected (Fig. 2.24A). Cell survival experiments indicated that transfection of Fus-1 reduced miR-378-mediated enhancement in cell survival (Fig. 2.24B).

To corroborate this result, we generated a siRNA construct containing two hairpin structures complementary to Fus-1 sequences (Fig. 2.5). Down regulation of Fus-1 was confirmed by RT-PCR (Fig. 2.5B) and Western blot (Fig. 2.5C). Cell survival was tested in U87 cells transfected with the siRNA against Fus-1 or a control vector expressing GFP. Result of this experiment indicated that transfection of Fus-1 siRNA reversed cell death induced by Fus-1 expression (Fig. S2.9), suggesting that a Fus-1-mediated pathway is essential for miR-378-enhanced cell survival.

To provide direct evidence that the 3' UTR of the Fus-1 mRNA is a target of miR-378, we generated a luciferase expression construct harbouring a fragment of the Fus-1 3'UTR containing the target sequence of miR-378 (Fig. 2.6). A mutant construct Luc-Fus-mu containing a mutation in the miR-378 target sequence was also made. Luciferase assays showed that the miR-378 construct co-transfected with the luciferase reporter construct Luc-Fus reduced luciferase activity significantly, while co-transfection of miR-378 with Luc-Fus-mu did not affect luciferase activity compared to a reporter construct harbouring a non-related sequence (Fig 2.25). This implies that nucleotides
Fig. 2.24 Comparing cell survivals in cells co-transfected with miR-378 and Fus-1

(A) miR-378- or vector-transfected U87 cells were transiently transfected with the Fus-1 or vector. Expression of Fus-1 was confirmed by Western blot probing with the anti-myc antibody.

(B) The cells were subjected to cell survival assay. Transfection of Fus-1 reduced miR-378’s effect on cell survival. Significant differences are indicated by asterisks. **, p < 0.01. Error bars, SEM (n=4).
**Fig. 2.25 Targeting of Fus-1 by miR-378 in luciferase activity assay**

COS-7 cells were co-transfected with the miR-378 construct and a luciferase reporter construct, which had been engineered with a fragment of the Fus-1 3’-UTR harbouring either the target sequence of miR-378-3p or mutated sequences. As a negative control, the luciferase reporter construct was also engineered with a non-related fragment of cDNA (Ctrl). Luciferase activities were determined. Significant differences are indicated by asterisks. **, p < 0.01. Error bars, SEM
748-770 in 3'UTR of Fus-1 are the interacting sequence of miR-378. The construct expressing two copies of miR-378 exhibited a greater effect on the reducing luciferase activities than that expressing one copy of miR-378 (Fig. S2.6B).

To confirm the down regulation of Fus-1 by miR-378, a fragment of the Fus-1 3'UTR was cloned and inserted into the Fus-1 construct following the translation stop codon, named FusUTR (Fig. 2.4B). Co-transfection of miR-378 with FusUTR resulted in lowered translation of Fus-1 than that of co-transfection of miR-378 with GFP vector (Fig. 2.26A). RT-PCR analysis of mRNA from duplicated samples produced similar levels PCR product (Fig. 2.26B), indicating that miR-378 only affected Fus-1 translation.

To conclude the effect of miR-378-regulated Fus-1 expression on cell survival, co-transfected cells were monitored in cell survival assay. U87 cells stably expressing miR-378 were then transfected with the Fus-1 or FusUTR construct, and cells were cultured on Petri dishes in serum-free conditions. Survived cells were examined under a light microscope and counted using trypan blue staining (Fig. 2.27A). This result indicated that expression of Fus-1 reversed the effect of miR-378-mediated enhancement in cell survival. Exogenous expression of Fus-1 was sufficient to cause cell death, suggesting that the effect of miR-378 on enhanced cell survival was partially taking place through repression of Fus-1 expression. To further confirm the targeting of the 3'UTR of Fus-1 by miR-378, the GFP- and miR-378-transfected cells were transiently transfected with the FusUTR construct. Survived cells were examined under a light microscope and surviving cells were counted (Fig. 2.27B). While expression of FusUTR in the GFP-transfected cells greatly reduced survival rate, expression of FusUTR in the miR-378-transfected cells did not, suggesting repression of FusUTR translation by miR-378.
Fig. 2.26 Western blot and RT-PCR analysis of Fus-1 and miR-378 co-transfection

(A) Cell lysate was prepared from U87 cells co-transfected with FusUTR and either miR-378 or a GFP vector, followed by Western blot probing with an anti-myc tag antibody, followed by anti-actin antibody to confirm equal loading.

(B) Transcription of Fus-1 was also determined by RT-PCR.
Fig. 2.27 Cell survival assay with co-transfected stable cells

(A) U87 cells expressing miR-378 were transfected with Fus or FusUTR. Survival assays indicated that transfection of Fus reversed the effect of miR-378-mediated enhancement in cell survival.

(B) GFP- and miR-378-transfected cells were transfected with FusUTR. While expression of FusUTR in GFP-transfected cells greatly reduced survival, expression of FusUTR in the miR-378-transfected cells did not. Significant differences are indicated by asterisks. **, p < 0.01. Error bars, SEM (n=5).
2.5 Conclusion

In summary, we have demonstrated that a construct harbouring a duplicate pre-microRNA sequence can be successfully expressed and processed to mature microRNA. With this approach, we have demonstrated that miR-378 functions as an oncogene by enhancing tumor cell survival, blood vessel expansion, and tumor growth. It acts on two tumor suppressors, SuFu and Fus-1. Other tumor suppressors may also be involved in miR-378 function. This study sheds light on the possibility of expressing or modulating miRNAs as means of gene therapy.
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**Table 2.1 List of primers used**
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Table 2.1 List of primers used- continued
Fig S2.1 Illustrating the RT-PCR reaction for mature microRNA mir-378

The mRNA was isolated with mirVana miRNA Isolation Kit (Ambion). RT-PCR was performed using Superscript II Reverse Transcriptase (Invitrogen). PCR was carried out at the temperature of 94 °C, 55 °C, and 72 °C for 30 cycles.
**Fig. S2.2 Stable MT1 cells under fluorescence microscope examination**

MT-1 cells were transfected with miRNA miR-378 or a control vector. The cells were maintained in serum-free conditions or in serum-containing conditions but allowed to cell to over grown. Cell survival was monitored with a fluorescent microscope. Transfection with the miR-378 construct enhanced cell survival as compared with the control cells transfected with GFP alone.
Fig. S2.3 Morphological change of miR-378-transfected U87 cells

U87 cells transfected with the mir-378 construct or a control vector were maintained in serum-free conditions in Petri dishes. Cell morphology changed after two days of culture.
Fig S2.4 Tumor formation affected by miR-378 over-expression.

(A) U87 cells transfected with mir-378 or control were injected subcutaneously into nude mice. 19 days after cell injection, large tumors were detected in cells transfected with mir-378 but not in the control group.

(B) The mice were sacrificed and the tumors were removed.

(C) Tumor sizes were measured and tumor growth curves were obtained.
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\[
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**Fig S2.5 Conservation of miR-378 target site in SuFu 3’UTR**

Comparison of mir-378 target sequence at the 3’UTR of SuFu mRNA across different species. Hs, Human; pt, chimpanzee, mm. mouse; rn, rat, cf, dog.
Fig S2.6 Correlative relationship between miR-378 and SuFu expression

RNA and cell lysates were collected from a variety of human cell lines. Isolated RNA were amplified for miR-378 expression by RT-PCR, and their protein lysates were probed by anti-SuFu antibody by western blot.
Fig S2.7 Luciferase activity with one-copy miR-378 construct

(A) The luciferase report constructs containing the 3'UTR of SuFu (Sufu) or mutant SuFu (Sufu-mu) were co-transfected with mir-378 (expressing two copies of pre-mir-378) or mir-378-1 (expressing one copy of pre-mir-378) in COS-7 cells, followed by luciferase activity assays. Mir-378 played a greater effect than mir-378-1 on reduction of luciferase activities normalized with Sufu-mu construct. Significant differences are indicated by asterisks p < 0.01. Error bars, SEM (n=4).

(B) The luciferase report constructs containing the 3'UTR of Fus (Fus) or mutant Fus (Fus-mu) were co-transfected with mir-378 or mir-378-1 in COS-7 cells, followed by luciferase activity assays. Mir-378 played a greater effect than mir-378-1 on reduction of luciferase activities normalized with Fus-mu construct. Significant differences are indicated by asterisks **, p < 0.01. Error bars, SEM (n=4).
miR-378-3p:

TGTGTCCTGG---------AC-CTCAGTCCTC-5’

Fig S2.8 Conservation of miR-378 target site in Fus-1 3’UTR
Comparison of mir-378 target sequence at the 3’UTR of Fus-1 mRNA across different species. Hs, Human; pt, chimpanzee, mm. mouse; rn, rat, cf, dog.
Fig S2.9 Effect of Fus-1 in cell survival

U87 cells stably transfected with an siRNA construct against Fus-1 were grown on Petri dishes in serum-free conditions. Cell survival was monitored and quantified by counting. Significant differences are indicated by asterisks. **, p < 0.01. Error bars, SEM (n=4).
Chapter 3

Versican and Fibronectin expression is targeted by miR-199a-3p

A version of this chapter is published in PLoS ONE (Lee DY, Shatseva T, Jeyapalan Z, Du WW, Deng Z, Yang BB. 4(2):e4527)
3.1 Abstract

Versican (VCAN) is an extracellular matrix proteoglycan, and expression of which is elevated in a variety of human tumors. Versican also serve as an early diagnostic and prognosis marker in several types of cancer. Computational analysis indicates that a large number of microRNAs can bind to 3’ untranslated region (3’UTR) of VCAN. miRNAs do not code for proteins and functions by regulating gene expression by base-pairing to 3’UTR of target mRNA. In the search of miRNAs that regulates VCAN, miRNA:UTR identification PCR is developed to assess the interaction of predicted miRNA and VCAN 3’UTR in silico. In which, miR-199a-3p is found to target both Versican and Fibronectin, which is another extracellular matrix molecule. The targeting of VCAN and FN is further confirmed by luciferase reporter assay. In this study, we also explore the possibility to regulate mature miRNA and thus its subsequent function. Here we report that expression of Versican 3’UTR induces organ adhesion in transgenic mice by modulating miR-199a-3p activities. Transgenic mice expressing a construct harboring the 3’UTR of Versican exhibits the adhesion of organs. Expression of VCAN and FN, two targets of miR-199a-3p, are up-regulated in transgenic mice, suggesting that the 3’UTR binds and modulates miR-199a-3p activities, freeing mRNAs of VCAN and FN from being repressed by miR-199a-3p. Enhanced adhesion by expression of the 3’UTR was confirmed by in vitro assays. This study proves that miR-199a-3p can regulate cell adhesion by targeting both VCAN and FN. Inhibiting miR-199a-3p function by expressing VCAN 3’UTR in transgenic mice results in elevation of VCAN and FN translation and occurrence of organ adhesion.
3.2 Introduction

Versican (VCAN) is a chondroitin sulfate proteoglycan located primarily in the extracellular matrix. It binds to hyaluronan and is involved in the assembly of extracellular matrix. VCAN has been shown to mediate a variety of cellular activities [167-169] and contributes to several pathogenesis [167, 170]. Extracellular proteins contribute to cancer progression by providing external signals to both transformed cells and their neighbouring healthy cells. Many studies have reported an elevated of VCAN expression in tumor formation. Patients with breast cancer containing versican-rich peritumoral stroma experienced shorter relapse-free intervals than patients with versican-poor tumors [171]. Furthermore, versican also serve as an early prognostic marker in prostate cancer progression [156]. Since miRNA expression is dysregulated in cancer, expression of VCAN may be influenced by miRNAs. This study is designed to investigate the potential miRNA or miRNAs that regulate Versican translation. We hypothesize that translation of VCAN mRNA is repressed by miR-199a-3p. Conversely, expression of VCAN 3’UTR can be used to modulate miRNA activities. As a result, translation of VCAN and another matrix protein, Fibronectin, will improve by arresting function of miR-199a-3p. This hypothesis will be tested in a cell model and also in transgenic animals expressing VCAN 3’UTR.

3.3 Materials and Methods

Construct Generation

To study the effect of versican 3’UTR on cell activities, we have cloned the 3’UTR by RT-PCR using two primers Huver-UTRNXbaI and Huver-UTRCApal. The PCR product was digested with restriction enzymes Xbal and Apal and inserted into Xbal- and
Apal-opened pcDNA3.1 vector. After transformation, colony selection, and DNA mini-preparation, and restriction enzyme digestion, the correct clones were sequenced to ensure identity of the 3'UTR (Fig. 3.10A).

A luciferase reporter vector (pMir-Report; Ambion) was used to generate the luciferase constructs. The 3’UTR of versican was cloned using 2 primers, HuverUTR-NSpel and HuverUTR-CHindIII, by PCR. The PCR products were then digested with Spel and HindIII and the fragment was inserted into a SpeI- and HindIII-digested pMir-Report Luciferase plasmid (Ambion), to obtain a luciferase construct, Luc-VCAN. Primers used in this study are listed in Table S1. A mutant construct was generated with two PCRs, one using two primers, HuverUTR-NSpel and HuverUTR-mu-RXbaI, the other using HuverUTR-mu-FXbal and HuverUTR-CHindIII. After restriction enzyme digestion, one with SpeI and XbaI, and the other with XbaI and HindIII, both fragments were ligated with pMir-Report vector digested with SpeI and HindIII.

