IDENTIFICATION OF CARE-BINDING MICRORNAS TARGETING VEGFA AND THE ROLE OF HNRNP L IN MODULATION OF THEIR ACTIVITIES

by

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To my parents, Javaher Vejdani and Karim Jafarifar
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<th>Full Form</th>
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<tr>
<td>Ago</td>
<td>Argonaute</td>
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<tr>
<td>ARE</td>
<td>AU-rich element</td>
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<tr>
<td>CARE</td>
<td>CA-rich element</td>
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<tr>
<td>CAT-1</td>
<td>Cationic amino acid transporter 1</td>
</tr>
<tr>
<td>CURE</td>
<td>CU-rich element</td>
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<tr>
<td>DND1</td>
<td>Dead end 1</td>
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<tr>
<td>dsRBP</td>
<td>Double-stranded RNA binding protein</td>
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<tr>
<td>ELAV</td>
<td>Embryonic lethal abnormal vision</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>FXR 1</td>
<td>Fragile X mental retardation protein</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
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<tr>
<td>hnRNP L</td>
<td>Heterogeneous nuclear ribonucleoprotein L</td>
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<td>HRP</td>
<td>Hypoxia regulated miRNA</td>
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<tr>
<td>HSR</td>
<td>Hypoxia stability region</td>
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<td>Huh 7</td>
<td>Hepatocarcinoma cells</td>
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<td>HuR</td>
<td>ELAV protein HuA</td>
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<td>ID</td>
<td>Immunodepletion</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
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<td>miRISC</td>
<td>miRNA-induced silencing complex</td>
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<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>NES</td>
<td>Nuclear export signal</td>
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<td>Abbreviation</td>
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<tr>
<td>nt</td>
<td>Nucleotide</td>
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<td>P body</td>
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<tr>
<td>pre-miRNA</td>
<td>Precursor miRNA</td>
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<td>pri-miRNA</td>
<td>Primary miRNA</td>
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<tr>
<td>PTB</td>
<td>Polypyrimidine tract binding protein</td>
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<tr>
<td>pVHL</td>
<td>Von hippel-lindau</td>
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<tr>
<td>RBP</td>
<td>RNA binding protein</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
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<tr>
<td>stRNA</td>
<td>Small temporal RNA</td>
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<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
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<td>TNFα</td>
<td>Tumor necrosis factor α</td>
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<td>3’-UTR</td>
<td>3’-Untranslated region</td>
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<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
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<tr>
<td>VPF</td>
<td>Vascular permeability factor</td>
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Identification of *CARE-binding MicroRNAs* Targeting *VEGFA* and the Role of *HnRNP L* in Modulation of their Activities

Abstract

by

FAEGHEH JAFARIFAR

Expression of vascular endothelial growth factor A (VEGFA) by tumor-associated macrophages (TAMs) is critical for tumor progression and metastasis. Hypoxic microenvironment of solid tumors induces VEGFA expression in TAMs by increased transcription and mRNA stabilization. VEGFA mRNA stabilization mediated by RNA binding proteins (RBPs) such as heterogeneous nuclear ribonucleoprotein L (hnRNP L) plays a major role in VEGFA regulation. hnRNP L binds to a specific CA-rich element (CARE) in the VEGFA 3’-untranslated region (3’-UTR). Our laboratory has identified an RNA conformational switch in human VEGFA 3’-UTR that integrates signals from interferon gamma (IFN-\(\gamma\)) and hypoxia to regulate VEGFA expression in human macrophage-like monocytic cells. The RNA conformational change in adjoining elements GAIT (IFN-\(\gamma\)-activated inhibitor of translation) and CARE is dictated by mutually exclusive, stimulus-dependent binding of proteins. These proteins include the heterotetrameric IFN \(\gamma\)-activated inhibitor of translation complex (GAIT) and hnRNP L. This dissertation is focused on the interplay between microRNAs (miRNAs) and RBP that regulates the expression of one of the most important angiogenic factors VEGFA. We identified a new mechanism of stress-dependent VEGFA regulation that involves
reversal of miRNA-mediated silencing of VEGFA expression by hnRNP L. The data indicate that the CARE element of human VEGFA 3’-UTR, the binding site of hnRNP L, is targeted by miRNAs. Among these miRNAs, miR-297 and -299 are endogenously expressed in monocytic cells and negatively regulate VEGFA expression. Our data revealed that the repressive effect of these miRNAs can be inhibited in hypoxia. We showed the direct role of hnRNP L and established a mechanism for this observation. In hypoxia, the hnRNP L expression does not change but rather its cellular localization does. Hypoxia induces translocation of nuclear hnRNP L to the cytoplasm in a VEGFA mRNA independent manner and markedly increases cytoplasmic hnRNP L concentration which competes away the CARE-binding miRNAs. These studies introduce a novel mechanism of VEGFA gene regulation and can contribute to development of miRNA-based anticancer therapeutic tools.
I. Introduction

A. History of microRNAs

microRNAs (miRNAs) are a family of 21- to 25-nt non-coding RNAs expressed in a variety of organisms ranging from plants to worms and humans. The first miRNA, lin-4 was identified in 1993 in the Ambros laboratory. They showed that lin-4 mutant worms lack a developmental switch which results in an abnormal repetition of certain larval stages. Cloning of the lin-4 gene revealed that it does not encode a protein product, but instead it produces short, ~22-nt transcripts that repress lin-14 expression, a protein involved in the developmental timing pathway [1]. Seven years later, the let-7 RNA, which likewise regulates developmental timing in worms, was discovered [2]. Similar to lin-4, let-7 RNA recognized sequences present in the 3’-untranslated region (3’-UTR) of its mRNA target, lin-41 and repressed its expression. lin-4 and let-7 RNAs were named small temporal RNAs (stRNAs) [2]. Further studies revealed that let-7 RNA was evolutionarily conserved and expressed in all bilaterians tested [3]. The discovery of a conserved genetic surveillance mechanism called RNA interference (RNAi) [4] suggested that other ~22-nt regulatory RNAs might exist in diverse organisms and could be generated like stRNAs. In the RNAi mechanism, due to the presence of double stranded RNA (dsRNA) corresponding to the targeted mRNA, the small 22-nt antisense RNAs degrade mRNAs. Soon after, the Tuschl, Bartel, Ambros and Dreyfuss laboratories reported the existence of more than 100 endogenous ~22-nt RNAs in different organisms. These RNAs are derived from longer hairpin-like precursors and were named miRNAs
stRNAs are thus the two founding members of this large class of small regulatory miRNAs.

**B. miRNA biogenesis**

Most miRNA genes are independent of protein coding genes and are located in intergenic regions. A sizable minority is located in the intronic regions of protein-coding genes in the sense or antisense orientation called mirtrons [8-9]. miRNA genes are located in all chromosomes and half of them are identified in clusters transcribed as polycistronic primary transcripts [10]. The miRNAs from independent genes are transcribed by RNA polymerase II as long primary transcripts pri-miRNAs with 5’ m7 G capping and 3’ poly (A) tails [11-12]. The several kb pri-miRNAs have local hairpin structures and are subsequently cleaved by the Drosha-DGCR8 complex in the nucleus. The cleaved product is a stem-loop structured ~70-nt precursor pre-miRNA. The mirtrons, on the other hand, are spliced-out introns corresponding precisely to pre-miRNAs, thus circumventing the requirement for Drosha-DGCR8 [13-15]. Drosha is an RNase III family endonuclease and together with its cofactor DGCR8, cleaves the pri-miRNA at the base of stem structure resulting in a 5’-phosphate end and 2-nt 3’ overhang. DGCR8 is a double- stranded RNA-binding protein (dsRBP) known as Pasha in *Drosophila* and *C. elegans*. The pre-miRNAs are recognized by nuclear export factor, exportin-5 that delivers pre-miRNAs through nuclear pores to the cytoplasm [16-17]. Once there, they are subjected to the second processing step by Dicer. Dicer is a highly conserved cytoplasmic RNase III that cleaves the pre-miRNAs into ~22-nt duplexes of mature miRNA and a miRNA passenger strand (miRNA/miRNA*) containing 2-nt overhangs at
the 3'-termini [18-19]. Like other RNase III family proteins, Dicer associates with dsRBP partners including RDE-4 in *C. elegans* [20], R2D2 and FMR1 in *D. melanogaster* [21-24] and Argonaute family proteins in various organisms including human[19, 25]. Dicer-associated proteins are not required for RNA cleavage activity [21, 26]. Instead, they have various roles in miRNA stability and miRNA-protein complex formation and action. One strand of the ~22-nt duplex (miRNA*) disappears whereas the other strand remains as the mature miRNA. The mature miRNAs are incorporated into protein complexes known as miRNA-ribonucleoproteins (miRNPs), miRNA-argonautes (mirgonauts) or miRNA-induced silencing complexes (miRISCs). Strand selection may be determined by the relative thermodynamic stability of the two ends of miRNA duplexes [27-28]. The miRISCs contain several proteins including Dicer, TRBP, PACT, and Gemin 3, but the components that directly associate with miRNAs are Argonaute (Ago) proteins. Four Ago proteins have been identified in mammalian cells and can bind endogenous miRNAs [29]. The role of various Ago proteins in mammalian RISC is unclear.