A fragment of the 3’UTR of fibronectin was also cloned using 2 primers, FN1-N3’Sacl and FN-199aC3’MluI, by RT-PCR. The PCR products were then digested with Sacl and MluI and the fragment was inserted into a Sacl- and MluI- digested pMir-Report Luciferase plasmid (Ambion), to obtain a luciferase construct, Luc-FN. A mutant construct, Luc-FN-mut, was generated with two primers FN1-N3’Sacl and FN-199aC3’MluI-Mut using similar approach.

To serve as a negative control, a non-related sequence was amplified from the coding sequence of the chicken versican G3 domain using 2 primers, chver10051Spel and chver10350Sacl. It is expected that there is no endogenous miRNA bind to this fragment as it is in the coding region. The PCR product was then inserted into a SpeI- and Sacl-digested pMir-Report Luciferase plasmid.
miRNA:UTR identification PCR

For in vitro binding of microRNAs with versican 3'UTR, a PCR method was developed. The pcDNA3.1 plasmid containing the 3'UTR of versican was used as the template in PCR. The forward primer located at the vector (pcDNA3.1hygro). The reverse primers for different miRNAs are listed in Table 3.1. In general, the PCR mixture contained: 100 mM KCl, 100 mM \((\text{NH}_4)_2\text{SO}_4\), 200 mM Tris HCl, pH 8.75, 1.0% Triton X-100, 1 mg/ml BSA, 200 M dNTPs, 2 M forward primer, 2 M reverse primer, 1 unit Taq DNA polymerase. The parameters for the PCR reaction were: one cycle at 95 °C for 10 min; 35 cycles at 95 °C for 2 min, 37 °C for 2 min, 72 °C for 2 min; and a final elongation step at 72 °C for 10 min. The PCR products were then visualized with a 1.5% agarose gel.

Generation and genotyping of 3'UTR transgenic mice

The transgene was released from the plasmid by digestion with ApaLI and StuI. The digested product was fractionated by agarose gel electrophoresis and the 3 kb transgene fragment was excised from the gel, purified by Elutip mini-column (Schleicher and Schuell, Keene, NH) and then resuspended in injection buffer (10 mM Tris, pH 8.0 and 0.1 mM EDTA) at a concentration of 1 to 2 ng/l. The transgene was microinjected into the male pronuclei of C57BL/6 X CBA F₂ mouse zygotes. Injected embryos were implanted into the oviducts of pseudopregnant recipient females using a standard protocol approved by the Animal Use Subcommittee of the University Council on Animal Care, The University of Western Ontario. Transgenic founder lines were maintained by backcrossing with C57BL/6 X CBA F₁ mice. Genotyping was performed by PCR, using primers EGFP-347F pairing with EGFP-668R (for CMV promoter and huver10861F
pairing with Huversican-UTRCApal (for versican 3'UTR), and tail snip or ear punch DNA as template. GAPDH served as a control using primers mo-Gapdh1F and mo-Gapdh250R.

**Cell adhesion assay**

Vector- or 3'UTR-transfected cells were plated onto culture dishes at a density of 4x10^5 cells/ml and incubated for 30 min with DMEM containing 5% FBS. After 30 min, cells were fixed with 3.7% paraformaldehyde. Adhering cells were counted and cell images were captured using a phase-contrast microscope. Ten different fields (100x) were used for cell counting.

**Western Blot**

Organs were weighted and homogenized with lysis buffer containing protease inhibitors (150 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.5 M EDTA, 20% Triton X-100, 8 M Urea, and 1x protease inhibitor cocktail). Protein concentration was measured by Bio-Rad Protein Assay kit (#5000-0006). The lysates were subjected to SDS-PAGE and then transferred to nitrocellulose membranes probed with a primary antibody against versican (Lifespan Biosciences, LS-C25140), fibronectin (BD, 610078), or β-actin (Sigma-Aldrich) overnight at 4°C. After incubation with corresponding HRP-conjugated secondary antibodies, the membranes were washed, followed by detection with the ECL kit.

**Tissue preparation and Immunohistochemistry**

Organs were freshly excised and fixed in formalin overnight, immersed in 70% ethanol, embedded in paraffin, and sectioned by a microtome (Leica RM2255). The
sections were de-paraffinized with xylene and ethanol and then boiled in a pressure cooker. After washing with Tris-Buffered-Saline (TBS) containing 0.025% Triton X-100, the sections were blocked with 10% goat serum and incubated with primary antibody against versican, fibronectin, or collagen Type1 (Santa Cruz, sc-25974) in TBS containing 1% bovine serum albumin (BSA) overnight. The sections were washed and labeled with biotinylated secondary antibody, followed by avidin conjugated horse-radish peroxidase provided by the Vectastain ABC kit (Vector, PK-4000). The slides were subsequently stained with Mayer’s Hematoxylin for counter staining followed by slide mounting.

**Luciferase Reporter Assay**

U343 cells were seeded onto 24-well tissue culture plates at a density of $3 \times 10^4$ cells/well in DMEM containing 10% FBS and maintained at 37°C for 24 hrs following the methods described by us recently [172]. The cells were co-transfected with the luciferase reporter constructs and the 3’UTR construct by using Lipofectamine 2000. The cells were then collected by trypsin and lysed with a luciferase specific lysis buffer from Luciferase Assay Kit (Promega). Cells were centrifuged at 3000 rpm for 5 min. The supernatants were transferred into a black 96-well plate (3x10 μl) for luciferase activity measurement and into a transparent 96-well plate (3x50 μl) for β-gal activity determination. For the luciferase activity measurement, 70 μl of luciferase assay reagent was added into each well and the luciferase activity was detected by using microplate scintillation and luminescence counter (Packard, Perkin Elmer). For the internal control of β-gal activities, 90 μl of assay reagent (4 mg/ml ONPG, 0.5M MgSO$_4$, β-mercaptoethanol and 0.4M sodium phosphate buffer) were added into each well. The plate was then incubated at 37°C for 60 min. The absorbance at 410 nm was measured
by using a microplate reader (Bio-Tek Instruments, Inc.).

**Statistical Analysis**

The results (mean values ± SD) of all the experiments were subjected to statistical analysis by *t*-test unless otherwise specified. The level of significance was set at *p* < 0.05.

### 3.4 Results and Discussion

#### 3.4.1 Versican 3’UTR interacts with multiple miRNAs

Versican (VCAN) is an extracellular matrix protein that is known to mediate various cellular activities such as proliferation, apoptosis, cell adhesion and differentiation. Increase in VCAN expression is also involved in tumor formation [156, 173]. In the previous chapter, the oncogenic potential of miR-378 was studied. Staining of tumors sectioned formed by vector-transfected cells showed that VCAN is accumulated in the peripheral border of tumor, where cells actively proliferate (Fig. 3.1). Distribution of VCAN spread much more evenly in the tumor generated by *miR-378*-transfected cells instead of forming a narrow proliferative zone in the control tumors. Down-regulating gene expression by siRNA has been proven to be an efficient way of modulating gene expression. However, the use of natural existing miRNAs to regulate gene expression can be as effective by targeting genes with similar functions. According to the results of our first study, miR-378 enhances cell survival by targeting SuFu and Fus-1. Therefore, we are interested in finding the miRNAs that regulate VCAN and potentially other matrix proteins at the same time.

Unlike some mRNAs, VCAN mRNA has a 3’UTR and hence it is possible to be
Fig. 3.1 Tumor sections stained with anti-VCAN antibody
Tumors formed by cells transfected with miR-378 or control vector were sectioned and stained with anti-VCAN antibody. In the tumor derived from cells transfected with miR-378, VCAN were distributed near blood vessels and proliferative zones of the tumor. On the other hand, VCAN appeared to be localized at borderline of tumor in the control tumor.
regulated by miRNA. In order to prove this assumption, we developed a number of experiments to analyze the effects of the 3'UTR. A conserved region of VCAN 3'UTR, right after the stop codon, was linked with the construct expressing versican G3 domain, producing the G3-UTR (Fig 3.2A). Cell lysate prepared from U343 cells transiently transfected with the G3 and G3-UTR constructs was subjected to Western blot analysis probed with anti-G3 and anti-actin antibodies simultaneously. While actin levels were similar, G3 levels were much lower in cells transfected with the G3-UTR construct (Fig. 3.2B). This result suggests that some endogenous miRNAs targeted the 3'UTR and repressed G3 translation. The 3'UTR was also linked to a GFP expression unit (Fig. 3.3A). Fluorescent levels of U87 and U343 cells stably transfected with the GFP and GFP-UTR constructs were monitored with flow cytometry (Fig. S3.1). Cells transfected with the GFP-UTR construct produced lower levels of fluorescence than cells transfected with the GFP construct. Cells transfected with the GFP and GFP-UTR constructs were also examined under a light and fluorescent microscope (Fig. 3.3B). This experiment was done in two different cell lines and the results were similar (Fig. 3.3C). These results suggested that translation of VCAN G3 domain was repressed due to the addition of 3'UTR in the mRNA sequence. Presence of 3'UTR allows miRNA to exert repressive pressure on translation.

Currently, there are few methods to predict miRNA:UTR interaction. Computational algorithms rely on miRNA's seed region match with the target sites located at the 3'UTR of the mRNA [6]. Interaction between the rest of the sequences of miRNA and its target sites also affect the thermodynamic stability of the miRNA:3'UTR complex [174]. Conservation of target sites between different species also improves the target recognition, but experimental proof is always the key. To indicate a direct interaction of miRNAs with the 3'UTR, we developed a PCR assay to test the potential binding
Fig. 3.2 Examining translational repression in the presence of 3'UTR by western blot

(A) Diagram of two generated constructs, linking VCAN C-terminal G3 domain with or without its 3'UTR in pcDNA3 vector.

(B) Cell lysate prepared from U343 cells transfected with various amount G3 or G3-UTR constructs was subjected to Western blot analysis by probing with anti-G3 and anti-actin antibodies. While actin levels were similar, G3 levels were much lower in cells transfected with the G3-UTR construct.
Fig. 3.3 Examining translational repression in the presence of 3’UTR by fluorescence microscope

(A) Diagram of two generated constructs, linking GFP coding region with or without VCAN 3’UTR in pcDNA3 vector.

(B) U343 or U87 cells transfected with GFP or GFP-UTR constructs were cultured and fixed in a slide chamber. Cells were then examined under fluorescence microscope. Typical pictures were shown.

(C) Number of fluorescent cells were counted from pictures randomly taken under microscope. There were less fluorescent cells transfected with GFP-UTR compared to ones transfected with GFP.
interactions of miRNAs with the 3’UTR in order to validate the target sites \textit{in silico}. This method assumes that the miRNAs targeting the 3’UTR could serve as a PCR-primer, and the PCR products could be generated by a vector-specific primer pairing with the miRNA primer (Fig. 3.4A). The nucleotide sequence of miRNA-X primer corresponds to the RNA sequence of miRNA-X but with the substitution of uridine for thymidine (Table 3.2). A conserved region of VCAN 3’UTR was cloned, generating VCAN-UTR (Fig. 3.10A). This construct was used as a PCR-template.

We analyzed the potential miRNAs that bind to VCAN 3’UTR. A number of candidates with low binding energy were detected. We tested 17 different miRNAs selected from the potential candidates for the 3’UTR of VCAN. As shown in Figure 3.4B and Supplementary Figure S3.2, only 14 out of the 17 miRNAs showed anticipated PCR products. There was no correlation between positive PCR results and the G/C content or the melting temperature of primers, which indicated the specificity of miRNAs toward the 3’UTR of VCAN. This method can be used to screen miRNAs that bind to the 3’UTR of interest. It is more efficient than the commonly-used luciferase assay and can be used to confirm the candidate binding sites as identified by several tools \textit{in silico}. Nevertheless, this is not to replace any existing methods. Rather, it adds an alternative approach to identify miRNAs targeting a 3’UTR of interest. It also produces a shorter list of candidates for validation by luciferase activity assays and transfection experiments.

It is expected that one 3’UTR contains many miRNA binding sites and one miRNA might target many 3’UTRs. This would create a balanced network composing of the synergy and counteraction of miRNA: UTR interactions. The homeostatis of miRNAs and miRNA-binding sites might be disrupted through changes in the expression of transcripts or miRNAs.
Fig. 3.4 miRNA:UTR Identification PCR

(A) Scheme of PCR method to identify the interaction of miRNAs with 3’UTR. An oligonucleotide corresponding to miRNA-X is used as a reverse primer. It binds to the potential targeting sites on the antisense strand of the 3’UTR construct, depending on the extent of complementation. One forward primer docked on different location of the vector was used to pair with the miRNA primer for PCR.

(B) PCR products were obtained showing different size of products corresponding to the forward primer and the miRNA sequences. The expected sizes of PCR products are indicated with arrows.
3.4.2 Versican and Fibronectin are targets of miR-199a-3p

Using the computation algorithm FindTar (http://bio.sz.tsinghua.edu.cn/findtar), we found a great number of miRNAs potentially targeting VCAN 3’UTR. One of the positive miRNAs that showed strong and promising interaction with the VCAN 3’UTR is miR-199a-3p. Using the online search engine TargetScan4.0 (www.targetscan.org), we found a great number of genes that potentially targets of miR-199a-3p including VCAN (Genbank access number NM_004385) and fibronectin (FN) variant 1-6 (Genbank access number NM_212482, NM_212475, NM_002026, NM_212478, NM_212476, and NM_212474).