**C. Mechanism of miRNA-mediated gene regulation**

In plants, miRNAs generally base pair to mRNAs with near perfect complementarity and trigger endonucleolytic mRNA cleavage by an RNAi-like mechanism [30]. Metazoan miRNAs, pair imperfectly with their targets, following a set of rules determined by experimental and bioinformatic analysis. The most stringent requirement is a perfect base-pairing of miRNA nt 2-8, representing the ‘seed region’ that nucleates the interaction. miRNA binding sites in metazoan mRNAs lie in the 3’- UTR and are usually present in multiple copies [31-35].
Different modes of miRNA-mediated regulation, including translational inhibition and mRNA deadenylation followed by mRNA degradation have been reported. The major pre-2005 hypothesis described miRNA-mediated repression of target gene expression by inhibition of miRNA translation at a stage after the initiation step [1, 36]. This idea was challenged by the publication of two papers describing miRNA-mediated inhibition at the level of initiation of protein synthesis [37-38]. Collectively, the studies of translational inhibition have revealed distinct mechanisms by which cap-dependent translation can be repressed. The ability of miRNAs to suppress translation promoted by internal ribosome entry sites (IRESs) is different. There are reports indicating that IRES-directed protein synthesis is unaffected by miRNAs, whereas other reports show that it is impaired. Based on the results obtained so far, it is likely that miRNA-mediated inhibition of translation acts in an mRNA-specific manner at distinct steps in the process of translation [39].

Recent papers demonstrate miRNA-mediated acceleration of target mRNA degradation, not by a siRNA-like mechanism of endonucleolytic cleavage, but rather, through the normal pathway of deadenylation, followed by decapping and subsequent degradation of mRNAs [40-43]. mRNA degradation and inhibition of translation, apparently both contribute to the overall repression of gene expression by miRNAs, but the relative importance of each mechanism may vary according to the cell type and the stability or configuration of the particular miRNA-mRNA pair [41].

On the other hand several groups have reported that miRNAs can also activate rather than repress their targets under certain conditions [44-49]. A miRNA can have either positive or negative regulatory impact on gene expression, depending on how miRNA base-pairing influences the structure and composition of the RNP. They can bind to protein
factors and recruit them to specific mRNAs or act as gatekeepers that alter mRNA secondary structure and thereby indirectly control the binding of other factors. miRNA targets may not be restricted to mRNAs; they can base-pair with and regulate noncoding RNAs as well. They can also compete with other RNAs for binding to a regulatory protein [50].

D. The biological function of miRNAs

miRNAs are among the most abundant gene-regulatory molecules in the animal cells, constituting almost 1-2% of the predicted genes in human, worms and flies [51-52]. Many are differentially expressed during the course of development and differentiation, leading to the prospect that each cell type might have a unique miRNA profile [1, 3, 5-6, 51, 53-56]. In addition, a link between miRNA alterations and human diseases, especially cancer, has been established [7, 57-66].

Considering the abundance, differential expression and potential promiscuity of metazoan miRNAs, the miRNA milieu, unique to each cell type can provide a crucial context for the evolution of all mRNA sequences and modulate the use of a substantial fraction of the metazoan transcriptome. For genes that should not be expressed in a particular cell type, protein output is lowered to inconsequential levels. For other genes, dosage should be adjusted in a manner that allows for customized expression in different cell types but a more uniform level within each cell type. The influence of miRNAs on mRNA expression is referred to as the micromanager model [67-68]. In this model, the miRNA targets fall into three categories. (1) Switch targets in which the protein targets are not expressed in a particular cell type and the result is equivalent to a discrete off-switch. (2)
Tuning targets in which the protein targets are optimally expressed at only low levels in a particular cell type. Such mRNAs could take advantage of the miRNA milieu, fine-tuning their complementarity to the relevant miRNAs to achieve optimal expression in each cell type. (3) Neutral targets in which the consequent downregulation of target proteins is tolerated or offset by feedback mechanisms. In neutral targets, the target mRNAs fortuitously pair with miRNAs and if there is no selective pressure to maintain or decrease pairing; these ‘bystander’ mRNAs are considered as neutral targets [67].

Recent findings of the impact of miRNAs on protein output revealed that hundreds of genes were directly repressed to a modest degree by individual miRNAs indicating that for most interactions, miRNAs make fine-scale adjustments to protein output [69-72]. For the more highly repressed targets (switch targets), mRNA destabilization is the major component of repression while for the tuning targets translational repression or a combination of translational repression and mRNA destabilization comprise the mechanism of repression [69].

E. Modulation of miRNA function

Recent findings indicate that under certain conditions, or in specific cells, miRNA-mediated repression can be effectively modulated. In most cases, a specific RNA-binding protein (RBP) alleviates or enhances the repressive activity of specific miRNAs. Here we describe examples of such modulations.

In human hepatocarcinoma cells (Huh7) subjected to various stress conditions, cationic amino acid transporter 1 (CAT-1) mRNA and reporters bearing its 3’-UTR can be relieved from miR-122-mediated inhibition. The derepression, which is accompanied by
HuR release from cytoplasmic processing bodies (p bodies) and its recruitment to polysomes, requires binding of HuR to an AU-rich element (ARE) in the 3’-UTR of the CAT-1 mRNA [73]. The derepression mechanism by HuR remains poorly understood. The interference of HuR with the function of miRNAs, which would result in enhanced translation or stability of the mRNA may be involved in this phenomenon. Leung and Sharp hypothesized that since HuR is required for Ago upregulation, HuR and other RBPs that bind to a site adjacent to the miRNA-binding site can interact with Ago proteins to modulate the function of miRNAs [74]. Another example of such proteins is fragile X mental retardation protein (FXR1) that interacts with Ago2 during serum starvation and associates with the ARE of tumor necrosis factor (TNF-α) to activate translation [44].

HuR interacting with Ago2 can also increase the repressive activity of miRNAs. HuR association with an ARE region next to let-7 miRNA-binding site in the c-Myc 3’-UTR, recruits let-7-loaded RISC to the c-Myc 3’-UTR. Let-7 miRNA requires HuR to inhibit c-Myc expression and vice versa [75].

The ability to alleviate miRNA-mediated repression was reported for an RNA-binding protein dead end 1 (Dnd1) as well [76]. The expression of Dnd1 counteracts the function of several miRNAs in human cells and in primordial germ cells of zebrafish. The effect of Dnd1 is mediated through U-rich regions present in the miRNA-targeted mRNAs. Since Dnd1 associates with mRNAs and not with miRISCs, Dnd1 derepression may be due to either a change in the RNA structure so that it is unfavorable for miRNA-binding or a change in mRNA localization so that it is inaccessible to miRNA-mediated silencing [76-78].
Another example of miRNA activity modulation is exposure of neurons to extracellular stimuli which prohibits the activity of a brain-specific miRNA, miR-134. By inhibiting Limk1 translation, miR-134 negatively regulates the size of dendritic spines in the mammalian nervous system. The mechanism for this modulation has not been identified [79].

Because of protein factors, miRNAs can be effective regulators of a target mRNA in one tissue but ineffective in another tissue as shown for miR-430. miR-430 reveals differential effects in somatic cells and primordial germ cells. In somatic cells, miR-430 targets the 3’-UTR of Nanos1 which encodes a protein required for germ line development in zebrafish, and inhibits its expression. The Nanos1 3’-UTR includes cis-acting elements that possibly recruit factors to the 3’-UTR and increase Nanos1 mRNA stability and translation in primordial germ cells even in the presence of mir-430 [80]. In another example of miRNA activity modulation, NHL-2, a C. elegans protein, functions as a cofactor for the miRISC and thereby enhances the post-transcriptional repression of several genetically verified miRNA targets. In fact, NHL-2 modulates the efficacy of miRNA-target interactions in response to physiological and developmental signals [81].

Blocking or enhancing the processing of miRNA transcripts (pri-miRNAs) by protein factors is another way of modulating miRNA function. lin28, a developmentally regulated RBP, selectively blocks the processing of pri-let-7 miRNAs in embryonic cells and acts as a negative regulator of miRNA biogenesis [82]. On the other hand, in response to DNA damage, p53, a tumor suppressor factor, enhances the post-transcriptional maturation of several miRNAs involved in growth suppressive function.
p53 interacts with the Drosha processing complex and facilitates the processing of pri-miRNAs to pre-miRNAs [83].

miRNAs can also modulate the function of RBPs. The translational inhibitory effect of heterogeneous nuclear ribonucleoprotein E2 (hnRNP E2) on the CEBPA mRNA is prevented by binding of miR-328, which acts as an RNA decoy and deactivates hnRNP E2 [84]. Overall, the crosstalk of miRNPs with RBPs and other factors emerges as a major theme in the modulation of miRNA/protein function in a cell-specific or state-specific manner.

**F. Tumors, macrophages and VEGFA**

Solid tumors are comprised of not only malignant cells, but also many other non-malignant cell types. This produces a unique microenvironment that can modify the neoplastic properties of the tumor cells. It is becoming apparent that the microenvironment has an important role in allowing the tumor to express its full neoplastic phenotype [85-86]. The tumor microenvironment contains many resident cell types such as adipocytes and fibroblasts as well as many migratory hematopoietic cells, most notably macrophages called tumor-associated macrophages (TAMs) [87-90]. A range of growth factors and chemokines recruit circulating monocytes. Once monocytes have moved across the tumor vasculature, many are attracted into the hypoxic area provided by tumors that are 150 µm from vessels. The hypoxic area stimulates monocytes to differentiate into TAMs [91]. TAMs are abundant in most forms of solid tumors, where they often display a relatively immature phenotype and are positively correlated with tumor angiogenesis and progression [92]. Oxygen tension regulates TAM
expression of angiogenesis factors. The key component is vascular endothelial growth factor A (VEGFA), a crucial regulator of angiogenesis [86, 93-94]. Both VEGFA and macrophages play a crucial role in tumorigenesis [95].

VEGFA, which was initially described as vascular permeability factor (VPF), is expressed by various cell types including tumor cells and monocytes [96-97]. It has five major isoforms: VEGF_{120}, VEGF_{144}, VEGF_{164}, VEGF_{188} and VEGF_{205} [98]. VEGF_{164} is the major isoform of VEGFA, forming a homodimeric heparin-binding glycoprotein with a molecular weight of 45 kDa. The secretory isoform is VEGF_{120}. The role of VEGFA in the maintenance of a highly permeable and extensive vasculature in multiple tumors has been established [99-100]. VEGFA production by TAMs not only stimulates angiogenesis but also tumor cell invasion which results in tumor progression and metastasis [85-86, 101].

G. Role of RBPs in regulation of VEGFA in hypoxia

Hypoxia is a significant pathophysiologic component of several diseases including cancer. Local hypoxia evokes local responses such as increased synthesis of VEGFA [102]. Regulation of VEGFA gene expression is controlled by both transcriptional and post-transcriptional mechanisms [103-104]. Hypoxia inducible factor (HIF-1α) plays a major role in VEGFA upregulation at the transcriptional level. HIF-1α binds to cis-acting hypoxia-response element within the VEGFA gene to activate transcription [105]. The post-transcriptional regulation of VEGFA is responsible for a major increase in VEGFA production under hypoxia [104, 106-110]. Post-transcriptional regulation is mediated by RBPs that act on defined cis-acting elements, usually found in the 3’-UTR
of the targeted mRNAs. While VEGFA mRNA has a half-life in the range of 15-40 min during normoxia, several investigators have shown that hypoxia increases it by as much as 3-4 fold [111].

Several RBPs involved in VEGFA mRNA stability have been identified: HuR (ELAV protein HuA), is one of four members of a family of human proteins that are highly homologous to a Drosophila nuclear protein known as ELAV. HuR binds with high affinity and specificity to an ARE in the 3’-UTR of VEGFA and increases VEGFA mRNA stability in hypoxia [112-113]. Hypoxia does not affect the total cellular concentration of HuR. Although, predominantly nuclear, hypoxia causes HuR translocation to the cytoplasm, which is linked to its ability to stabilize VEGFA mRNA [114]. pVHL, von Hippel-Lindau protein, modulates the regulation of VEGFA mRNA stability [108]. The increased VEGFA mRNA stability in cells lacking pVHL is due to a similar regulation of an RBP [115]. The absence of pVHL in cells grown in normoxic conditions is associated with a hypoxic phenotype that expresses increased levels of VEGFA. The mechanism by which pVHL regulates mRNA stability remains unclear. CSD/PTB complexes bind to VEGFA mRNA 3’- and 5’-UTRs and play a role in VEGFA mRNA stability in both non-induced and induced conditions, demonstrating a general stabilizing function [116]. PTB, known as hnRNP I, is a member of hnRNP family and shuttles between nucleus and cytoplasm [117-118]. hnRNP L is an abundant nucleoplasmic 68 kDa protein with 4 loosely conserved RNA recognition motifs (RRMs) containing several glycine- and proline-rich regions [119]. RRM"s 1, 2 and 3 are involved in the localization of hnRNP L. The RRM"s critical for RNA binding have not been characterized yet [120]. hnRNP L acts as a splicing enhancer which promotes the removal
of introns or exon skipping [121-122]. Like several hnRNPs, *hnRNP L* shuttles between the nucleus and the cytoplasm [119, 123-124]. It binds directly and with high affinity to CA-repeats and CA-rich clusters. *hnRNP L* also autoregulates its own expression at the level of alternative splicing using a highly conserved dense bipartite cluster of CA-rich motifs spread over 800-nt of intron 6 [125]. hnRNP L regulates human VEGFA mRNA stability in the hypoxia condition [109]. A 125-nt AU-rich element in the 3’-UTR of VEGFA, termed the hypoxia stability region (HSR), forms seven hypoxia-inducible RNA-protein complexes with apparent molecular masses ranging from 40 to 90 kDa in UV cross-linking assay [106, 109]. Protein purification of the 60 kDa complex identified the associated protein hnRNP L. The RNA binding site of hnRNP L, located in the HSR, is a CA-rich, 29-nt element (5’-CACCCACCCACAUACAUACAU-3’). The VEGFA mRNA half-life is significantly reduced when cells are transfected with an antisense oligonucleotide to the hnRNP L RNA-binding site [109] demonstrating the importance of hnRNP L in VEGFA mRNA stability. The *in vivo* specific interaction of *hnRNP L* with VEGFA HSR has been identified in hypoxia, however the mechanism is not clear yet [109]. Recently, the Fox laboratory reported an RNA switch in the VEGFA 3’-UTR that integrates signals from interferon (IFN-γ) and hypoxia to regulate VEGFA expression in myeloid cells. Analogous to riboswitches, the VEGFA HSR undergoes a binary conformational change dictated by mutually exclusive, stimulus-dependent binding of proteins, namely, the GAIT complex and hnRNP L in response to environmental signals [126].
**H. Role of miRNAs in regulation of VEGFA in hypoxia**

A group of hypoxia regulated miRNAs (HRMs) has been documented in certain cell types [127-130]. A potential miRNA target of particular importance is VEGFA. For this gene a group of candidate regulatory miRNAs were recently identified [131]. Most of these miRNAs were found to respond to hypoxia. Among these, miR-16, mir-15b, mir-20a and mir-20b repress VEGFA expression and are referred to as anti-angiogenic miRNAs which are downregulated in hypoxia [131-133]. miR-20b regulates VEGFA and HIF-1α, and is regulated by HIF-1α keeping tumor cells adapted to different oxygen concentrations [134]. miR-210 was identified as hypoxia inducible in all the cell types tested and is overexpressed in most cancer types. Hypoxic induction of miR-210 is dependent on a functional HIF-1α and associated with adverse prognosis in breast tumors and lymphoma [135-136]. miR-126 restoration downregulates VEGFA and inhibits the growth of lung cancer [137].

**I. Research plan**

There are two major questions that I sought to address in this thesis. The first has focused on the role of hnRNP L as a modulator of miRNA activity which also suggests a new mechanism of VEGFA regulation. The second centers around the physiological significance of the miRNAs that are modulated by hnRNP L in human monocyte-derived macrophages. To address the first question, I began by investigating whether the hnRNP L-binding site of VEGFA mRNA can be targeted by miRNAs, using bioinformatics and experimental approaches including gain of function studies. Since during hypoxia, hnRNP L binds the same site as the miRNAs, the possible crosstalk
between miRISCs and hnRNP L under both conditions of hypoxia and normoxia were investigated. I utilized IP followed by RT-PCR and luciferase reporter assays to investigate the possible competition mechanism that might occur due to a shared target by both miRISCs and hnRNP L.

The second question was addressed by investigating the endogenous levels of these miRNAs in human monocytic cells U937, THP 1 and primary blood monocytes (PBM). Northern blot and quantitative real time PCR demonstrated the endogenous presence of miRNAs that target the hnRNP L-binding site. I also conducted quantitative real time PCR and polyribosome profile analysis to reveal the mechanism of VEGFA repression by these miRNAs. I then utilized anti-miRNA inhibitors to block the activity of these miRNAs and investigated the level of VEGFA expression in human macrophages. I focused on human macrophages because of the important role of macrophages and VEGFA produced by macrophages in the hypoxic microenvironment of tumors, which is critical for tumor progression and metastasis. I utilized a luciferase reporter assay to further establish the physiological relevance of these miRNAs in regulating VEGFA level.

Few studies have been conducted to introduce miRNAs with a physiological role in VEGFA regulation. Results from our work identify miRNAs that have a physiological significance in VEGFA regulation. Our results also provide a therapeutic application for cancer using miRNA-based approaches.

On the other hand, previous reports indicated the role of nuclear hnRNP L in splicing of several mRNAs and cytoplasmic hnRNP L in mRNA stability. Our studies revealed a new role for cytoplasmic hnRNP L as a modulator of miRNA activity. Unlike the
previously reported modulators of miRNA activity, our work proposes a definite mechanism and suggests that the cis-element binding sites of RBPs in 3’-UTRs can be a hot spot for being targeted by miRNAs. Our work confirmed that miRNA regulation is dynamic and that crosstalk between miRNA and RBP may play a significant role in regulation of gene expression.
II. Materials and methods

A. Cells and lysates

Human monocytic cells, i.e., U937 cells (CRL-1593.2, ATCC), THP1 cells (TIB-202, ATCC), and primary blood monocytes (PBMs) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human PBM were isolated by leukapheresis, followed by countercurrent centrifugal elutriation [138], under an Institutional Review Board-approved protocol that adhered to American Association of Blood Bank guidelines. Human HEK 293T (CRL-11268, ATCC), HepG2 (HB-8065, ATCC) and A375 (CRL-1619, ATCC) cells were cultured in DME medium supplemented with 4.5 mg/ml glucose and 10% heat-inactivated FBS. HUVEC cells (CRL-2873, ATCC) was cultured in DME medium supplemented with 20% FBS. Cells were treated under either normoxic (21% ρO$_2$) or hypoxic (1% ρO$_2$) conditions in a humidified incubator (5% CO$_2$) for 24 hr. Cell lysates were prepared in phosphosafe extraction buffer (Novagen) containing protease inhibitor cocktail (Thermo Scientific). Cytoplasmic and nuclear extracts were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents as described in the brochure (Thermo Scientific).