To test whether VCAN was a target of miR-199a-3p, U343 cells were co-transfected with a luciferase construct containing VCAN 3’UTR (Luc-VCAN) or mutant construct (Luc-VCAN-mut), in which the miR-199a-3p target site was mutated by nucleotide replacement (Fig. 3.5A). Luciferase activity assays showed that while presence of the VCAN 3’UTR reduced luciferase activities, mutation of the miR-199a-3p target site partially rescued luciferase activities (Fig. 3.5B). This suggests that miR-199a-3p repressed VCAN expression by targeting its 3’UTR. Furthermore, incomplete rescue of luciferase activities suggests that the versican 3’UTR is also targeted by other endogenous miRNAs.

To test whether FN was a target of miR-199a-3p, we cloned the fragment of FN 3’UTR containing the miR-199a-3p target site. The fragment was inserted into the luciferase reporter vector producing a construct Luc-FN. The potential target site of miR-199a-3p was mutated generating Luc-FN-mut (Fig. 3.6A). U343 cells were co-transfected with Luc-FN or the mutant Luc-FN-mut. Luciferase activity assays showed that insertion of the Fibronectin 3’UTR repressed luciferase activities as compared with the control (Fig. 3.6B). Mutation of the miR-199a-3p target site partially
Fig. 3.5 miR-199a-3p regulates VCAN expression by luciferase activity assay

(A) Versican 3’UTR (nucleotides 275-299) was cloned and inserted into the luciferase reporter vector pMir-Report. Mutations were generated on the hypothetical target sequence (red color).

(B) U343 cells were co-transfected with the miR-199a and the luciferase reporter construct Luc-VCAN or the mutant construct Luc-VCAN-mut. Results indicated that miR-199a-3p repressed luciferase activities when it harbored the versican 3’UTR, which was abolished when its target site was mutated. Significant differences are indicated by asterisks. ** Error bars, SEM (n=3), ** p<0.01
Fig. 3.6 miR-199a-3p regulates FN expression by luciferase activity assay

(A) Fibronectin 3'UTR (nucleotides 663-683 of the 3'UTR, Upper) was found to be the potential target of miR-199a-3p. A fibronectin 3'UTR was cloned and inserted into the luciferase reporter vector pMir-Report. Mutations were generated on the potential target sequence (red color)

(B) U343 cells were co-transfected with miR-199a and luciferase reporter construct harboring fibronectin 3'UTR (Luc-FN) or mutant (Luc-FN-mut). Results indicated that miR-199a-3p repressed luciferase activities when it harbored the fibronectin 3'UTR, which was abolished when the potential miR-199a-3p target site was mutated. Significant differences are indicated by asterisks. ** Error bars, SEM (n=3), ** p<0.01
rescued luciferase activities. This suggests that miR-199a-3p repressed FN expression by targeting its 3'UTR. Incomplete rescue of luciferase activities suggests that the FN 3'UTR is also targeted by other endogenous miRNAs.

3.4.3 Inhibit miR-199a-3p function by expression of Versican 3’UTR

From the above results, miR-199-3p exerts repressive pressure on VCAN and FN translation. Therefore, removal or antagonizing miR-199a-3p will eliminate its repression on VCAN and FN. Over-expression of the VCAN 3’UTR would reduce the levels of available miR-199a-3p by forming into a non-functional duplex (Fig. 3.7). When less miR-199a-3p is available to bind to its target mRNAs, translation efficiency of these mRNAs will be restored. This interaction is expected to affect protein expression, leading to the functional consequence.

A series of in silico experiments by were performed to examine this possibility. Luciferase reporter system was used in validation. Cells were transfected with incremental concentrations of Luc-ctrl or Luc-VCAN and luciferase activity were normalized by using cells transfected with control luciferase. Results showed that cells transfected with Luc-VCAN always generated lower levels of luciferase activities than cells transfected with a control construct (Fig. S3.3), suggesting that repression of luciferase expression was due to endogenous miRNAs. Nevertheless, with increased concentrations of Luc-VCAN, the relative luciferase activities increased when normalized with the control construct. This suggests that higher concentrations of Luc-VCAN diverted the repression from endogenous miRNAs and allow more Luc-VCAN to be translated. No increase in expression of miRNA in respond to the increasing luciferase constructs transfected.

To corroborate this finding, Luc-VCAN construct was co-transfected with the VCAN
Computational analysis showed that miR-199a* potentially targeted both versican and fibronectin 3'UTRs. Overexpression of versican 3'UTR would attract endogenous miR-199a* and thus freeing versican mRNA and fibronectin mRNA for translation.

**Fig. 3.7 Scheme of using 3’UTR to antagonize miR-199a-3p**
3'UTR construct. Increased 3'UTR concentrations in the transfection generated higher levels of luciferase activities in U343 cells (Fig. 3.8) and U87 cells (Fig. S3.4). In addition, Luc-FN was also co-transfected with VCAN-UTR, luciferase activities increased along with incremental abundance of non-coding VCAN 3'UTR (Fig. 3.9), suggesting the competing of miRNAs, such as miR-199a-3p, between VCAN-UTR and Luc-FN. Presence of VCAN 3'UTR bound to available pool of miR-199a-3p, relieving the translational repression on luciferase constructs.

3.4.4 Expression of Versican 3'UTR affects cell morphology and cell adhesion in vitro

Presence of abundant VCAN 3'UTR is expected to boost VCAN and FN expression in vitro. Fibronectin is an extracellular glycoprotein which plays key roles in cellular activities such as cell adhesion and migration, and also an interactive role in fundamental processes such as wound healing, embryogenesis and tissue maintenance [175, 176]. Our previous studies indicated that VCAN regulates cell adhesion through its interaction with integrins [177]. Thus, elevation in VCAN and FN protein expression is expected to influence cell activities, such as cell adhesion.

To study how VCAN 3'UTR affected adhesion, the human astrocytoma cell line U343 was stably transfected with the 3'UTR construct or an empty vector (Fig. 3.10B). The cells were resuspended in normal culture conditions, and were examined under a light microscope and photographed. The 3'UTR-transfected cells attached to tissue culture plates slower than the vector-transfected cells (Fig. S3.5). After cell attachment, the UTR-transfected cells tended to adhere together and appeared less elongated (Fig. 3.11A). Two days after cell inoculation, 3'UTR-transfected cells proliferate, forming an island-like morphology (Fig. 3.11B). To confirm the direct effect of the 3'UTR, 4 different
**Fig. 3.8 Antagonize miRNAs with 3’UTR in luciferase reporter assay**

Luciferase reporter vector harboring the versican 3’UTR was co-transfected with the versican 3’UTR construct at different amount combined with a control vector in U343 cells. When twice amount of 3’UTR was co-transfected, luciferase activity significantly increase by 15% compared to cells transfected with control vector alone.
**Fig. 3.9 Antagonize miRNAs with 3’UTR in luciferase reporter assay**

Luciferase reporter vector harboring the versican 3’UTR was co-transfected with the FN 3’UTR construct at different amount combined with a control vector in U343 cells. Luciferase activity elevated along with increasing proportions of 3’UTR that was co-transfected.
Fig. 3.10 Generation of VCAN 3’UTR expression construct and clones

(A) Diagram of a construct expressing a fragment of VCAN 3’UTR and a hygromycin selection marker in pcDNA3.1.

(B) RNA was prepared from one pooled cell line and two individual clones stably transfected with VCAN-UTR or the empty vector, and were subjected to RT-PCR. Expression of 3’UTR was confirmed by gel electrophoresis.
Fig. 3.11 Morphological change of U343 cells transfected with 3’UTR

(A) Vector- or the 3’UTR-transfected cells were inoculated in tissue culture plates overnight. Cell morphology was examined under a light microscope.

(B) Two days after cell inoculation, 3’UTR-expressing cells exhibiting island-like morphology
siRNAs complementary to the 3'UTR were synthesized. Down regulation of the 3'UTR was confirmed by real-time PCR amplifying a fragment of the 3'UTR (Fig. S3.6). Cell adhesion assays showed that transfection with the siRNAs partially rescued the reduced adhesion in the 3'UTR-transfected cells (Fig. 3.12B). The number of cells were counted by trypan blue staining and compared for significance (Fig. 3.12C). Furthermore, transfection with siRNA against VCAN 3'UTR also reversed the altered cell morphology (Fig. 3.12A).

### 3.4.5 Transgenic expression of VCAN 3’UTR causes organ adhesion

To study the effect of VCAN 3’UTR on modulating miRNA functions *in vivo*, the 3’UTR transcription unit was cropped from VCAN-UTR construct for the generation of transgenic mice. Four VCAN 3’UTR transgenic founder animals were obtained subsequently. All of them were bred with wild-type mice and a total of twelve litters of F1 mice were attained. Genotyping was performed by PCR using two different pairs of primers and the result from one litter is shown in Figure 3.13A. Expression of the transgene in a number of organs was also confirmed by RT-PCR (Fig. 3.13B). Real-time PCR analysis indicated that the levels of the 3’UTR were 15-fold higher than that of wild-type (Fig. 3.13C).

To test whether the expression of VCAN 3’UTR affected VCAN expression, we prepared protein lysates from the brain, heart, kidneys, lungs, and spleen and analyzed VCAN expression by western blotting. Results from western blot showed that the 3’UTR transgenic mice expressed higher levels of VCAN compared with the wild-type mice (Fig. 3.14). The organs were also subjected to histological analysis probed with anti-VCAN antibody. In the reproductive system, VCAN levels were much higher in the 3’UTR mice as compared with the wild-type mice (Fig. 3.15, upper left
Fig. 3.12 Knock-down of VCAN 3’UTR reverses changes in cell adhesion and morphology

(A) Vector- and 3’UTR-transfected cells were transfected with siRNA against VCAN 3’UTR or control scramble sequence. Morphological change by 3’UTR was reverted.

(B) Transfection of 3’UTR reduced cell adhesion in cell adhesion assay, and transfection of siRNA against 3’UTR rescue the number of cell adhered to tissue culture plates.

(C) The number of adhered cells were counted by tryphan blue staining.
Fig. 3.13 Genotyping VCAN 3’UTR transgenic mice

(A) Genotyping PCR was performed on tail DNA extracted from the same litter of F1 using two pairs of primers amplifying CMV promoter region and the downstream transcript VCAN 3’UTR.

(B) Expression of the transgene was analyzed by RT-PCR using RNAs isolated from different organs of UTR transgenic mice.

(C) By RT-qPCR, expression level of 3’UTR was quantified.
Fig. 3.14 VCAN expression in organs of 3’UTR transgenic mice by western blot

Protein lysate was prepared from different organs and subjected to western blot analysis probed with anti-VCAN antibody. Detection of beta-actin on the same membranes served as a loading control. Increased VCAN expression was detected in the organs harvested from the transgenic mice.
panel). In the lung tissues examined, VCAN level was much higher in the airways and pulmonary blood vessels of the transgenic lung compared with the wild-type (Fig. 3.15, upper right panel). Similar observations were made in the livers (Fig. 3.15, lower left panel), the connective tissues (Fig. 3.15, middle right panel), and the ribs of the UTR mice (Fig. 3.15, lower right panel). Both western blot and immunohistochemistry confirm an elevation of VCAN expression in the organs of 3'UTR mice. These results reinforced our finding in vitro with the luciferase system.

To test whether expression of VCAN 3'UTR affect FN expression level, we also subject the protein lysate from the brain, heart, kidneys, lungs, and spleen to western blot analysis by probing with anti-FN antibody. Results showed that the 3'UTR transgenic mice expressed higher levels of FN compared with the wild-type mice (Fig. 3.16). The organs were also subjected to histological analysis by probing with anti-FN antibody. In the spleen, FN level was higher in the connective tissue of 3'UTR mice when compared to the same tissues in wild-type mice. In addition, FN level was higher in the brain, connective tissues, liver, and ribs of 3'UTR mice compared to organs of wild-type (Fig. 3.17). Elevated expression of FN in vivo reinforced the results obtained in vitro with the luciferase reporter system.

In a number of the positive F1 mice were examined, organ adhesion was observed. We observed that in some 3'UTR transgenic mice, the livers were strongly adhered to the stomach (Fig. 3.18A, left panel). In some others, the livers adhered with connective tissues sticking to the body (Fig. 3.18A, middle panel) or directly adhered to the body (Fig. 3.18A, right panel). In a different transgenic line, stomach adhesion (Fig. S3.7, left panel) and liver adhesion (Fig. S3.7, right panel) were both observed. These adhering organs were subjected to histological analysis by Hematoxylin and Eosin (H&E) staining. Connection between liver and connective tissues could be clearly seen (Fig.
Fig. 3.15 VCAN expression in organs of 3’UTR transgenic mice by immunohistochemistry

Paraffin sections of tissues from reproductive system, lung, liver, connective tissues, and rib of the 3’UTR-transgenic and wild-type mice were stained with anti-VCAN antibody. In all sections shown, VCAN expression levels in the transgenic mice were higher as compared with the wild-type (arrows).
Fig. 3.16 FN expression in organs of 3’UTR transgenic mice by western blot

Protein lysate was prepared from different organs and subjected to western blot analysis probed with anti-FN antibody. Detection of beta-actin on the same membranes served as a loading control. Increased FN expression was detected in the organs harvested from the transgenic mice.
Fig. 3.17 FN expression in organs of 3’UTR transgenic mice by immunohistochemistry

Paraffin sections of tissues from spleen, brain, connective tissue, liver, and rib of the 3’UTR-transgenic and wild-type mice were stained with anti-FN antibody. In the transgenic spleen, the connective tissues expressed higher levels of FN than the wild-type tissues did (arrows). In the transgenic brain section, FN expression was higher in the blood vessels (arrows). In the connective tissues of the transgenic mice, some areas expressed higher levels of FN compared to wild-type tissues (arrows). In the transgenic liver, FN expression was higher along the edges of the liver than the wild-type liver.
Fig. 3.18 Organ adhesion in Hematoxylin and Eosin staining
(A) Photograph showing adhesion of liver to stomach (left panel), liver to diaphragm (middle), and liver to internal body wall (right).
(B) Paraffin sections of adhesion tissues were stained by Hematoxylin and Eosin (H&E). A picture of adhesion between liver and connective tissue was shown.
In the adhering organs of livers and pancreas, VCAN expression in the 3'UTR pancreas was much higher compared with the wild-type (Fig. 3.19A). Normally, the liver does not adhere to any organ. Surprisingly, in the 3'UTR transgenic mice, the liver adhered tightly to other organs such as pancreas and connective tissues of the body wall (Fig. 3.19B). In the extreme case, the liver lost its smooth edges (Fig. 3.19B, middle) and even merged with the connective tissue (Fig. 3.19B, bottom). More staining of VCAN in the adhesion junction between liver and stomach is presented in supplementary figure S3.9.