B. Plasmid construction, transfection, and dual luciferase activity assay

The pSP64-FLuc-VEGFA 3’-UTR (nt 11-900) construct was generated as described before [139]. The VEGFA 3’-UTR HSR (nt 332-456) was PCR-amplified and inserted downstream of FLuc in pcDNA3 vector (Invitrogen) to generate pcDNA3-FLuc-VEGFA HSR. The VEGFA HSR was also amplified using a forward primer with the C and A
residues in the hnRNP L binding site mutated to G and T, respectively. The sequence was inserted downstream of FLuc in pcDNA3 to generate pcDNA3-FLuc-VEGFA HSR hnRNP L site-mutant. pcDNA3-c-Myc-hnRNP L (rat) plasmid was a gift from N. Kataoka.

U937 cells (2 x 10^6 cells per well) were transfected with oligomers including pre-miR miRNA precursors, miRNA-negative control, anti-miR miRNA inhibitors, anti-miR-negative control, anti-miR mutants (200 nM, Ambion), siRNA against hnRNP L and siRNA control (300 nM, Santa Cruz), using GenomONE-Neo EX HVJ Envelope Vector Kit (Cosmo Bio). In the anti-miR mutants, nt 3 and nt 4 of the seed regions of miR-297 (G and U to A and A) and miR-299 (U and G to A and A) were mutated. siRNA against hnRNP L was a pool of three target specific siRNAs against hnRNP L. The same kit was used to transfec U937 cells (2 x 10^6 cells) with plasmid DNA (40 µg). For HEK 293T cells, 2 x 10^6 cells were transfected with pre-miR miRNA precursors or miRNA-negative control (100 nM) using lipofectamine 2000 (Invitrogen). For the dual luciferase assay, oligomers were co-transfected into U937 cells with FLuc reporter plasmid (40 µg) and RLuc-expressing vector pRL-SV40 (20 µg) (Promega) to normalize for transfection efficiency. Relative Luc activities were measured using Dual Luciferase Assay kit (Promega). In HEK 293T cells, oligomers were cotransfected with FLuc reporter plasmid (2 µg), and pRL-SV40 (0.5 µg) as internal transfection control, using lipofectamine 2000 (Invitrogen) and the same Promega kit was used to measure luciferase activity.
C. Bioinformatic analysis

The potential miRNA candidates that target the CA-rich element of human VEGFA 3′-UTR were predicted by TargetScanHuman5.1 software (http://www.targetscan.org); miRanda software (http://www.microrna.org) and MicroCosm Targets version 5 software (formerly miRBase Targets) (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5).

D. Immunocytochemistry

U937 cells (about 50% confluency) on glass slides were washed three times with phosphate-buffered saline (PBS) and fixed in 1% paraformaldehyde for 30 min. Fixed cells were washed with PBS and permeabilized with 0.1% Triton X-100 for 15 min. After further washing, the cells were incubated with rabbit polyclonal anti-hnRNP L antibody (Santa Cruz) for 2 hr at room temperature. After thorough washing, anti-rabbit Alexa fluor 488 (Invitrogen) was added for 60 min at room temperature. DAPI (Sigma-Aldrich) was used to stain nuclei. The slides were then mounted with 90% glycerol in PBS and immunofluorescent images were acquired by confocal microscopy with LaserSharp software.

E. Immunoblot analysis

Cell lysates were subjected to SDS- PAGE (12%). The transferred blot was probed with rabbit anti-human VEGFA polyclonal antibody (Santa Cruz) and HRP-conjugated anti-rabbit secondary antibody (GE Healthcare), and detected with ECL Plus (Amersham).
Immunoblotting with a monoclonal anti-GAPDH-peroxidase antibody (Sigma) provided a loading control. Band intensities were measured using Image J software (http://rsbweb.nih.gov/ij/).

For detecting VEGFA in conditioned media, we used IP [140]. Briefly, protein-balanced medium was kept overnight at 4°C on a rocker with addition of 2 µg/ml rabbit anti-human VEGFA polyclonal antibody (Santa Cruz). Next 15 µl of GammaBind plus Sepharose beads (GE Healthcare) were added for another 4 hr. The Sepharose beads were collected by centrifugation, washed twice in RIPA buffer (Thermo Scientific) and one time in TSA buffer (0.01 M Tris, 0.14 M NaCl, pH 8.0). One additional wash was performed with 0.05 M Tris, pH 8. After boiling for 5 min in SDS gel loading buffer, supernatants were collected and electrophoresed using 12% SDS polyacrylamide gel. Separated proteins were blotted and probed using mouse anti-human VEGFA monoclonal antibody (Santa Cruz). Other antibodies used for immunoblotting were mouse monoclonal anti-c-Myc (Santa Cruz), mouse monoclonal anti-α-tubulin (Sigma), rabbit polyclonal anti-hnRNP L (Santa Cruz) and polyclonal anti-HIF1α (Boster Biological Technology) antibodies.

F. Analysis of RNA by PCR

To analyze miRNAs, total small RNA was extracted with miRVana miRNA isolation kit (Ambion), and the quality and quantity were determined using a nanodrop spectrophotometer. miRNA was assessed by real-time PCR using taqman probe and primer sets in an ABI PRISM 7000 system (Applied Biosystems), and normalized with
hsa-miR-17-5p as a control [141]. Briefly, 10 ng of total small RNA was reversed transcribed using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) and amplified using Taqman 2x Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems).

To determine VEGFA and hnRNP L mRNAs, first-strand cDNAs were synthesized with SuperScript III Reverse Transcriptase (Invitrogen) using total RNA (2 µg) extracted with Trizol (Invitrogen), and amplified with SYBR Green PCR Master Mix (Applied Biosystems) in an ABI PRISM 7000 system. GAPDH mRNA was used as internal normalization control.

Primers for 120-nt VEGFA PCR product were: TATGCGGATCAAACCTCAC (forward) and CTCGGCTTTGTCACATTTTTCTTGTCTTG (reverse); primers for 180-nt hnRNP-L PCR products were TTCTGCTTATATGGCAATGTGG (forward) and GACTGACCAGGCATGATGG (reverse); primers for 96-nt GAPDH product were TGCACCACCAAACCTGCTTAGC (forward) and GGCATGGACTGTGGTCATGAG (reverse).

**G. Northern blot analysis**

Northern blot analysis using locked nucleic acid probes was used to determine microRNA [142]. Briefly, total RNA (100 µg) was mixed with an equal volume of gel loading buffer II (Ambion), heated at 80 °C for 5 min, snap-cooled, and fractionated by 15% denaturing acrylamide gel electrophoresis. RNA was transferred to Hybond N+ (GE Healthcare) by capillary blotting using 20 x SSC buffer (Invitrogen) and fixed using a
UV crosslinker. miRCURY LNA detection probes (10 pmol, Exiqon) complementary to the CARE-binding miRNAs were radiolabeled with 1 µl T4 polynucleotide kinase (New England Biolabs) and 1 µl [γ-32P] ATP (0.4 MBq) for 1 hr at 37 °C. Labeled LNA probes were heated at 95 °C for 1 min, ice-cooled, diluted 1:1000 in pre-warmed (50 °C) PerfectHyb Plus hybridization solution (Sigma) with denatured salmon sperm DNA (20 µg/ml, Ambion), and added to the pre-hybridized UV crosslinked membrane at 50 °C for 1 hr. Membranes were washed 3 times in 2x SSC, 0.1% SDS at 50 °C. Decade Marker (Ambion) was used as the molecular weight markers. The membranes were stripped with boiled 0.1% SDS, 5 mM EDTA for 30 min, and re-probed with radiolabeled LNA-modified U6 as loading control.

H. Interaction of hnRNP L with VEGFA mRNA

In vivo interaction of hnRNP L with VEGFA mRNA was determined by IP followed by RT-PCR as described [109, 143]. Briefly, nuclear and cytoplasmic extracts (500 µg) prepared in NE-PER Extraction Reagent (Thermo), were incubated with Halt Protease Inhibitor Cocktail (EDTA-free, Thermo), RNase inhibitor (Promega), mouse IgG (4 µg) and 50 µl of GammaBind Plus Sepharose beads (GE Healthcare) for 1 hr at 4 °C. The supernatant was incubated overnight at 4 °C with 4 µg of mouse monoclonal hnRNP L (Santa Cruz), mouse monoclonal hnRNP A1 as a positive control (Santa Cruz), or with anti-Flag M2 monoclonal antibody (Sigma) as a negative control, and then with protein G Sepharose beads (50 µl) for 4 hr at 4 °C. The supernatant was discarded and the beads were washed 4 times with NE-PER cytoplasmic or nuclear extraction buffers, resuspended in the same buffers, and RNA was extracted with an equal volume of Trizol.
reagent (Invitrogen) and 1/5 volume of chloroform. Total RNA was also isolated from the same extracts using Trizol reagent. mRNAs and pre-mRNAs were detected by RT-PCR with SuperScript Reverse Transcriptase III (Invitrogen) using oligo d(T) and Taq DNA polymerase (Invitrogen).

For in vivo cytoplasmic interactions, PCR primers for VEGFA [109] generated a 716-nt product resolved by 1.5% agarose gel electrophoresis. A 96-nt PCR product generated from GAPDH primers served as a control.

VEGFA forward primer: TATGCGGATCAAACCTCAC
VEGFA reverse primer: ATAACATTAGCACTGTTAATTT
GAPDH forward primer: TGCACCACCAACTGCTTAGC
GAPDH reverse primer: GGCATGGACTGTGGTCATGAG

For in vivo nuclear interactions, PCR primers for VEGFA exon (exons 1-8) and intron (introns 3-4) recognitions generated PCR products of 600-nt and 300-nt, respectively, and a 96-nt GAPDH PCR product served as a control (the same as above).