Expression of FN was also examined in the sections of adhesion junctions. In the adhering junctions between liver and pancreas, FN expression in the transgenic pancreas was clearly detected (Fig. 3.20A, upper left). In the adhesion junction between liver and connective tissues, FN was strongly expressed in the connective tissues adhering to the liver (Fig. 3.20A, lower left). In the extreme cases, liver lost its smooth tissue boundaries and grew along with connective tissue (Fig. 3.20A, right panel). In this area, FN expression was greatly promoted. Furthermore, FN levels were much higher in the adhesion areas between liver/liver, liver/stomach, liver/muscle, and pancreas/stomach (Fig. 3.20B). More staining of FN in the adhesion junction associated with liver is presented in supplementary figure S3.10.

3.5 Conclusion

In this study, a miRNA:UTR screening PCR was developed in the purpose of screening for potential interacting miRNAs from a large list of candidates. From this method, miR-199a-3p was found to target both VCAN and FN, confirmed by both in vitro
Fig. 3.19 VCAN stain of adhesion junction between liver and pancreas or connective tissues

(A) The levels of VCAN expression were higher in the pancreas that adhered to liver (arrows). VCAN expression in wildtype was also shown.

(B) The levels of versican expression were higher in the junctions between liver and the surrounding connective tissues (arrows). In the lower panel identified by the circle, some liver tissues (solid arrow) and connective tissues (open arrow, stained with anti-VCAN antibody) expanded into each other. No clear border were identified between the different tissues.
Fig. 3.20 FN stain of adhesion junction between liver and pancreas or connective tissues

(A) The levels of FN expression were higher in the 3'UTR pancreas that adhered to the liver (arrows). The connective tissues that adhered to the livers also expressed high levels of fibronectin. In the areas identified by the circles, some liver tissues (solid arrow) and connective tissues (open arrow, stained with anti-fibronectin antibody) were completely merged.

(B) Adhesion of different organs was identified between liver/liver, liver/muscle, liver/stomach, and pancreas/stomach. The junctions between the organs expressed high levels of FN (arrows).
and in vivo. Expression of a non-coding transcript, VCAN 3’UTR, was able to antagonize the function of miR-199a-3p, leading to elevated expression of both VCAN and FN in silico and in vivo. As a result of increased VCAN and FN expression, there was a change in cell morphology, increase in cell-cell adhesion, and loss in cell adhesion in cell adhesion assay. In addition, organ adhesion was observed in the 3’UTR transgenic mice. This result does not only suggest that a non-coding transcript is functional. It also points out a potential method of gene therapy by targeting miRNA involved in the pathological process.
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**Table 3.1 List of primers used**
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Table 3.2 List of primers used- continued
Fig. S3.1 FACS analysis of GFP intensity with or without VCAN 3’UTR in transfected cells

Typical fluorescent levels of U87 and U343 cells transiently transfected with GFP or GFP-UTR constructs were examined by FACS analysis.
**Fig. S3.2 miRNA:UTR identification PCR performed at 35 degree**

PCR was performed at a different temperature (35 °C). PCR products visualized showed different sizes corresponding to the predicted length using the particular forward primer and miRNA sequences. Expected sizes of PCR products are indicated with arrows.
Fig. S3.3 Increasing reporter expression alleviate miRNA repressive pressure in luciferase activity assay

U343 cells were transiently transfected with luciferase reporter vector harboring the VCAN 3'UTR (Luc-VCAN) or a control sequence (Luc-ctrl). Luciferase activities were normalized using the control as 100%. The luciferase activities of Luc-VCAN never reached the control, suggesting endogenous miRNAs targeting the versican 3’UTR. Nevertheless, the activities increased with higher doses of plasmids, suggesting that some versican 3’UTR absorbed some endogenous miRNAs freeing the others to translate.
**Fig. S3.4 Antagonize miRNAs with 3’UTR in luciferase reporter assay**

Luciferase reporter vector harboring the versican 3’UTR was co-transfected with the versican 3’UTR construct at different amount combined with a control vector in U87 cells. Increase amounts of VCAN 3’UTR bound more endogenous miR199a-3p and thus freed luciferase construct for translation, resulting in higher levels of luciferase activities.
Fig. S3.5 Effect of 3’UTR in cell adhesion in cell adhesion assay
Vector- or 3’UTR-transfected cells were inoculated in tissue culture dishes at normal culture condition. Cell adhesion was examined at 1 hour and 2.5 hour time points under a light microscope, and views were photographed.
**Fig S3.6 Effect of siRNA against VCAN 3’UTR by RT-qPCR**

The 3’UTR-transfected cells were transiently transfected with siRNA targeting the 3’UTR or a control sequence (ctrl), followed by real-time qPCR analysis of the 3’UTR levels in the transfected cells.
Fig. S3.7 Additional examination of organ adhesion
Photographs showing organ adhesion occurred between liver and stomach, also between liver and internal body wall or intestines in a different transgenic line of mice
Fig. S3.8 Expression of Type-I Collagen at adhesion junctions

The tissues at adhesion junctions were sectioned and immunostained with anti-type I collagen that normally deposits in wound healing areas. Collagen was expressed at high levels in the areas of tissue adhesion.
Fig. S3.9 More examination of VCAN expression at adhesion junctions
Photographs showing organ adhesion took place between stomach and connective tissues. Sections were stained with anti-VCAN antibody showing that versican was deposited in the adhesion junction areas.
Fig S3.10 More examination of FN expression in adhesion junctions
Paraffin sections of adhesion organs from a different transgenic line of mice were stained with anti-FN antibody. The levels of FN were higher in the adhesion junctions between liver and pancreas, between liver and connective tissue, and also between liver and liver.
Chapter 4

Versican 3’UTR modulates miRNA functions and tumor growth

A version of this chapter has been prepared as a manuscript for submission (Lee DY, Lee MH, Du WW, Shatseva T, Jeyapalan Z and Yang BB.)
4.1 Abstract

In our previous study, we have shown that a non-coding transcript, Versican 3'UTR, antagonizes miR-199a-3p function in regulating expression of two matrix proteins, Versican (VCAN) and Fibronectin (FN), resulting in enhanced cell-cell adhesion and organ-adhesion. Here, we report that the expression of VCAN 3'UTR does not only antagonize miR-199a-3p but also lower its steady state expression. VCAN 3'UTR was stably expressed in a mouse breast carcinoma cell line, 4T1. Reduction of miR-199a-3p levels in the cancer cells was found two-fold more than that of primary tissues. The regression of miRNA function consequently translated into differences in tumor growth. Computational analysis indicated miR-199a-3p and miR-144 both target a cell cycle regulator, Retinoblastoma 1 (Rb1). In addition, miR-144 and miR-136, another miRNA which has been shown to interact with VCAN 3'UTR, target another negative cell cycle regulator, Phosphatase and Tensin homolog (PTEN). Protein expression of Rb1 and PTEN were up-regulated synergistically in vitro and in vivo, suggesting that the 3'UTR binds and modulates miRNA activities, freeing mRNAs of Rb1 and PTEN from synergistic translational inhibition by several miRNAs. In tumor formation assays, cells transfected with 3'UTR formed smaller tumors compared with the control, due to decreased cell proliferation. This study suggests that miRNAs in the cancer cells are more susceptible for regulation, due to its interaction with VCAN 3'UTR. This
non-coding transcript has evolved to be targeted by groups of miRNAs with distinct functions, and thus can be used retrospectively to modulate miRNA activities.

4.2 Introduction

As a negative gene regulator, miRNA regulates about 30% of genes [178], and has been vigorously studied for its functions in various cell activities and involvement in many fundamental processes. Recently, proteomics studies have shown that a single miRNA impacts translation of hundreds of mRNAs [179, 180]. Moreover, most 3' Untranslated Regions (3'UTR) of these affected mRNAs harbour target sites that match the seed region of the miRNAs, suggesting that miRNAs with similar seed regions may have overlapping functions. In addition, there also have been evidence demonstrating that one miRNA can regulate expression of multiple genes of related functions in order to fine tune cell activities, as discussed in the previous two chapters. Conversely, multiple miRNAs aiming at the 3'UTR of a particular mRNA may target a set of mRNAs with similar functions as well. Many studies relate a cellular function with the regulation of a gene by a miRNA. However, we will attempt to investigate the commonality of different miRNAs that are targeting the same 3'UTR. Using the previously developed PCR method, potential miRNAs binding to 3'UTR can be screened. In this chapter, a fragment of versican 3'UTR is expressed in an in vitro cell model, and its effect on miRNAs levels and the changes in cell activities are investigated. The role of 3'UTR other than being a cis-element of mRNA is then unveiled.
4.3 Materials and Methods

Construct and 3’UTR transgenic mice generation

The versican 3’UTR expressing construct, VCAN-UTR that transcribes a conserved region of versican 3’UTR, was previously described (Fig. 3.10A). A list of primers used during construct generation is included in Table 4.1. A luciferase reporter vector (pMir-Report; Ambion) was used to generate the luciferase constructs. The 3’UTR of Rb1 was cloned using forward primer, huRB1-Sacl, and reverse primers, huRB1-miR144MluI and huRB1-miR199a*MluI, by PCR. The PCR products were digested with Sacl and MluI and then inserted into a Sacl- and MluI-digested pMir-Report Luciferase plasmid, to obtain luciferase constructs, Luc-RB1-144 and Luc-RB1-199a3p. Primers used in this study are listed in the SI Figure 6. Mutant constructs were generated with PCR by the same forward primer but different reverse primers, huRB1-miR144MluI-mut and huRB1-miR199a*MluI-mut. After double restriction enzyme digestion by Sacl and MluI, both fragments were ligated with pMir-Report vector opened with Sacl and MluI.

PTEN luciferase constructs were generated similarly. The 3’UTR of PTEN was cloned by PCR using a forward primer, huPTEN-Sacl, and two reverse primers, huPTEN-miR144MluI and huRB1-miR136MluI. The PCR products were double digested with Sacl and MluI and ligated with the Sacl- and MluI-opened pMir-Report Luciferase plasmid, to obtain luciferase constructs, Luc-PTEN-144 and Luc-PTEN-136. Their mutant constructs were generated with PCR by reverse primers, huPTEN-miR144MluI-mut and huRB1-miR36MluI-mut. After restriction enzyme digestion by Sacl and MluI, both fragments were ligated with the pMir-Report vector opened with Sacl and MluI.
To serve as a negative control, a non-related sequence was amplified from the coding sequence of the chicken versican G3 domain as previously described in Chapter 2. No miRNAs are expected to bind to this fragment as it is in the coding region.

Transgenic mice were generated by microinjecting a DNA fragment excised from the versican 3’UTR expressing construct. Details of generation and genotyping methods were previously described in Chapter 3. Tissue harvest and analysis have been approved by the Animal Care Committee of Sunnybrook Research Institute, Ontario, Canada.

**Tumor Formation Assay and Immunohistochemistry**

Mice of six-week-old strain BALB/c mice were injected with VCAN-UTR- and control-transfected 4T1 cell lines (5x10^5 cells) subcutaneously. Tumor growth was monitored weekly and sizes were recorded using a caliper by determining the length (L) and width (W), where \( V = (L \times W^2)/2 \). Tumors were retrieved by end of fourth week, fixed by formalin and then sectioned. After antigen retrieval, the sections were blocked with 10% goat serum and incubated with primary antibodies against PTEN (clone 6H2.1, Millipore), or Rb1 (ab32199, ABCAM) in TBS containing 1% bovine serum albumin (BSA) overnight. The sections were washed and labeled with biotinylated secondary antibody, followed by avidin conjugated horse-radish peroxidase provided by the Vectastain ABC kit (Vector, PK-4000). Staining was developed according to the manufacturer's protocols. The slides were subsequently stained with Mayer's Hematoxylin for counter staining followed by slide mounting.

**Cell Proliferation Assay and Cell Cycle Analysis**

Cells were plated onto a 6-well plate at 5 x 10^4 cells/well in 1.5% FBS containing
medium. Cell numbers were counted by trypan blue staining daily for a period of four
days. For cell cycle analysis, the cells were spun down at 1200 rpm for 3 minutes
repeatedly after initial fixation with 70% cold ethanol and following washes by PBS. In
the final step, 1x10^6 cells were resuspended in 1 ml PBS with the addition of 100 μl
ribonuclease (100 μg/ml, Sigma) and 400 μl propidium iodide (50 μg/ml, Sigma). After
leaving the samples in room temperature for five minutes, they were acquired by a
FACScan flow cytometer (BD Biosciences), and data was analyzed using CellQuest
software.