Forward VEGFA exon recognition primer:
ATGCGGATCCATGAACTTTCTGCTCTTTGGG

Reverse VEGFA exon recognition primer:
ATGCAAGCTTGCTATGGGTAGTTCTGTG

Forward VEGFA intron recognition primer: GTGTCATCGCCTCTCATGCAG

Reverse VEGFA intron recognition primer: CCACTTCCCCAAAGATGCCAC
I. Sucrose gradient fractionation for polysome analysis

Ribosomal fractions were prepared as described [144]. Briefly, 24 hr-transfected HEK293T cells were lysed in TMK lysis buffer containing cycloheximide (0.1 mg/ml) and the cytosolic extract was obtained by centrifugation at 10,000 × g for 20 min. The extract was overlaid on a 10-50% (w/v) sucrose gradient and centrifuged at 100,000 × g for 4 hr at 4 °C. Absorbance was measured at 254 nm. RNA was isolated from each fraction using Trizol reagent (Invitrogen) and used for RT-PCR. The specific primers for FLuc generated a 96-nt product. GAPDH was used as control (primers the same as above).

Forward primer for FLuc: GCCTGAAGTCTCTGATTAAGT
Reverse primer for FLuc: ACACCTGCCTCGAAAGT

J. Statistical analysis

All quantitative data are expressed as mean ± s.d. for n=3 independent experiments. An asterisk (*) indicates a significant difference, *P* < 0.05, two-tailed t-test.
III. Activity of the miRNAs targeting CA-rich element (CARE) of human VEGFA 3’-UTR is modulated by hnRNP L during stress

A part of this chapter has been published in the EMBO Journal [145].

VEGFA produced by TAMs is a critical angiogenic factor for tumor progression and metastasis. Several studies have revealed VEGFA regulation in hypoxic microenvironment of tumors. The CARE of VEGFA 3’-UTR, through binding to hnRNP L, is one of the important regulatory elements involved in VEGFA induction during hypoxia. The role of miRNAs as negative regulators of gene expression in several cellular processes has been well established. We investigated the cross-talk between hnRNP L and miRNAs targeting the CARE of VEGFA 3’-UTR. We used several approaches, including bioinformatics, gain of function studies and a reporter assay to test this hypothesis. The data were utilized to produce a model for the modulation of miRNA activity by hnRNP L. With that we introduced a novel stress-dependent regulatory mechanism for VEGFA expression. We have proposed a novel role for hnRNP L as modulator of miRNA activity and suggest that cis-regulatory elements on 3’-UTRs can be a target for the interplay between RBPs and miRNAs. Furthermore, our studies suggest a potential cancer therapeutic agent based on miRNA-deliveries.

A. CARE of human VEGFA mRNA is a potential target for miRNAs
The proximal VEGFA 3’-UTR contains a 126-nt HSR that confers hypoxia-mediated stability to the VEGFA mRNA [106]. hnRNP L binds to a 21-nt CARE within HSR and
stabilizes VEGFA mRNA during hypoxia [109]. We wanted to know whether CARE can also be targeted by miRNAs. In order to find the putative miRNA candidates, we employed computer prediction programs (Table 1). Current prediction methods are diverse, both in approach and performance. Nonetheless, agreements are emerging on some conclusions, which are each reassuringly consistent with a growing body of experimental data. The most important determinant is a conserved Watson-Crick pairing in the 5’ region of the miRNA centered on nt 2-7 called the miRNA “seed” region which markedly reduces the occurrence of false-positive predictions [32-33, 68]. We mainly used TargetScan [33] and compared the results with miRanda [146] and MicroCosm Targets (miRBase Targets) [8] to predict putative miRNAs that target CARE. An in silico approach provides an important tool for miRNA target detection and together with experimental validation, reveal regulated targets of miRNAs. At least four miRNAs: miR-299, -297, -567 and -609, which we call “CARE-binding miRNAs”, were detected by the above approach (Figure 1) (Table 1).

B. CARE-binding miRNAs directly target hnRNP L-binding site and repress reporter expression

To investigate whether the hnRNP L-binding site of VEGFA 3’-UTR can be directly targeted by CARE-binding miRNAs, we engineered luciferase reporters bearing the 3’-UTR of either wild-type CARE (WT HSR) or an hnRNP L site-mutant (Mut. HSR), in which ‘C’ and ‘A’ residues in the hnRNP L binding site were mutated to ‘G’ and ‘T’, respectively. Reporter constructs such as these are widely used to provide experimental evidence for direct repressive effect of miRNAs on their target sites. In human HEK cells
Figure 1. Schematic of elements in human VEGFA 3’-UTR. The VEGFA 3’-UTR (open box) contains a hypoxia stability region (HSR) [57, 71] and Hur-binding site [107] (gray boxes) that are involved in mRNA stabilization. The HSR (expanded below) contains an hnRNP L-binding site (CA-rich element) (gray) and an adjacent GAIT element (black) that binds to the heterotetrameric inflammatory GAIT complex [126]. The sequence of the CA-rich element and seed regions of predicted miRNA candidates are depicted [145].
Table 1. List of CARE-binding miRNA candidates that target hnRNP L-binding site of human VEGFA 3’-UTR. Their seed positions, seed matches, conservations and the target prediction programs are depicted [145].

<table>
<thead>
<tr>
<th>miRNA</th>
<th>conservation</th>
<th>seed position</th>
<th>seed match</th>
<th>target prediction program</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-609</td>
<td>human</td>
<td>336-342</td>
<td>ACACCCA; 7mer-1A</td>
<td>TargetScan</td>
</tr>
<tr>
<td>miR-299</td>
<td>mammals</td>
<td>343-350</td>
<td>CCCACAU; 8mer</td>
<td>TargetScan; miRanda</td>
</tr>
<tr>
<td>miR-297</td>
<td>human</td>
<td>346-352</td>
<td>ACAUACA; 7mer-m8</td>
<td>TargetScan; miRanda; MicroCosm Target</td>
</tr>
<tr>
<td>miR-567</td>
<td>human</td>
<td>346-352</td>
<td>ACAUACA; 7mer-1A</td>
<td>TargetScan</td>
</tr>
</tbody>
</table>
The luciferase reporters were cotransfected with pre-miR™ miRNA precursors. These are small, chemically modified, double-stranded RNA molecules designed to mimic endogenous mature miRNAs. Use of the pre-miR miRNA precursors enables miRNA functional analysis by upregulation of miRNA activity. miR-control (Cont.), a random precursor with no identifiable effects and no homology to the human genome was used to control for nonspecific effects. The random precursor did not affect the reporter activities.

The pre-miRs mimetics of miR-299, -297, -567 and -609 reduced the activity of the firefly luciferase reporter bearing the wild-type VEGFA HSR, by 50-80% as compared with the control (p<0.008) (Figure 2, left panel). Pre-miRs mimetics of miR-410 and -369, predicted to target the GAIT element, were used as negative controls as they did not reduce luciferase activity (p>0.05). The activity of a luciferase reporter bearing hnRNP L site-mutant VEGFA HSR was not reduced by CARE-binding miRNAs (p>0.06), indicating that CARE directly mediates the repressive effect on luciferase expression (Figure 2, right panel).

We conclude that the CARE in the VEGFA 3’-UTR is a miRNA target site and ectopic miR-299, -297, -567 and -609 directly target the hnRNP L-binding site and significantly reduce the reporter expression.

C. CARE-binding miRNAs repress the endogenous VEGFA expression in U937 cells

Since VEGFA produced by macrophages has a critical role in cancer progression and metastasis, we examined whether the CARE-binding miRNAs are able to repress the endogenous VEGFA expression in human U937 monocytic cell line. We upregulated CARE-binding miRNAs by transient transfection (gain-of-function approach), and
Figure 2. CARE-binding miRNAs specifically target hnRNP L-binding site of VEGFA HSR. pcDNA3 vectors expressing firefly luciferase (FLuc) bearing HSR 3’-UTR with either wild-type CA-rich element (WT HSR) (left panel) or hnRNP L site mutant (Mut. HSR) (right panel) were co-transfected with CARE-binding miRNA candidates, random control (Cont.), miR-410 and -369 as negative controls and RLuc-expressing vector pRL-SV40 as an internal transfection control. Relative luciferase levels \( \frac{[\text{sample FLuc/sample RLuc}]/[\text{control FLuc/control RLuc}]}{\times 100} \) were measured after 24 hr of transfection in HEK 293T cells. The error bars (SD) and p values were calculated based on three independent experiments. The p values were compared to the random control. The significant differences (asterisk) were depicted (p< 0.05, two-tailed t-test) [145].
studied their effects on the endogenous VEGFA protein level after 24 hr of transfection. We used miR-16 as a positive control. It has been previously reported that miR-16 downregulates VEGFA expression [147]. Western blot analysis of total cell lysates using anti-VEGFA antibody showed that the protein level of VEGFA was reduced by miR-16 as well as by CARE-binding miRNAs by 50-70% compared to the control, while miR-410 and -369 did not change the VEGFA level (Figure 3). These findings indicate that endogenously expressed VEGFA in human monocytes can be post-transcriptionally controlled by ectopic CARE-binding miRNAs.

D. Hypoxic induction of VEGFA expression in human U937 cells

It is well established that during hypoxia, hypoxia inducible factor (HIF) is upregulated and regulates transcription of many genes including VEGFA [148]. We tested the hypoxia-mediated HIF1α and VEGFA upregulation in human U937 monocytic cells. Cells were incubated in either hypoxia or normoxia conditions for 24 hr and cell lysates were prepared for immunoblotting using anti-VEGFA and anti-HIF1α antibodies (Figure 4A, C). Total RNAs were also prepared from hypoxic and normoxic U937 cells and RT-PCR was performed using specific primers for VEGFA and GAPDH (Figure 4B). As we expected, the HIF1α protein expression and the VEGFA mRNA level significantly increased under hypoxia in U937 cells. Unexpectedly, VEGFA protein of total cell lysates did not show a significant increase in hypoxia compared to normoxia (Figure 4C). We then measured the amount of secretory VEGFA in conditioned media using VEGFA IP followed by immunoblotting using anti-VEGFA antibody. Interestingly, the secretory VEGFA increased significantly in hypoxia when compared to normoxia (Figure 4D).
Figure 3. Ectopic CARE-binding miRNAs negatively regulate endogenous VEGFA expression. Total U937 cell lysates after 24 hr of transfection with CARE-binding miRNAs, random control, miR-369, -410 and -16, were prepared. 40 µg of total cell lysates were processed in Laemmlli gel-loading buffer and subjected to electrophoresis on 12% SDS-PAGE followed by immunoblotting with anti-VEGFA antibody (top panel) and anti-GAPDH antibody (middle panel). Protein quantification was performed using Image J and normalized to control (100%) (bottom panel). GAPDH was used as loading control. The p values were compared to Control. The significant differences (asterisk) were depicted (p< 0.05, two-tailed t-test) [145].
A

HIF1 alpha

IB, anti-HIF1 alpha

GAPDH

IB, anti-GAPDH

B

VEGFA mRNA

RTPCR, VEGFA specific primers

GAPDH mRNA

RTPCR, GAPDH specific primers

C

VEGFA

IB, anti-VEGFA

GAPDH

IB, anti-GAPDH

D

Normoxia

Hypoxia

VEGFA

IB medium: anti-VEGFA

VEGFA fold change

4.5

4.0

3.5

3.0

2.5

2.0

1.5

1.0

0.5

0.0
Figure 4. Hypoxic regulation of VEGFA expression in U937 cells. (A) HIF1α protein induction in hypoxic U937 cells. 35 µg of total cell lysates of either hypoxic (Hpx.) or normoxic (Nmx.) U937 cells were immunoblotted using anti-HIF1α and anti-GAPDH antibodies. GAPDH was used as control. (B) VEGFA mRNA induction by hypoxia. After 24 hr of incubation of U937 cells in either Hpx. or Nmx. total RNA was prepared. The VEGFA and GAPDH mRNA levels were measured by RT-PCR using specific primers for VEGFA and GAPDH. GAPDH was used as control. (C) Hypoxic regulation of total VEGFA protein level in U937 cells. Lysates were prepared and 30 µg of total protein was immunoblotted using anti-VEGFA and anti-GAPDH antibodies. GAPDH was used as control. (D) Hypoxia-mediated secretory VEGFA induction in U937 cells. Anti-VEGFA polyclonal antibody was used to immunoprecipitate VEGFA from conditioned media of hypoxia- and normoxia-treated U937 cells. The immunoprecipitated VEGFA was measured by immunoblotting using monoclonal anti-VEGFA antibody (top panel). Fold-change of the secretory VEGFA level in hypoxia was measured using Image J and compared with normoxic level that was normalized to 1. This observation may indicate that the rate of VEGFA secretion in human U937 cells is high so that we could clearly see the upregulation of VEGFA protein due to hypoxia in conditioned media compared to the total cell lysates.
E. Role of hnRNP L in hypoxia-mediated VEGFA regulation in human monocytes

There are many reports regarding the role of RBPs in VEGFA mRNA stabilization during hypoxia [106-107, 109]. hnRNP L is one of the RBPs that stabilizes VEGFA mRNAs during hypoxia in human melanoma cells. We wanted to test the effect of hnRNP L on VEGFA expression in hypoxic macrophage-derived monocytic cells. We transfected U937 cells with siRNAs against hnRNP L for 24 hr in both conditions of hypoxia and normoxia. Total cell lysates were prepared and VEGFA levels were measured using immunoblotting with an anti-VEGFA antibody. Our results indicate that while downregulation of hnRNP L has no effect on VEGFA protein expression during normoxia, in hypoxia it downregulates VEGFA level by almost 40%, confirming that hnRNP L is also involved in VEGFA regulation in human monocytes during hypoxia (Figure 5).

F. Hypoxia specifically relieves CARE-binding miRNA-mediated repression of VEGFA

Recent findings indicate that miRNA activity can be modulated in a state-specific manner such as cellular stress [73, 76, 81, 149-150]. Therefore, we next investigated whether hypoxia can modulate the observed inhibitory effect of CARE-binding miRNAs on VEGFA expression. To test that, we upregulated CARE-binding miRNAs and miR-16 in U937 cells under both conditions of normoxia and hypoxia. miR-16 targets the distal region of VEGFA 3’-UTR (nt 1793-1818) and downregulates VEGFA expression [147]. After 24 hr, the effects of the miRNAs on the U937 endogenous VEGFA level were measured by Western blot of total cell lysates (Figure 6).
Figure 5. Hypoxic regulation of VEGFA by hnRNP L in U937 cells. The cells were transfected with siRNAs against hnRNP L, siRNA 1 (from Santa Cruz) and siRNA 2 [126] as well as an siRNA control (Santa Cruz). The VEGFA expression was analyzed with immunoblot using anti-VEGFA antibody after 24 hr of incubation in either hypoxia or normoxia. GAPDH was used as loading control and hnRNP L was measured using anti-hnRNP L antibody. All quantitative data are expressed as mean ± s.d. for n=3 independent experiments. An asterisk (*) indicates a significant difference, $P<0.05$, two-tailed t-test.
Figure 6. The specific effect of hypoxic conditions on the activity of CARE-binding miRNAs in human U937 cells. Hypoxia overcomes CARE-binding miRNA-mediated inhibition of VEGFA expression. U937 cells were incubated with microRNAs under normoxic (21% pO2, N) or hypoxic (1% pO2, H) conditions for 24hr. 40 µg of prepared cell lysates were processed in Laemmlli gel loading buffer, electrophoresed on 12% SDS-PAGE and immunoblotted with anti-VEGFA (top) and anti-GAPDH (middle) antibodies. The amount of VEGFA for each treatment was measured using Image J and normalized to the normoxic control (100%) (bottom). miR-16 was used as positive control. All quantitative data are expressed as mean ± s.d. for n=3 independent experiments. An asterisk (*) indicates a significant difference, \( P < 0.05 \), two-tailed t-test [145].
Interestingly, the repressive effect of the CARE-binding miRNAs was alleviated under hypoxia, whereas the miR-16 repression remained unchanged. In normoxia, the endogenous level of VEGFA was decreased by 50-70% compared to the control (p<0.007). In contrast hypoxia caused a 30-50% relief in the repression by CARE-binding miRNAs so that the endogenous level of VEGFA remained unchanged when compared to control (p>0.08). The hypoxic effect was specific to miRNAs targeting the hnRNP L-binding site since miR-16 targeting a distal region of VEGFA 3’-UTR showed the same repressive activity (~70% repression) in both conditions of hypoxia and normoxia. Similar results were observed with other previously reported miRNAs that negatively regulate VEGFA (data not shown). Our bioinformatic studies showed that none of these miRNAs target CARE (nt 336-357 of VEGFA 3’UTR). miR-20a and -20b [131, 134] target nt 183-190 of VEGFA 3’-UTR. miR-15b [131] target nt 277-284 and miR-126 [137] targets nt 1212-1232. These data explain that hypoxia specifically modulates the function of miRNAs that target hnRNP L-binding site and suggest that hnRNP L may play a role in this phenomenon.

G. Hypoxic effect on hnRNP L expression in human monocytes
Since hypoxia specifically modulates the function of the miRNAs that target the hnRNP L-binding site, we then investigated hnRNP L expression in U937 cells under both conditions of hypoxia and normoxia. Total RNA was extracted from U937 cells exposed to either hypoxia or normoxia for 24 hr and the level of hnRNP L mRNA was measured by quantitative real time RT-PCR using specific primers for hnRNP L (Figure 7A). Total cell lysates were also prepared and the protein level of hnRNP L was investigated using
immunoblotting with an anti-hnRNP L antibody (Figure 7B). Our data indicated that hypoxia does not change the expression of hnRNP L at either transcriptional or translational levels in U937 cells. Previous reports showed that a minor induction of hnRNP L occurs under hypoxia in melanoma M21 cells [109]. It is tempting to think that the stress-dependent regulation of hnRNP L is cell specific.