**Luciferase Activity Assay**

Luciferase activity assay was performed using a dual-luciferase reporter system
developed by Promega (E1960). In brief, U343 cells were seeded onto 24-well tissue
culture plates at a density of 3x10^4 cells/well in 10% FBS containing medium for 24 hrs.
Cells were co-transfected with the luciferase reporter constructs, corresponding miRNA
mimics, and Renilla luciferase construct by Lipofectamine 2000. The cells were then
lysed by 100 μl of passive lysis buffer per well on a shaker for 2 hours, and lysates were
centrifuged for supernatant collection. 20 μl of lysates were then mixed with 100 μl of
LAR II, and then firefly luciferase activity was measured by a single-sample luminometer.
For the internal control, 100 μl of Stop & Go reagent was added to the samples. Renilla
luciferase activities were then measured in the same tube. Luciferase activities between
different treatments were compared after normalization with Renilla luciferase activity.

For the VCAN-UTR competition experiments, the same luciferase system was used.
Besides 10 μg luciferase construct containing Rb1 or PTEN 3’UTR was transfected in
each well, cells were also transfected with increasing concentrations of VCAN-UTR and
corresponding amount of control vector to a total of 2 μg plasmid per well.
**Western Blot**

Cells were seeded onto a 6-well plate at 2 x 10^5 cells per well overnight. They were then transfected with 1 μg of VCAN-UTR or the control vector in combination with scramble RNA or siRNA against VCAN-UTR. Proteins were extracted 48 hours after transfection by lysing in 60 μl of lysis buffer containing protease inhibitors (150 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.5 M EDTA, 20% Triton X-100, 8 M Urea, and 1x protease inhibitor cocktail). Tissues were grinded in a quantity of lysis buffer according to weight. All samples were subjected to SDS-PAGE and then transferred to nitrocellulose membranes followed by incubating with a rabbit monoclonal antibody against PTEN (ab32199, ABCAM) by 1:1000, or mouse monoclonal antibody against RB1 (ab24, ABCAM) by 1:500 overnight at 4ºC. The secondary antibody used was goat anti-mouse IgG at 1:2000 dilution for 1 hour at room temperature. After detection of the protein bands, the blot was stripped and re-probed with mouse monoclonal antibody against β-actin (A5316, Sigma) to confirm equal sample loading. After secondary antibody incubation, the blot was washed and detected by ECL kit (Millipore) in autoradiography.

**RT-PCR and RT-qPCR**

Stably or transiently transfected cells (2.5 x 10^6) were harvested. Organs from transgenic mice were grinded and lysed with lysis buffer. Total RNA was extracted with the mirVana miRNA Isolation Kit (AM1560, Ambion) according to the manufacturer’s instructions. RT-PCR assays were performed as previously described in Chapter 2. Briefly, 2 μg of total RNA was used to synthesize cDNA by reverse transcription, and the primers used for RT-PCR is listed in Table 4.1. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. For miRNA
RT-qPCR, miScript PCR system (Qiagen) was performed following manufacturer’s protocol. miRNAs were amplified by miRNA-sepecific primers and poly-T primer. Comparisons between samples were made after normalization with U6 RNA level. RT-qPCR was carried out and the measurements were made by Roche Lightcycler 2.0.

**Statistical Analysis**

The results (mean values ± SD) of all the experiments were subjected to statistical analysis by t-test. The level of significance was set at p < 0.05.

**4.4 Results and Discussion**

**4.4.1 Expression of 3’UTR reduces cell proliferation and tumor growth**

An expression construct is required to study the function of 3’UTR. Thus, a conserved region of versican 3’UTR (2285-3000 bp, NM_001126336.1) was cloned and expressed under CMV promoter named VCAN-UTR (Fig. 3.10A). In order to monitor the effect of 3’UTR, VCAN-UTR was stably expressed in a mouse breast carcinoma cell line, 4T1, and its expression was confirmed by RT-PCR (Fig. 4.1). 4T1 cell is chosen because it is compatible with BALB/c mice without host rejection. Spreading of 4T1-induced tumor growth and metastatic in mice very closely mimics to human breast cancer, and is an established animal model for stage IV human breast cancer. In a tumor formation assay, transfected cells were subcutaneously injected into BALB/c mice. In two weeks, there was a noticeable difference in tumor sizes (Fig. 4.2). Tumors generated by cells transfected with the 3’UTR were consistently smaller compared with controls. Colony formation assay also revealed similar results (Fig. S4.1). Statistical analysis showed that the difference in tumor sizes was significant two weeks
**Fig. 4.1 Positive expression of VCAN 3’UTR in 4T1 cells**

VCAN-UTR, a construct containing a fragment of Versican 3’UTR (700bp), or control vector was transfected into mouse breast carcinoma, 4T1. RNA was isolated from pooled stably transfected cells and the expression of 3’UTR was confirmed by RT-PCR.
Fig. 4.2 Tumor growth curve of stably transfected 4T1 cells

Pooled cells were injected subcutaneously into BALB/c mice. Tumor size was recorded weekly and tumor growth curve was obtained for a period of four weeks. Asterisks indicate signficances. *, P<0.05. Error bars indicate SEM (n=3).
post-injection, and collagen was not the cause for the difference in tumor sizes (Fig. S4.2). Since VCAN 3'UTR antagonizes miRNAs, this result suggests that unknown miRNAs are important in supporting tumor growth. The expansion of tumor was limited due to the neutralized miRNA function by the 3'UTR.

A similar disparity in tumor growth was repeatedly observed in cell proliferation. In a cell proliferation assay, cells that were transfected with 3'UTR or control vector were cultured in low serum medium. Pooled cells that were transfected with the 3'UTR exhibited reduced proliferation compared with control cells (Fig. 4.3). In a further step, cell cycle was analyzed on these transfected cells by staining with Propidium Iodide. FACS analysis showed that there were approximately two-fold more control cells undergoing G2/Mitosis phase compared to the 3'UTR-transfected cells (Fig. 4.4). More 3'UTR-transfected cells were stalled in the G1 phase where cells were not committed into DNA synthesis. This data suggests that the presence of 3'UTR reduced cell proliferation by interfering with miRNA functioning. To take a further step in tracing the genes that are targets of these miRNAs, protein expression of proliferation-related candidate genes were inspected.

The tumors that were retrieved in the tumor formation assay were fixed and sectioned for immunohistochemistry. Staining with Ki67, a proliferation marker, showed strong staining in tumor formed by vector-transfected cells, especially at the peripheral edges of the tumors where cells have room to actively proliferate and expand (Fig. 4.5, lower panel). On the other hand, there were also some stainings of Ki67 in the tumors formed by the 3'UTR-transfected cells. The staining appeared to be in small patches throughout the tumor sections, possibly the cells expressing low level of 3'UTR in the pooled cells population. Ki67 staining suggests that the reason for difference in tumor growth is that control cells actively proliferate and thus excel in tumor growth. Therefore,
**Fig. 4.3 Cell proliferation of stably transfected 4T1 cells**

VCAN-UTR-transfected and vector-transfected cells were counted and plated in low serum culture condition for a period of four days. Cell proliferation assay differentiate proliferation rate between cells transfected with control vector or 3’UTR. The number of cells were counted with tryphan blue staining. *, P<0.05. Error bars indicate SD (n=5).
Fig. 4.4 Cell cycle analysis of stably transfected cells by FACS

(A) Cell cycle analysis by FACS confirmed that there is more G2/M population within the pooled control cells. Cells transfected with control vector are dividing faster than 3’UTR-transfected cells. Representative data is shown. (n=3).

(B) Cell populations were fitted into different stages of cell cycle by computer software, FlowJo. Percentage of cells in different cell cycle phases were approximated in percentages.
Fig. 4.5 Examination of PTEN and KI67 expression in tumor sections
Retrieved mice tumors were fixed and sectioned. Staining with Ki67, a proliferation marker, and anti-PTEN antibody showed tumor originated from control cells expanded at a faster rate than tumor derived from 3’UTR-transfected cells. Increased expression of PTEN was found in tumor generated from 3’UTR-transfected cells, resulting reduced tumor growth.
tumor samples were examined with two negative cell cycle regulators, PTEN and Rb1, which could affect the proliferation of the 3’UTR-transfected cells. Staining with anti-PTEN antibody showed a strong elevation of PTEN expression ubiquitously throughout the tumors formed by the 3’UTR-transfected cells (Fig. 4.5, upper panel). Tumors formed by the control cells displayed very little expression of PTEN in different regions of the tumors. Interestingly, there were still tumor cells expressing PTEN in the core of the 3’UTR tumors where is usually room to pack with cell debris and patches of surviving cells. It is possible that impaired miRNA function reduced cell proliferation but enhanced cell survival. However, the differences in tumor sizes cannot be ruled out as the cause of this observation. Smaller 3’UTR tumors may be supplied with comparatively more adequate and sufficient small blood vessels. Since miR-199a-3p had previously been shown to target versican (VCAN) and fibronectin (FN), tumor sections were also stained with anti-VCAN and FN antibodies. Results of the staining showed that there is indeed an elevation of VCAN and FN expression in the tumors formed by the 3’UTR-transfected cells, strongly suggesting that miR-199a’s function was likely impaired (Fig. 4.3). Transfection with the 3’UTR has enabled smooth translation of VCAN and FN. Tumors were grinded and lysed, and the expression of PTEN and Rb1 were further examined by western blot analysis (Fig. 4.6). Compared to tumors formed by control cells, tumors formed by the 3’UTR-transfected cells exhibited folds of increased expression of PTEN and Rb1. The elevated expression of these two negative cell cycle regulators may have synergistically reduced tumor cell proliferation and thus tumor growth.

4.4.2 Rb1 is targeted by miR199a-3p and miR-144

Currently, miR-199a-3p is the only miRNA that has been proven to regulate VCAN
Fig. 4.6 PTEN and Rb1 expression in tumor sections by western blot

Mice tumors were also lysed and subject to western blot analysis. Tumor lysates were probed with anti-Rb1, anti-PTEN, and anti-actin antibodies for sample loading control. Results showed a significant elevation of Rb1 and PTEN protein expression in the 3’UTR-generated tumor compared to tumor comprised of control cells.
by binding to its 3'UTR. However, VCAN 3'UTR has also been shown to interact with a number of other miRNAs [181]. By computational algorithms, a list of genes that are both potential targets of these miRNAs and are negative regulators of cell cycle were predicted. A few of these genes are of particular interest because of their potential targeting by multiple VCAN 3'UTR-bound miRNAs, and Retinoblastoma1 (Rb1). Rb1 is one of the earliest reported tumor suppressors studied and causes primary cancer when Rb1 protein is lost or dysfunctional [182]. It plays the role of a negative cell cycle regulator by postponing cells from passing G1 interphase [183]. Expression of Rb1 will result in reduced cell proliferation and tumor growth.

Cell lysates of pool cells that were transfected with VCAN-UTR or control vector were analyzed for their protein expression by western blot. Probing with anti-Rb1 antibody showed that there was an elevated expression of Rb1 (Fig. 4.7A). Primary lung and kidney tissues were collected from 3'UTR transgenic and wildtype mice, and similar results were observed (Fig. 4.7B). It appeared that presence of 3'UTR induced the expression of Rb1. To confirm this result, a rescue experiment by siRNA against 3'UTR was performed. In this experiment, 4T1 cells were transfected with 3'UTR together with a control sequence or siRNA molecules against VCAN 3'UTR. As a negative control, another set of 4T1 cells were transiently transfected with vector and control sequence. Transfected cells were then analyzed for Rb1 protein expression by western blotting. Results from this experiment showed that there was an increased Rb1 expression in the 3'UTR-transfected cells, reinforcing earlier findings (Fig. 4.8). Transfection with siRNA instead of scramble control knocked down the cytoplasmic level of 3'UTR, and thus successfully lowered the expression of Rb1 to the level that is comparable to the negative control. Apparently, removal of 3'UTR could readily reduce the expression of Rb1, indicating that change of Rb1 expression was not a secondary effect. RNA of a
Fig. 4.7 Expression of Rb1 in stably transfected cells and transgenic organs

(A) Cell lysate from pooled 3’UTR- or vector-transfected cell lines was analyzed by western blot for Rb1. Rb1 expression was elevated in the abundant transcription of Versican 3’UTR and the staining for actin confirmed equal loading.

(B) Lysates prepared from lung and kidney of Versican 3’UTR transgenic and wildtype mice were probed with anti-Rb1 antibodies during western blot analysis.
Fig. 4.8 Knock-down of VCAN 3’UTR by siRNA directly affects Rb1 expression level

4T1 cells were transiently transfected with VCAN 3’UTR and control sequence or siRNAs against 3’UTR. For comparison, cells were also transfected with empty vector and control sequence, followed by culturing in low serum condition. Protein lysates were prepared from transfected cells and subject to western blot analysis by probing with anti-Rb1 and anti-actin antibody.
duplicated set of transfected cells was isolated, and RT-PCR amplified with Rb1 specific primers showed that there was no significant difference in Rb1 mRNA levels. Results of this series of experiments strongly relate the translational efficiency of Rb1 mRNA with the presence of 3'UTR.

Two versican 3'UTR-bound miRNAs were hypothesized to inhibit Rb1 translation. These miRNAs included miR-199a-3p and miR-144. To confirm that these miRNAs target Rb1, luciferase experiments were performed. The potential binding sites of miR-199a-3p and miR-144 on Rb1 3'UTR (nucleotide 654-675; GeneBank Accession No. NM_000321.2) was found. Target site of miR-199a-3p was strongly conserved in human and mice genome, suggesting its importance in regulating Rb1 translation (Fig. S4.4A, upper panel). Additional target site was found in the 3'UTR of mouse Rb1 gene (Fig. S4.4B, upper panel). miR-199a-5p, the other arm of precursor miR-199a, also targets the 3'UTR of human Rb1 gene (Fig. S4.4B, lower panel). Therefore, a region of Rb1 3'UTR containing this binding site was cloned into a luciferase vector. Another luciferase construct with mutated nucleotide in the miRNA-binding site was also generated (Fig. 4.9A). Luciferase activity assays were performed by co-transfecting miR-199a-3p with luciferase constructs containing original or mutated target sites. The experiments showed that mutated binding site alleviated luciferase from translation inhibition by miR-199a-3p and restored luciferase activity by 20% (Fig. 4.9B).

Rb1 is also hypothesized to be targeted by miR-144, another miRNA that binds to versican 3'UTR. The potential binding site of miR-144 on Rb1 3'UTR (nucleotide 674-695) was found to be very to close to the miR-199a target site. Moreover, miR-144 site is also highly conserved in human and mice genome (Fig. S4.4A, lower panel). Strong conservation of this region of Rb1 3'UTR implied its important role in miRNA interaction. This region of Rb1 3'UTR including the potential miR-144 binding site or its
**Fig. 4.9 Rb1 is target by miR-199a-3p in luciferase assay**

(A) A hypothetical region of Rb1 that is a potential target of miR-199a was cloned into a luciferase vector. The same binding site was mutated (in red) and cloned into another luciferase reporting vector, generating the constructs Luc-Rb1-199a and Luc-Rb1-199a-mut.

(B) U343 cells were co-transfected with miR-199a-3p and luciferase constructs with or without mutation. Luciferase activity assays showed that miR-199a repressed luciferase activities when they harbored Rb1 3'UTR. Repression was abolished when mi-199a binding sites were mutated. **, p<0.0001. Error bars, SD (n=3)
mutated version was cloned into a luciferase vector (Fig. 4.10A). Luciferase activity assays were performed by co-transfecting miR-144 with luciferase construct with or without mutated binding site. Results show that luciferase activity was significantly repressed in the construct harboring the miR-144 target sequence, compared with the control vector harboring a non-related fragment (Luc-Ctrl). When the target site was mutated, luciferase activity was restored nearly as control (Fig. 4.10B). This result pointed out the direct targeting of miR-144 on 3'UTR of Rb1.

In a luciferase activity competition assay, VCAN-UTR was co-transfected with luciferase construct inserted with Rb1 3'UTR (Fig. S4.5). In this assay, we monitor the effect of increasing antagonized miRNA functioning by 3'UTR. As increased quantities of 3'UTR was co-transfected with luciferase construct, more and more miRNAs bound to 3'UTR and thus luciferase activities kept rising. However, luciferase activity nearly plateaus when 50-folds of 3'UTR compared to luciferase construct were co-transfected. This result suggests that translation of endogenous Rb1 can potentially be up-regulated by 2.5-fold without changing the transcription of the gene. Moreover, presence of VCAN 3'UTR enhances Rb1 translation.

4.4.3 miR-144 and miR-136 repress PTEN translation

Another gene of interest was Phosphotase and Tensin Homolog, or PTEN. It has been shown to function as a tumor suppressor, where loss or mutation of this gene leads to cancer predisposition [184]. Similar to Rb1, PTEN has also been known for its role as a negative cell cycle regulator by arresting cells in G1 phase [185]. Increased expression of PTEN can further slow down cell proliferation. To confirm earlier results from tumor western blot, pooled cells transfected with VCAN-UTR or control vector were lysed and probed with anti-PTEN antibody. Western blot results showed an elevation of
Fig. 4.10 Rb1 is targeted by miR-144 by luciferase assay

(A) A region of Rb1 containing binding site for miR-144 was cloned and mutated (in red), generating Luc-Rb1-144 and Luc-Rb1-144-mut. U343 cells were co-transfected with miR-144 and luciferase constructs with or without mutation.

(B) Luciferase activity assays showed that mutation of miR-144 binding site removed the translational inhibition exerted by miR-144. **, p<0.0001. Error bars, SD (n=3).
PTEN expression in 3'UTR-transfected cells (Fig. 4.11A). When primary tissues were examined for their PTEN expression, similar results were obtained but less significantly in kidney tissues (Fig. 4.11B). In order to show that 3'UTR antagonize PTEN-targeting miRNAs, a knockdown experiments using siRNA against VCAN 3'UTR was carried out. Western blot results again exhibited an elevation of PTEN expression in cells transfected with VCAN-UTR and scramble control sequence (Fig. 4.12). Knockdown of the 3'UTR by siRNA abolished the increased PTEN expression to the same level of cells transfected with vector control. Since removal of 3'UTR quickly improved the translation of PTEN mRNA, the effect of 3'UTR was not a secondary effect. RNA of cells that were transfected in a similar method was isolated, and was subjected to RT-PCR to inspect the levels of PTEN mRNA. There was no significant difference in PTEN mRNA levels (Fig. 4.12). Therefore, VCAN 3'UTR affects the translational efficiency of PTEN mRNA.

Luciferase experiments were performed to prove the targeting of PTEN by two proposed miRNAs. miR-144, which was proved above to regulate Rb1, is also predicted to regulate PTEN. The hypothetical binding site of miR-144 on the 3'UTR of PTEN (nucleotide 2906-2925 bp, GeneBank Accession No. NM_000314.4) was highly conserved between human and mice genome (Fig. S4.6A), and was cloned into a luciferase vector (Fig. 4.13A). Seed sequence matching site on the PTEN 3'UTR was mutated and placed into a luciferase vector as well. Luciferase assay showed that luciferase activity restored significantly when target sequence was mutated (Fig. 4.13B). By computational predictions, another versican-bound miRNA, miR-136, can potentially target PTEN through three binding sites on the PTEN 3'UTR (nucleotides 389-411; 490-511; 2759-2781). Two of these three sites are strongly conserved in human and mouse genome except one (Fig. S4.6B). An additional target site in mouse PTEN gene was also predicted (Fig. 4.6C). Apparently, miR-136 is likely to play a key role in
Fig. 4.11 Expression of PTEN in stably transfected cells and transgenic organs

(A) Cell lysates prepared from pooled cell lines were analyzed by western blot. Staining with anti-PTEN antibody showed an increase in PTEN expression, while protein loading is equal as exhibited by actin staining.

(B) Lysates prepared from lung and kidney of Versican 3’UTR transgenic and wildtype mice were probed with anti-PTEN antibodies during western blot analysis.
Fig. 4.12 Knock-down of VCAN 3’UTR by siRNA directly affects PTEN expression level

4T1 cells were transiently transfected with Versican 3’UTR and siRNAs against 3’UTR or control sequence. As a negative control, 4T1 cells were also transfected with control vector with control sequence, followed by low serum culture. Lysates were prepared from transfected cells and analyzed by western blot by probing with anti-PTEN and anti-actin antibody.
**Fig. 4.13 PTEN is target by miR-144 in luciferase assay**

(A) Hypothetical targeting site by miR-144 in the 3'UTR of PTEN was cloned and mutated, generating two constructs Luc-PTEN-144 and Luc-PTEN-144-mut.

(B) In a luciferase activity assay, U343 cells were co-transfected with Luc-PTEN-144 or Luc-PTEN-144-mut with miR-144. Luciferase activity of mutated construct was higher than that of the construct expressing original 3'UTR.
targeting PTEN. A 3'UTR harbouring multiple target sites by the same miRNA is possible, and this was proven in another study where CD44 3'UTR was regulated by miR-328 through multiple target sites [172]. A segment of PTEN 3'UTR harboring one of the binding sites (nucleotide 2759-2781) and its mutated version were cloned into a luciferase vector (Fig. 4.14A). Luciferase activity showed that miR-136 reduced luciferase activity significantly by more than 60%, but had almost no effect when the target sequence was mutated (Fig. 4.14B).

In a luciferase competition assay, increased amount of VCAN-UTR was co-transfected with a luciferase construct containing PTEN 3'UTR (Fig. S4.7). As increased amount of 3'UTR was transfected to antagonize endogenous miRNAs, luciferase activity elevated along. Elevation of luciferase activity was due to increased translation of luciferase. Although 200-folds of 3'UTR copies was present in the cell compared to luciferase mRNA tailing a PTEN 3'UTR, luciferase activity was 2.3-fold higher than original activity. Maximum level of translation was not reached in this experiment. It is possible that many miRNAs regulate PTEN or that the expression of miRNAs that target PTEN is very abundant in the cell.

4.4.4 miRNA level is affected in the presence of 3'UTR

In this study, we expected to interfere miRNA targeting by expressing the 3'UTR as a decoy. Since there are no studies using exogenous 3'UTR as a decoy [186], it is of further interest to examine how 3'UTR affects miRNA expression in vitro and in vivo. We have chosen to inspect the expression of miR-199a-3p and miR-136 because miR-144 is an erythroid lineage-specific miRNA [187]. By RT-qPCR, expression of miR-199a-3p and miR-136 were found and affirmed in both cells and several primary tissues but not miR-144.
Fig. 4.14 PTEN is target by miR-136 in luciferase assay

(A) miR-136’s potential targeting sequence in the 3’UTR of PTEN was cloned and mutated, generating two constructs Luc-PTEN-136 and Luc-PTEN-136-mut.

(B) In a luciferase activity assay, U343 cells were co-transfected with luc-PTEN-136 or luc-PTEN-136-mut with miR-136. Repression on luciferase activity was removed when miR-136 targeting site was mutated.
In Chapter 2, miR-199a-3p was found to regulate two extracellular matrix proteins, VCAN and FN [181], and it has also been found to be important during development [188]. Primary lung and kidney from wildtype and 3'UTR transgenic mice were compared on their level of miR-199a-3p. Different tissues may exhibit different levels of miR-199a-3p expression, but more interestingly, different tissues also respond differently to the presence of 3'UTR in this experiment. Reduction in miRNA expression was observed in these primary tissues. There was a 39% reduction of miR-199a-3p expression in lung tissues but no significant changes were found between the transgenic and wildtype kidney tissues (Fig. 4.15). The same samples were compared by their miR-17-5p expression, and there were no significant differences observed because miR-17-5p does not bind to versican 3’UTR (Fig. 4.16). The specificity and accuracy of miR-17-5p quantification by RT-qPCR was confirmed by northern blot in another study related to miR-17 and growth [189]. miR-199a-3p has been shown to contributes oppositely in different types of cancer [190-193] but there are currently no reports specifically related to breast cancer. Using primary liver tissues as another example, we found a reduced expression of miR-199a-3p by 29% in presence of 3’UTR (Fig. 4.17). In addition, cells transfected with 3’UTR lost 79% of their pool of miR-199a-3p compared to that of control cells. Level of miR-17-5p was not influenced as well.

Expression of miR-136 was also inspected in cells and primary tissues. In primary lung tissues, there was a significant reduction of miR-136 expression by 40%, while there was no significant difference between breast cancer cells transfected with 3'UTR and the control vector (Fig. 4.18). Expression levels of miR-328 in these samples were similar and nearly unchanged (Fig. 4.19). Kidney tissues were also examined by their miR-136 levels and there was still no significant changes observed as well (Fig. 4.20).
Fig. 4.15 VCAN 3’UTR enhances miR-199a-3p degradation

In the lung tissues, the level of miR-199a was about 39% lower in the 3’UTR transgenic mice compared to wildtype. In contrast, there was no significant differences between transgenic and wildtype kidney tissues. At least three pairs of mice were compared and representative data was shown. **, p<0.01. Error bars, SD (n=3)
Fig. 4.16 Level of miR-17-5p was not affected in the presence of VCAN 3’UTR

Tissues from Lung and kidney were inspected for their level of miR-17-5p, which does not bind to VCAN 3’UTR. No significant changes were observed between different organ samples. Expression level of miR-17-5p was compared to ensure subjects are comparable and the levels of other miRNAs were not affected.
**Fig. 4.17 VCAN 3’UTR enhances miR-199a-3p degradation in cells and transgenic organ**

The level of miR-199a was compared between primary tissues and cancer cell lines. There is a 29% reduction in miR-199a in the liver tissues of transgenic mice compared to that of wildtype. In the 4T1 cell line, cells transfected with Versican 3’UTR had 73% less miR-199a compared to cells transfected with control vector. **, p<0.01. Error bars, SD (n=4)
Fig. 4.18 VCAN 3’UTR enhances miR-136 degradation in cells and transgenic organ

The level of miR-136 was significantly lowered in the lung tissues of transgenic mice compared to that of wildtype by 40%. There was no significant difference in miR-136 expression level between the cells transfected with either Versican 3’UTR or control vector. *, p<0.05. Error bars, SD (n=3).
There is likely a tissue-specific degradation of miRNA regardless of miRNA abundance, since the levels of miR-136 were high in both lung and kidney.