H. Hypoxic effect on hnRNP L cellular localization in human monocytes

We showed that hypoxia has no effect on the expression level of hnRNP L in human monocytes. It has been shown that several hnRNPs including hnRNP L shuttle between the nucleus and the cytoplasm [119, 123-124]. Hypoxia differentially regulates the distribution of hnRNP L in different cellular compartments in human melanoma cell lines [109]. The intracellular localization of hnRNP L in human lung cancer cell lines depends on the type of the cell line. In SBC-3 and H69 lung cancer cells, hnRNP L is expressed equally in the nucleus and cytoplasm, whereas in PC-14 lung cancer cells, hnRNP L is expressed predominantly in the nucleus [120]. We wanted to investigate the status of hnRNP L cellular distribution in U937 monocytic cells under both conditions of hypoxia and normoxia. We first performed immunofluorescence staining of cells with an anti-hnRNP L antibody after exposure to either hypoxia or normoxia for 24 hr. The immunostaining data revealed that the cytoplasmic mobilization of hnRNP L increased in hypoxia when compared to normoxia (Figure 8). To further confirm the hypoxia-mediated subcellular localization of hnRNP L, human monocytic cells including U937, PBM and THP1 (data not shown) cells, were exposed to hypoxia or normoxia for 24 hr. Nuclear and cytoplasmic lysates were prepared and immunoblotted using anti-hnRNP L
Figure 7. Hypoxia does not alter hnRNP L expression. (A) U937 cells were incubated under normoxia (Nmx.) or hypoxia (Hpx.) for 24 hr and total RNA was subjected to quantitative real time RT-PCR using primers specific for hnRNP L and GAPDH. (B) Cell lysates were subjected to immunoblot analysis with anti-hnRNP L and -GAPDH antibodies [145].
Figure 8. Hypoxia-mediated immobilization of hnRNP L in cytoplasm of human monocytes. Immunofluorescent staining of hnRNP L in human monocytic U937 cells after incubation in normoxia (Nmx.) or hypoxia (Hpx.) for 24 hr. Anti-hnRNP L antibody was used as the primary antibody and fluorescent anti-rabbit IgG as the secondary antibody[145].
and anti-α-tubulin antibodies (Figure 9). α-tubulin was used as a cytoplasmic marker. Hypoxia increased the cytoplasmic level of hnRNP L by 3- to 5-fold. Therefore, hypoxia does not induce hnRNP L expression level in human monocytes. Rather, it alters the cellular localization of hnRNP L leading to an enhanced level of cytoplasmic hnRNP L. The molecular mechanism regulating hnRNP L localization is not known; however, another miRNA modulator, HuR, undergoes stress-dependent translocation to the cytoplasm following dephosphorylation, while its expression level remains unchanged [114].

I. Cytoplasmic hnRNP L association with VEGFA mRNA

Previous work from the Claffey laboratory showed that cytoplasmic hnRNP L specifically interacts with VEGFA mRNA in hypoxic human melanoma cells in vivo and regulates VEGFA mRNA stability [109]. In order to test the in vivo association of hnRNP L with VEGFA mRNA in hypoxic U937 monocytic cells, hnRNP L was immunoprecipitated from cytoplasmic fractions of 24 hr hypoxic and normoxic cultured U937 cells using anti-hnRNP L monoclonal antibody. RNAs were isolated from these samples and analyzed by RT-PCR using specific primers for human VEGFA mRNA. The VEGFA primers used in RT-PCR contained part of VEGFA coding region and the entire HSR [109]. A unique PCR product of 700 nt appeared with hnRNP L-immunoprecipitated hypoxic U937 cytoplasm; while the normoxic samples did not show any products (Figure 10). Neither IP with anti-FLAG tag antibody followed by VEGFA RT-PCR (data not shown) nor IP with anti-hnRNP L antibody followed by RT-PCR using specific primers for GAPDH showed any product, demonstrating the specificity of
Figure 9. U937 and PBM cells were incubated in either hypoxia or normoxia for 24 hr. The nuclear and cytoplasmic fractions of each treatment were prepared and immunoblotted with anti-hnRNP L and anti-alpha tubuline antibodies. Alpha tubuline was used as cytoplasmic marker [145].
the result. The presence of VEGFA and GAPDH mRNAs were also tested before IP. We conclude that the interaction of cytoplasmic hnRNP L with VEGFA mRNA occurs only during hypoxia.

J. Nuclear hnRNP L association with VEGFA mRNA

Because hnRNP L is a nucleocytoplasmic protein, we wanted to know whether the interaction between hnRNP L and VEGFA mRNA occurs in the nucleus followed by transport to the cytoplasm as would be expected for a shuttling factor or in the cytoplasm after transport. hnRNPs such as hnRNP A1 that interact with nuclear intron-dependent mRNAs remain bound to mRNAs while transported to the cytoplasm [151]. Such nuclear mRNA-associated hnRNPs contais nuclear export signals (NES) [119]. Since hnRNP L lacks a NES, it probably does not have any role in intron-dependent mRNA export [152]. In mammals, hnRNP L is associated with most nascent transcripts (pre-mRNAs) and is considered a global regulator of alternative splicing [153]. In intron-less mRNAs, however, in the absence of splicing, hnRNP L is involved in mRNA export [154]. We studied the in vivo nuclear association of hnRNP L with VEGFA mRNA and pre-mRNA in U937 cells, using an IP approach followed by RT-PCR. The nuclear extracts of hypoxic and normoxic U937 cells were immunoprecipitated with a monoclonal anti-hnRNP L antibody. After total RNA extractions, the association of hnRNP L with VEGFA pre-mRNA and mRNA was investigated using specific primers (Figure 11). hnRNP A1 which does not interact with hnRNP L [155] was used as a positive control [151]. The presence of VEGFA pre-mRNA and VEGFA mRNA in the nucleus was tested before IP using the same primers. As expected, a unique 300 nt PCR product of VEGFA
Figure 10. Hypoxia induces cytoplasmic hnRNP L association with VEGFA mRNA in U937. Cytosolic lysates from U937 cells were subjected to IP with anti-hnRNP L monoclonal antibody (right panels) and bound VEGFA and GAPDH mRNAs were determined by RT-PCR. The presence of VEGFA and GAPDH mRNAs in cytosolic extracts were also determined before IP using RT-PCR with the same primers (input) [145].
pre-mRNA and a unique 600 nt PCR product of VEGFA mRNA were observed when the hnRNP A1-immunoprecipitated nuclear fraction was utilized, indicating that hnRNP A1 is associated with both VEGFA pre-mRNA and mRNA. On the other hand, when hnRNP- L-immunoprecipitated nuclear fractions were utilized, only the 300 nt PCR product of the VEGFA pre-mRNA was observed, indicating that hnRNP L is only associated with VEGFA pre-mRNA and stress-mediated hnRNP L shuttling from nucleus to cytoplasm of U937 cells is VEGFA mRNA independent. IP with an anti-flag tag antibody was used to confirm the specificity of the experiment (data not shown). We conclude that since hnRNP L is only associated with VEGFA pre-mRNA, it is probably involved in VEGFA splicing events and since it is not associated with mature VEGFA mRNA, it likely dissociates from the VEGFA mRNA after the maturation process in the nucleus. During hypoxia, hnRNP L is transferred to the cytoplasm independent of VEGFA mRNA and associates with the transcript in the cytoplasm. We take into consideration that different arrays of hnRNP proteins are assembled on different transcripts and nuclear association of hnRNP L with mRNAs might be transcript specific [156-157].

**K. Association of cytoplasmic hnRNP L with VEGFA mRNA acts in a concentration-dependent manner**

We showed that the cytoplasmic concentration of hnRNP L increases in hypoxic U937 cells and that the cytoplasmic hnRNP L becomes associated with VEGFA mRNA only in hypoxia. These observations and the fact that hnRNP L has a high affinity for the CA-rich element led us to hypothesize that if we increase the cytoplasmic concentration of
Figure 11. hnRNP L binds mature VEGFA mRNA in the cytoplasm. Nuclear lysates were prepared from normoxic and hypoxic U937 cells, and VEGFA pre-mRNA and mRNA were determined by RT-PCR using intron (I)- and exon (E)-specific primers, respectively (top). The nuclear lysates were subjected to IP with anti-hnRNP L and anti-hnRNP A1 monoclonal antibodies, and bound VEGFA pre-mRNA and mRNA were determined by RT-PCR (middle); RT-PCR of GAPDH mRNA was used as specificity control (bottom) [145].
hnRNP L in normoxic condition, the increased level of hnRNP L may cause hnRNP L to become associated with the VEGFA mRNA during normoxia as well. To test this hypothesis, we aimed to mimic the hypoxic condition by overexpressing hnRNP L in normoxia. U937 cells were transfected with either pcDNA3-c-Myc-hnRNP L or pcDNA3 empty vector for 24 hr in normoxia. The overexpression of c-Myc-hnRNP L was confirmed by immunoblotting using anti-c-Myc antibody (Figure 12). Interestingly, after IP with a monoclonal anti-hnRNP L antibody and RT-PCR using specific primers for VEGFA, the 700 nt product was only observed in hnRNP L-overexpressed samples indicating that the cytoplasmic hnRNP L concentration has a role in VEGFA mRNA-hnRNP L association. Neither IP with anti-FLAG tag antibody followed by VEGFA RT-PCR (data not shown) nor IP with anti-hnRNP L antibody followed by RT-PCR using specific primers for GAPDH showed any product, demonstrating the specificity of the result. Since increased levels of cytoplasmic hnRNP L led to VEGFA mRNA/ hnRNP L association, we conclude that hypoxic hnRNP L association with VEGFA mRNA follows the law of mass action. The finding that ectopically expressed hnRNP L binds VEGFA mRNA suggests hypoxia or other hypoxia-inducible factors are not required, but rather the cytoplasmic concentration of hnRNP L determines its association with VEGFA mRNA.