4.5 Conclusion

In this study, a more thorough examination of VCAN 3’UTR was proceeded. Besides miR-199a-3p, two other miRNAs- miR-144 and miR-136 were both found to bind to VCAN 3’UTR. These miRNAs were shown to target two negative cell cycle regulators, Rb1 and PTEN. Expression of non-coding 3'UTR resulted in up-regulation of Rb1 and PTEN, which in turn reduced cell proliferation and also tumor growth. We anticipate the study of 3’UTR will aid in identifying true targets of miRNA and its application in amplifying the effect of coding transcript by regulating miRNA activities.
Fig. 4.19 Level of miR-328 was not affected by the presence of VCAN 3′UTR

The same samples were quantified for level of miR-328, which does not bind to VCAN 3′UTR. Results showed that the level of miR-328 was not affected. Degradation rate of other miRNA levels were not affected.
Fig. 4.20 VCAN 3’UTR enhances degradation of miR-136
Tissues from lung and kidney of wildtype and transgenic mice were analyzed for the level of miR-136 by RT-qPCR. Expression of miR-136 in lung and kidney tissues of the same mice was inspected. Representative data is shown. *, p<0.05. Error bars, SD (n=3)
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<th>Sequence</th>
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<tr>
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<tr>
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<td>mo-Gapdh250R</td>
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**Table 4.1 List of primers used**
In colony formation assays, pooled cell lines were mixed in soft agarose gel and cultured in 2% FBS-containing medium for a prolonged period of time. Cells were monitored and photographed periodically. Results showed that cells transfected with control vector formed larger but less colonies than cells transfected with Versican 3’UTR.

**Fig. S4.1 Colonies formed by stably transfected cells in colony formation assay**

In colony formation assays, pooled cell lines were mixed in soft agarose gel and cultured in 2% FBS-containing medium for a prolonged period of time. Cells were monitored and photographed periodically. Results showed that cells transfected with control vector formed larger but less colonies than cells transfected with Versican 3’UTR.
Fig. S4.2 Collagen staining in tumor sections

Trichrome staining was applied to tumor paraffin sections. Keratin and muscle fibers are stained red, collagen and bone are stained with blue or green, cytoplasm are light red or pink, and cell nuclei are dark brown to black. Within the tumor peripheral area, there are less collagen staining in the 3’UTR tumor because of slower cell proliferation and stronger cell-cell adhesion. In contrast, the area occupied by connective tissues was crowded with cancer cells in the control tumor.
Fig. S4.3 Inspecting VCAN and FN expression in tumor sections by immunohistochemistry

Paraffin tumor sections were stained with anti-Versican, Fibronectin, and CD34 antibodies. Conformed to previous studies, there was an increased staining of Versican and Fibronectin in the tumor comprised of cells transfected with 3'UTR and lots of small blood vessels were also identified. Control tumor has few but larger blood vessels spanning at the peripheral edge.
A

3' auugGUUACACGUCUGAGACa 5' hsa-miR-199a-3p
::| | | || | ||||| |
654:5' augaUAcUAU-CAUACUACUGa 3' Human
614:5' augaUAcUG-CuUACUACUGa 3' Mouse

3' ucaugUAGUGAUGACAu 5' hsa-miR-144
||| ||||| |
574:5' uuggaAUCgAUUAACUGUg 3' Human
603:5' uauaAUCauaUGUAACUGuc 3' Mouse

B

3' auuGGUUACACGUCUGAGACa 5' mmu-miR-199a-3p
:::| | | || | ||||| |
692:5' gcuUUGAACUGAAGACUAUUGa 3' Mouse Rb1

3' cuuGUC-CAUCAGACUUGUGACCc 5' hsa-miR-199a-5p
||| |:||:| | | | | | |
1117:5' aguCAGUAUGGUCU-AACACUGGc 3' Human Rb1

**Fig. S4.4 Conserved target sites in Rb1 by miR-199a-3p and miR-144**

(A) Target sequences in the Rb1 3’UTR by miR-199a and miR-144 are conserved in human and mice. Conserved nucleotides are in red and nucleotides that are complementary to miRNA are capitalized.

(B) Mouse miR-199a-3p has an additional target site in mouse Rb1 3’UTR from nucleotide 695-713. Human miR-199a-5p, the other arm of miR-199a, also has an additional site in human Rb1 from nucleotide 1120-1139.
Fig. S4.5 VCAN 3’UTR antagonizes miRNAs that regulate Rb1
Luciferase reporter vector harboring the Rb1 3’UTR was co-transfected with Versican 3’UTR construct in increasing amount complemented with a control vector in U343 cells. Increased abundance of Versican 3’UTR promotes the translation of luciferase construct, resulting in elevation of luciferase activity.
Fig. S4.6 Conserved target sites in PTEN by miR-144 and miR-136

(A) Binding site of miR-144 located within PTEN 3'UTR are conserved between human and mice. Conserved nucleotides are in red.

(B) miR-136 has more than one binding sites located within the 3'UTR of PTEN and the sequences of these target sites are very conserved.

(C) Mouse miR-136 has an additional binding target in mouse PTEN from nucleotide 5530-5546, which is not present in human PTEN 3'UTR.
**Fig. S4.7 VCAN 3’UTR antagonizes miRNAs that regulate PTEN**

Luciferase reporter vector harboring the PTEN 3’UTR was co-transfected with Versican 3’UTR construct in increasing amount complemented with a control vector in U343 cells. Increased rations of Versican 3’UTR bound more endogenous miRNAs and thus freeing the translation of luciferase protein, resulting in higher levels of luciferase activities.
Fig. S4.8 miR-16 potentially targets negative cell cycle regulators

(A) Computer algorithms predicted a target site for miR-16 in 3'UTR of mouse Rb1. Target site is located form nucleotide 246-265.

(B) miR-16 has two target sites in human PTEN which were not found in mouse PTEN. The targets sites are located in nucleotide 940-959 and 1023-1039.
Chapter 5

General Discussions and Future Directions
5.1 Role of microRNAs in tumorigenesis

The genomic location of microRNA genes often situate in the fragile regions and cancer susceptible loci, and the dysregulated miRNA expression in tumors becomes a marker for cancer diagnosis and prognosis. Since miRNA is a regulator of gene expression, over or under expression of this regulator affects many other genes. It is generally believed dysregulated miRNA activity can contribute to transformation of a normal cell. However, not all miRNAs are capable of inducing transformation. The studies reported in Chapter one are valuable not only because an oncogenic miRNA has been found, but also is one of the first few miRNAs that have been shown to target multiple genes at the same time, leading to tumorigenesis (Figure 5.1).

This work began by investigating the cellular changes exerted by miR-378, a miRNA that is over-expressed in primary tumors. In our in vitro cell model, presence of miR-378 promotes cell survival by reducing caspase 3 activities. The same phenomenon was observed in different cell types, suggesting an universal effect exerted by miR-378. Results from tumor formation assays also corroborate with the finding in cell survival assays, demonstrating an oncogenic role of miR-378 during tumorigenesis. Further investigations then revealed expression of two tumor suppressor genes were regulated by miR-378. Abundant presence of miR-378 led to the reduced translation of SuFu and Fus-1 without affecting their transcription. Expression of either
**Fig. 5.1 Targeting of genes by miRNAs and its consequences**

Targeting of both tumor suppressors- SuFu and Fus-1 by miR-378 promotes cell survival. miR-199a-3p regulates Versican and Fibronectin expression. Antagonizing by expressing Versican results in elevated translation of Versican and Fibronectin. In addition, Rb1 and PTEN are also translated more efficient without miR-199a-3p and miR-136 regulation.
tumor suppressors was shown to lower cell survival in our study. In a further step, targeting of SuFu and Fus-1 were confirmed by co-transfection with miR-378 and luciferase reporter assay. Results from both experiments showed that miR-378 significantly reduce translation of SuFu and Fus-1. SuFu was later suggested to act as a co-repressor in the nucleus and an inhibitor to the hedgehog pathway. Fus-1 was found to promote apoptosome assembly and to induce P53 degradation. Therefore, down-regulation of SuFu and Fus-1 by miR-378 promotes tumorigenesis. This finding may lead to the targeting of miR-378 in future cancer therapy rather than the targeting of genes regulated by miR-378.

Oncogenic miRNAs, such as miR-378, promote tumorigenesis by inhibiting expression of tumor suppressors. On the other hand, miRNAs, which function as tumor suppressors, reduce oncogene expression. Versican is an extracellular matrix protein that is known to promote tumorigenesis and angiogenesis through structural support and external signaling. The work described in Chapter two began by investigating the miRNA or miRNAs that regulate Versican expression. Through the miRNA:UTR PCR, more than one miRNA was found to bind to Versican 3'UTR and potentially regulate its expression. Additionally, another extracellular matrix protein, Fibronectin, was also found to be regulated by miR-199a-3p, which binds to Versican 3'UTR. By luciferase reporter assay, targeting of both matrix proteins by miR-199a-3p was confirmed. These
results reinforced our hypothesis that miRNA often operates by targeting multiple genes of similar function. In this study, both Versican and Fibronectin expression were regulated by miR-199a-3p.

In order to modulate miR-199a-3p activity, the 3'UTR of Versican was expressed to antagonize this miRNA. Loss of miR-199a-3p targeting was expected to relieve translational pressure of its target genes. In a cell adhesion assay, expression of Versican 3'UTR in cells induce cell-cell adhesion due to the increase translation of Versican and Fibronectin. Elevated expression of Versican and Fibronectin was confirmed in vivo, by analyzing transgenic mice expressing Versican 3'UTR. In addition, organ adhesion was observed in some of the transgenic mice. In these mice, liver was adhered to nearby organs and internal body wall. Adhesion junction displayed strong staining of both Versican and Fibronectin.

The effect of Versican 3'UTR in tumorigenesis was also investigated and described in Chapter four. Expressing Versican 3'UTR inhibited activities of several miRNAs in a late stage breast cancer cell line. As a result of Versican 3'UTR expression, cells formed smaller tumors compared with the tumors formed by the control cells. Induction of Versican expression was observed but expression of Retinoblastoma 1 (Rb1) was also induced due to the diminished miR-199a-3p activity. Another negative cell cycle regulator, Phosphatase and Tensin Homolog (PTEN), was also promptly translated. The
targeting of Rb1 by miR-199a-3p and PTEN by miR-136 was confirmed by luciferase reporter assays. Expression of Rb1 and PTEN in the stable cell lines also reinforced the finding in tumor experiments and has negatively regulated cell cycle progression.

Initially, this observation surprised us since elevated expression of Versican is expected to promote tumor formation. There are several possibilities have led to the staggered tumor growth. First, Versican can be expressed in four isoforms. Versican isoform V1 has been shown to promote tumorigenesis but isoform V2 has been shown to exert an opposite effect on tumor formation [169]. The cell line used in this study is likely to expressed Versican V2 isoform, which leads to the reduced tumor growth. Another reason is that abundant expression of PTEN and Rb1 interrupts the stimulatory signal from Versican. Versican has been known to exert proliferative signal through Receptor Tyrosine Kinase (RTK) pathway through its binding with Epidermal Growth Factor Receptor (EGFR). Cytoplasmic PTEN turns off Phosphatidylinositol-3 Kinase (PI3K) pathway and nuclear PTEN down-regulate MAPK/ERK leading to cell cycle arrest [194]. Consequently, internal signals exerted by cytoplasmic PTEN and Rb1 may offset the stimulatory signal released by Versican externally.

The studies reported in this thesis have provided new insights on the role of microRNA in tumorigenesis, and have also made an initial step in developing a method in gene therapy which modulates miRNA activities. At the time when this project was
initiated, there are very few resources for miRNA analysis due to its instability and sizes. Various tools are made and developed in order to study miRNA functions. These tools will also be useful not just in cancer research but will also benefit other field of studies involving miRNA regulation.

5.2 miR-378 contributes to tumor formation

Elaborated methods were used to uncover the oncogenic properties of miR-378. It has been found to enhance tumor cell survival, tumor growth, and tumor angiogenesis, by targeting the tumor suppressors, SuFu (Suppressor of Fused) and Fus-1 (Tumour Suppressor Candidate 2, TUSC2). The role of miR-378 in promoting tumorigenesis and angiogenesis makes it an attractive target in reducing tumor size. However, a more systematic approach will be required to assess the potential therapeutic benefits of anti-miR-378 treatment. This goal can be reached by generating transgenic mice expressing miR-378. I suspect that these mice will develop more cases of tumor-spawning organs compared with wildtype mice, suggesting greater susceptibility of these tissues to the oncogenic effects induced by miR-378. In another project of mine, miR-378 is found to affect osteoblast differentiation by regulating Nephronectin expression (Appendix B) [195]. It is likely that miR-378 play various roles during development, which can be investigated further with the transgenic mice. Since miR-378 can potentially affect development, tissue-specific or chemical-induced promoters can be engineered to drive miR-378 expression. Another possibility is to study existing animal models that spontaneously generate tumors, possibly expressing high levels of miR-378. It would be interesting to deliver antisense miR-378 to tumors in these animals. Single stranded RNA molecules such as this antisense, which is complementary to
miR-378, can be chemically modified for stability and cholesterol conjugated for better delivery. The purpose of this experiment is to determine whether or not antisense miR-378 could reduce tumor growth in these animals. More clinical samples should also be examined to establish the value of anti-miR-378 therapy. RT-qPCR would help to determine the level of miR-378 in tumors and nearby healthy tissues. Tumors with varying sizes or tumors in their primary or metastatic stages could also be assessed in order to complement aspects that are not covered in this initial study.

Furthermore, other functions of miR-378 that are yet to be explored include their role in tumor angiogenesis and drug resistance. Recently, miR-378 has been found to target Cytochrome P450 2E1 (CYP2E1). CYP2E1 metabolizes and metabolically activates numerous small molecular weight compounds (molecular weight < 100), such as aliphatic, aromatic and halogenated hydrocarbons [196]. Unlike other hepatic P450s, CYP2E1 metabolizes a few pharmaceutical agents, solvents and industrial monomers, and some of which are cancer suspect agents. Normally, CYP2E1 mRNAs are stable but CYP2E1 proteins are usually degraded by ubiquitination in liver tissues [197]. The presence of substrates contributes to CYP2E1 protein stability. Depending on the pharmacokinetic nature of the cancer drug used, down-regulation of CYP2E1 by miR-378 can affect its efficacy. Administration of miR-378 with cancer drug can potentially affect its bioactivity and half-life in the human body.

5.3 Use of 3’UTR to screen regulating miRNAs

Microarrays are extensively used as a powerful tool in miRNA profiling. Consequently, most miRNA studies proceed by searching for the targets of differentially expressed miRNAs. However, results from above experiments point out that multiple
miRNAs target a single mRNA. Another approach to search for potential mRNA targets of specific miRNAs is to study the 3’UTR of relevant mRNAs. Single Nucleotide Polymorphisms (SNPs) located in miRNA-binding sites has been suggested to affect miRNA target expression and function [198]. In this study, twelve SNPs in miRNA-binding sites of target genes exhibit cancer-associated aberrant allele frequencies, and are confirmed by genotyping these SNPs in tumors. For example, it was found that a SNP found in the 3’UTR of KRAS [199] prevented let-7 from binding to KRAS mRNA, resulting in KRAS over-expression in lung cancer. This SNP is also associated with an increase risk in developing non-small cell lung cancer (NSCLC).

Focusing on the 3’UTRs of interesting genes allow us to experimentally validate their potential interaction with hypothetical miRNA predicted based upon miRNA seed region match with conserved mRNA target sites. Initial screening can be readily performed by PCR, which is commonly used and can be automated. We developed a specialized PCR system that allows vast detection of miRNAs binding to the 3’UTR of a specific mRNA. Since certain miRNAs are degraded through binding to 3’UTR, RT-qPCR can be carried out to determine miRNA level. Measuring the level of miRNA not only determines its significance in vivo, but can also provide clues on their functionality in specific cell types. Lastly, confirming that the gene of interest is indeed a target of a specific miRNA can be confirmed by luciferase reporter assays. Above methods will aid in identifying gene-targeting miRNAs and can also help to significantly limit the number of potential miRNA candidates.

5.4 3’UTR as one of the methods to modulate miRNAs

In the past few years, different tools to regulate miRNA activity were trialed,
including decoys [200], sponges [201], locked nucleic acids (LNA) [202], and antagonirs [203]. Most of them are chemically modified antisense oligonucleotides and hence usually led to miRNA antagonism but not miRNA degradation. They are meant to antagonize specific miRNAs, so they are often designed with perfect seed region match and in multiple repeats. Therefore they are very effective against specific miRNA or members from a miRNA family such as let-7 or miR-17-52 cluster. However, many evidences have shown that multiple miRNAs can target a gene, and antagonizing one miRNA may not relieve enough translational repression exerted by other miRNAs targeting the same gene. miRNA activity relies on a threshold level of expression [204], and certain miRNAs may not be expressed abundantly in certain cell types. It is also possible that a combination of miRNAs is targeting a single gene in the cell, and thus strategy that aims at antagonizing specific miRNA may not be effective.

The current theory is that over-expression of oncogenic miRNAs hinders the translation of tumor suppressors. Hence, different approaches were developed to limit the specific oncogenic miRNAs. Usage of tumor suppressor 3'UTR may achieve similar effect as antagonizing the oncogenic miRNAs. In a further step, oncogenic miRNAs may be degraded through binding with 3'UTR, which will protect target mRNAs from translational inhibition. Furthermore, antagonizing the miRNAs that hinders translation of the tumor suppressor mRNA will greatly improve tumor suppressor translation. Expression of tumor suppressor will thus promote cell cycle arrest. For now, it is uncertain as to how 3'UTR will affect the functionality of other miRNAs. The main goal is to restore proper cell cycle rest in the cancer cells. Thus, 3'UTR expression may be an approach that could be applied to gene therapy.
5.5 3’UTR’s role in addition to translational regulation

When mRNA is translated, protein is the main executor of biological functions. With the discovery of miRNAs, various cis-elements that enable miRNA-mediated translational control were discovered within mRNA 3’UTR. Its secondary structure that consists of regions flanking target sites determine miRNA accessibility [205]. Rich uridine regions in the 3’UTR can be bound by Dead End 1 (Dnd1), which prohibits miRNA from associating with their target site [206]. The AU-rich element in the 3’UTR can even lead to translational activation by miRNAs [103]. These cis-elements are evolutionarily conserved to serve the seemingly complex regulation of miRNA. Through computer analysis, 3% of genes are alternatively spliced within their 3’UTR, potentially escaping miRNA regulation in various biological activities [207]. It has also been reported that a 3’UTR mutation located within the binding site of miR-189 resulted in Tourette’s syndrome [208]. Accompanied by the discovery of miRNA, the interplay between 3’UTR and miRNA during translational regulation is gradually being revealed.

Our study suggests that while 3’UTR enables translational regulation by miRNA, it also regulates miRNA activity by binding to miRNAs. This in turn means that transcription of a gene can relieve translational suppression on its own mRNA and others that are regulated by the same miRNA. For example, since miR-199a-3p regulates translation of both VCAN and FN, elevated transcription of VCAN will contribute to miR-199a-3p-mediated suppression by allowing increased translation of both VCAN and FN mRNAs. In another project that is carried out during my studies, FN is found to be regulated by another miRNA- miR-17 (Appendix C) [189]. This provides additional evidence that multiple miRNAs target on the same gene. Therefore, the translational pressure on FN mRNA is not relieved completely. Both miR-17 and
miR-199a-3p may play important role in laying down FN during development.

In terms of biological function, this study suggests that miR-199a-3p provides VCAN more diverse functions by fine-tuning functions of different VCAN isoforms. Versican V1 and V2 isoforms are known to exhibit opposite functions on cell proliferation [169]. When anti-proliferative Versican V2 is transcribed, translational repression on Rb1 and PTEN is relieved. Synergistic combination of Versican V2, Rb1, and PTEN will work strongly against proliferation. On the other hand, when proliferation-favored Versican V1 is transcribed, increased translation of Rb1 and PTEN will hinder the proliferative property of V1 but this would be less effective in comparison. Nevertheless, the adhesive function provided by Versican V1 isoform remains. This cellular function of VCAN can be particularly important for cell survival when nutrients are scarce. In conclusion, translation of VCAN mRNA not only exerts autocrine and paracrine effects on cells, but it also influences effective level of miRNAs in the cytoplasm. The reduction in miRNA-mediated repression can immediately affect translation of other mRNAs. The dual effect of a single mRNA may alter gene expression immediately by translation, and also by transcription in a comparatively longer period of time.

5.6 Degradation of miRNA base-pairing with imperfect sequences in the 3’UTR

Notably, levels of miR-199a-3p and miR-136 are consistently reduced in lung tissues but not kidney tissues. The abundance of miRNA is not the issue because miR-136 is expressed at similar level in both lung and kidney (Fig. 4.20). Clearly, other factors beyond expression level, such as cell type, are involved in 3’UTR-mediated miRNA degradation. A previous study suggested that an imperfect miRNA target is a
better decoy compared with a perfect target [209]. Naturally evolved 3’UTR contains imperfect binding sites for miRNAs, and thus are expected to antagonize miRNAs efficiently. In addition, we find that antagonizing miRNA by imperfect match with a long strand of non-coding RNA also leads to degradation. Human Argonaute 2 (Ago2) has been proven to mediate endonucleolytic cleavage of mRNA when perfect complementary is matched [210]. This may present a model to study mechanisms involved in miRNA degradation.

Our results showed a general loss of miRNA abundance by 30% depending on the types of primary tissue, while other miRNAs are not affected because their targets are translated at the same efficiency. Although we only examined the abundance of two miRNAs, other miRNAs that bind to that 3’UTR are very likely affected as well (Fig. S4.8). Transient or stable transfection of 3’UTR into breast cancer cells both reduced the level of miR-199a-3p, suggesting that the degradation process is immediate and can be prolonged. We also observed that cancer cells tend to lose miRNA by nearly two-fold more than various primary tissues examined. It is well accepted that genomic instability is common during cancer progression, and non-coding transcripts can be generated easily during this process. Binding of miRNAs to these transcripts is likely to have contributed to the global reduction of miRNAs. Currently, degradation of miRNA is not as well understood as its biogenesis. An exoribonuclease found in Arabidopsis named Small RNA Degrading enzyme 1, or SDN1, is the only enzyme proven to degrade miRNA [69]. More work is required to investigate the underlying mechanisms of miRNA degradation. Our data has shown that only certain miRNAs are degraded in the 3’UTR-transfected cells or specific 3’UTR-expressing tissues. It is likely that a RNA-binding protein that participates as a subunit of RISC, aids in the recognition of the specific miRNA and the recruitment of degrading enzymes. In order to purify this
RNA-binding protein, the miRNA degradative complex will need to be isolated. One approach will be to co-immunoprecipitate this protein by antibodies recognizing members of SDN family in mammals. This protein complex can be dis-assembled and analyzed by Mass Spectrometry in search for this RNA-binding protein. Since active degradation of certain miRNAs occurs in the 3'UTR-transfected or –expressing tissues, the proteins associated with the miRNA degradative complex will be different from the ones that are responsible for normal maintenance of miRNA levels. After excluding proteins that participate in RISC, the remaining candidates can be further filtered by the domains present in the proteins. Further ways to confirm its involvement in miRNA decay includes knocking down this protein using siRNA to prevent miRNA degradation.

5.7 Importance of non-coding transcripts

Our experiments indicated that the functions of miRNA can be regulated by a fragment of non-coding transcript. Genomic deletion/truncation leading to translational silencing produces mutant phenotypes and is accompanied by existing non-coding/mutated transcripts [211, 212]. This strategy has been extensively used to knock-out the gene of interest in studying gene functions. After gene knock-out, the protein is no longer expressed, but it is conceivable that the mutated genes are still able to produce non-coding transcripts. Sometimes, no detectable phenotypes are obtained, and it is said that the mutated/lost proteins may be compensated by other proteins. Although compensation by other proteins is possible, the non-coding transcript may play an important role in compensation and balancing the mutant entity. Our results do indicate a dramatic functional alteration induced by expressing non-coding transcript. Expression of 3'UTR altered the translation of other mRNAs that are targeted by the
same miRNA. It is possible that the 3'UTR can play more diverse roles than the protein expressed by the same transcript, even though proteins are known to be executors of biological activities. The human genome contains a large number of pseudogenes, which are nearly as abundant as functional genes and therefore appear to be an important component of the genome. It has been reported that there are approximately 20,000 putative pseudogenes in the human genome [213]. An analysis of chromosome 22 indicates that approximately 20% of the pseudogenes are potentially transcribed [214]. Pseudogene transcription has also been reported in other species including flies, mice, cows, and chimpanzees [215]. The assumption that pseudogenes are dysfunctional is based on the finding that pseudogenes do not code for proteins. It is possible that these non-coding pseudogenes play important roles in maintaining homeostasis, specifically as modulators of miRNA.

5.8 Genetic exchange of miRNAs between cells

The first strong report of exosome-mediated transfer of miRNAs was found in mast cells [216]. This exosome is likely prepared for antigen presentation purpose, and it contains functional mRNAs from 1300 genes that are not translated in donor mast cells. Exosome-mediated transfer has other physiological roles, including in neuron communication within synapses [217] and in maintenance of pluripotency in stem cells [218]. Exosomes can be formed through inward budding of endosomal membranes, giving rise to intracellular Multivesicular Bodies (MVB) [219]. MVB is packed with genetic materials and proteins, such as Ago2 and miRNAs [220]. As an exosome, it fuses with the plasma membrane and is released to the extracellular environment. However, normal cells excrete negligible amount of exosomes compared with cancer cells [221].
Exosomes from glioblastoma are shown to contain mostly proteins that stimulate angiogenesis in primary endothelial cells [222]. Messenger RNAs provide a lasting effect by prolonged production of proteins. Gene ontology shows that most mRNAs are involved in cell proliferation and in immune responses. And hence, translation of these mRNAs will initiate angiogenesis in endothelial cells, and suppress immune functions in NK cells [223]. Compared with cytosolic composition, many miRNAs, such as let-7a, are packed with little Ago2 [220]. The presence of a loaded miRNA effector complex is expected to immediately exert translational regulation.

Since exosomes are utilized by cancer cells to influence neighbouring cells, the presence of exosomes can potentially serve as a marker for cancer detection. The earliest report on finding circulating miRNAs in the blood revealed the exciting possibility of using blood to non-invasively perform prenatal diagnosis [224], as miRNA profiling in blood could potentially reflect the progress of pregnancy [225]. The same idea was transferred to cancer research. In one case, it was found that xenografting human prostate cancer cells into mice enables the detection of human-specific miRNA in blood of the mice [226]. In patients, the level of miR-141 can be distinguished between patients bearing prostate cancer and healthy controls. Similar quantities of miRNAs were found in serum and plasma. There have been multiple reports that detected trends in circulating miRNA expression in different types of cancer. Systemic miR-195 is high in patients having breast cancer, and its level dropped altogether with let-7a in patients post-operatively [227]. In a ovarian cancer study, protein profiles in exosomes closely relate to proteome of ovarian cancer [228]. Eight miRNAs, that are previously reported to elevate in ovarian cancer (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214), are detected in exosomes collected from patients with benign or malignant ovarian cancer. Interestingly, expression of these miRNA is
presented in different intensities between the two groups. Therefore, circulating miRNAs can potentially be non-invasive biomarkers for cancer diagnosis and prognosis. The close similarity between miRNA profiles in the exosomes and cancer cells can also aid in understanding the molecular mechanisms of tumorigenesis and cancer development.
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