L. Direct role of hnRNP L in overcoming miRNA-mediated repression of VEGFA

Since hnRNP L binds to VEGFA mRNA only in hypoxia and CARE-binding miRNAs target the hnRNP L-binding site on VEGFA mRNA, we next investigated the direct role
of hnRNP L in the modulation of the CARE-binding miRNA activity observed in hypoxia. We took advantage of the previous experiment, which demonstrated that hnRNP L overexpression in normoxia mimics hypoxic conditions and allows hnRNP L association with VEGFA mRNA (Figure 12). We performed luciferase assays in which the CARE-binding miRNA-mediated repression of luciferase reporter bearing hnRNP L-binding site was investigated in the presence of overexpressed hnRNP L. CARE-binding miRNAs were cotransfected with pcDNA3-c-Myc-hnRNP L, pcDNA3 empty vector and luciferase reporter into HEK293T cells and the relative level of luciferase was measured after 24 hr of transfection (Figure 13A). The presence of overexpressed hnRNP L was investigated using anti-c-Myc antibody. Our results indicated that ectopic hnRNP L overrides the miRNA-mediated repression of reporter expression significantly when compared to the control (p>0.09). We then examined whether the overexpressed ectopic hnRNP L is also able to override the miRNA-mediated repression of endogenously expressed VEGFA in human monocytes. We cotransfected pcDNA3-c-Myc-hnRNPL, pcDNA3 empty vectors and CARE-binding miRNAs into normoxic human U937 cells. The endogenous VEGFA level was measured after 24 hr by immunoblotting of total cell lysates using anti-VEGFA antibody and normalized to the miR-control (100%). Our results showed that hnRNP L overexpression relieved the miRNA-mediated repression by 40-60% in normoxic U937 cells as compared to control (p>0.09) (Figure 13B). Based on these data we conclude that hnRNP L has a direct role in alleviating CARE-binding miRNA-mediated repression of VEGFA and cytoplasmic hnRNP L concentration plays a major role in this phenomenon.
Figure 12. Overexpressed hnRNP L binds VEGFA mRNA in normoxia. U937 cells were transfected with pcDNA3-c-Myc-hnRNP L or empty vector (Vect.) under normoxia for 24 hr. Cytosolic lysates were immunoblotted with anti-c-Myc tag and anti-GAPDH antibodies (top two panels). Cytosolic lysates were subjected to IP with anti-hnRNP L monoclonal antibody. Expression of VEGFA and GAPDH mRNAs in lysates (input, left panels) and after IP (right panels) was determined by RT-PCR (bottom two panels) [145].
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IB, anti-c-Myc

IB, anti-GAPDH

B

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IB, anti-c-myc

IB, anti-VEGFA

IB, anti-GAPDH

VEGFA expression (% nmw. control)
Figure 13. hnRNP L relieves inhibition of VEGFA by CARE-binding miRNAs. (A) Overexpression of hnRNP L in normoxia prevents miRNA-mediated repression of HSR-bearing reporter. FLuc reporter bearing the VEGFA HSR was cotransfected into HEK293T cells with CARE-binding miRNAs (or control miRNA, Cont.), pcDNA3-c-Myc-hnRNP L or vector, and RLuc as transfection efficiency control. Lysates were subjected to immunoblot analysis with anti-c-Myc tag (top) and -GAPDH antibodies (middle). The relative level of FLuc was normalized by RLuc expression and expressed as % of control (bottom). (B) Overexpressed hnRNP L alleviates miRNA-mediated repression of endogenous VEGFA in normoxia. U937 cells were cotransfected with pcDNA3-c-Myc-hnRNP L or vector and CARE-binding miRNAs for 24 hr under normoxia. Lysates were subjected to immunoblot analysis with anti-c-Myc tag, VEGFA, and GAPDH antibodies. VEGFA expression was quantitated by densitometry and reported as percent of normoxic control. All quantitative data are expressed as mean ± s.d. for n=3 independent experiments. An asterisk (*) indicates a significant difference, \( P< 0.05 \), two-tailed t-test [145].
M. Effect of hnRNP L downregulation on the activity of CARE-binding miRNAs

We showed that overexpression of hnRNP L in normoxia can modulate CARE-binding miRNA activity to the same effect that we observed in hypoxia. In order to further manipulate the concentration of cytoplasmic hnRNP L, we downregulated hnRNP L expression in hypoxia to see if we can restore the repressive effect of CARE-binding miRNAs on VEGFA expression. We cotransfected miR-299 with siRNA against hnRNP L into U937 cells under both conditions of hypoxia and normoxia and after 24 hr of incubation, the level of VEGFA was measured in the prepared total cell lysates (Figure 14). The downregulation of hnRNP L was tested using immunoblotting with anti-hnRNP L antibody. As expected, the downregulation of hnRNP L in normoxia had no effect on the miR-299-mediated repression of VEGFA. In addition, in the normoxic cells treated with siRNA against hnRNP L only, we did not observe any change in the VEGFA expression level. On the other hand, in the hypoxic samples, downregulation of hnRNP L caused downregulation of VEGFA in the miR-299-transfected cells. Unfortunately, we could not conclude that the downregulation of VEGFA in hypoxic miR-299-transfected cells is due to the release of the hnRNP L effect on modulation of miR-299 activity because in the hypoxic cells treated with siRNA against hnRNP L only, VEGFA expression was downregulated as well. This result is expected as the previously published data showed that hnRNP L plays a major role in VEGFA regulation during hypoxia. Overall the observed effect of hnRNP L in regulation of VEGFA in hypoxia did not allow us to investigate the effect of CARE-binding miRNAs in the absence of hnRNP L during hypoxia.
Figure 14. The effect of hypoxic downregulation of hnRNP L on miRNA-mediated regulation of VEGFA. U937 cells were cotransfected with miR-299, siRNA against hnRNP L, miR-control or siRNA control. After 24 hr transfection, the VEGFA level was measured in total cell lysates using immunoblotting with anti-VEGFA antibody (top panel). The amount of hnRNP L in each treatment was detected using immunoblotting with anti-hnRNP L antibody (second panel from top). GAPDH was used as loading control (third panel from top). The amount of VEGFA expression in each treatment was measured using Image J and compared to the control siRNA/miR-control treatment (100%). All quantitative data are expressed as mean ± s.d. for n=3 independent experiments. An asterisk (*) indicates a significant difference, P< 0.05, two-tailed t-test.
Since both hnRNP L and CARE-binding miRISCs share the same target site and the modulation of miRNA activity by hnRNP L depends on hnRNP L concentration, we propose a mechanism in which hnRNP L and miRISCs compete with each other to bind to their shared target site, CARE. In order to confirm this mechanism, we first wanted to rule out the possibility of hnRNP L interaction with RISC components. We wanted to confirm that the modulation of miRNA activity is not due to interaction of hnRNP L with RISC components and binding of miRISCs and hnRNP L to the CARE is mutually exclusive. Ago proteins, especially Ago 2 are the major components of the mammalian RISC complex [158-160]. In order to study whether hnRNP L can interact with Ago 2, we prepared hypoxic U937 cell lysates and immunoprecipitated hnRNP L and Ago 2 using monoclonal anti-hnRNP L and anti-Ago 2 antibodies respectively. Anti-Flag antibody was used as specificity control. The immunoprecipitated samples were used for immunoblotting to detect the associated hnRNP L in each sample using anti-hnRNP L antibody (Figure 15A). Immunoblotting of anti-Ago 2-immunoprecipitated samples using anti-hnRNP L antibody showed that hnRNP L is not associated with Ago 2. The hnRNP L- and Ago 2-immunodepleted (ID) samples were used also to detect the presence of hnRNP L in each sample (Figure 15B). Only in the Ago 2-immunodepleted samples the presence of hnRNP L was confirmed. Thus, hnRNP L does not appear to interact with RISC indicating that binding of hnRNP L and miRISC to the CARE is mutually exclusive which supports a competition mechanism as ruling the hnRNP L modulation of miRNA activity.
Figure 15. hnRNP L does not interact with the major component of RISC, Ago 2. (A) Total cell lysates from hypoxia-treated U937 cells were immunoprecipitated with monoclonal anti-hnRNP L, anti-Ago 2 and anti-Flag antibodies and then probed with polyclonal anti-hnRNP L antibody using immunoblot analysis. (B) The hnRNP L- and Ago2- immunodepleted lysates were also probed with ant-hnRNP L antibody using immunoblot analysis. GAPDH was used as control.
O. Competition between hnRNP L and CARE-binding miRISCs in hypoxia

Since hnRNP L and CARE-binding miRISCs share the same binding site and binding of hnRNP L and miRISCs to the CARE is mutually exclusive, we suggest a mechanism for the modulation of miRNA by hnRNP L. We hypothesized that there is equilibrium between cytoplasmic hnRNP L, and the CARE-binding miRNAs in normoxia, which is shifted toward the CARE-miRISC interaction. In hypoxic stress, following an increase in the amount of cytoplasmic hnRNP L, due to the law of mass action which is determined by the concentrations of the reactants, hnRNP- L competes with miRISCs in binding to the CARE, and the equilibrium is shifted toward CARE-hnRNP L interaction:

\[
\begin{align*}
\text{Normoxia} & \quad \text{CARE-miRISCs} + \text{hnRNP L} \leftrightarrow \text{CARE-hnRNP L} + \text{miRISCs} \\
\text{Hypoxia} & \quad \text{CARE-miRISCs} + \text{hnRNP L} \leftrightarrow \text{CARE-hnRNP L} + \text{miRISCs}
\end{align*}
\]

Results of the experiments performed in normoxia using hnRNP L overexpression (Figures 13A, B) are in line with our hypothesis that increased amount of hnRNP L shifts the equation toward its interaction with CARE. The miRISC/hnRNP L competition was further established by measuring the activity of our luciferase reporter bearing HSR after 24 hr of transfection of the same amount of HEK 293T cells (2 x 10^6) with the same amount of luciferase reporter (1.0 µg pcDNA3-FLuc-HSR) and with the same amount of miR-299 (100 nM). An hnRNP L titration of 2, 4, 6 and 8 µg pcDNA3-c-Myc-hnRNP L was performed (Figure 16A). The increasing amounts of hnRNP L progressively derepressed miRNA-mediated luciferase expression in HEK293T cells. The hnRNP L
amount was measured using c-Myc antibody. We plotted the amount of hnRNP L-mediated derepression of luciferase activities against the concentration of hnRNP L. The plot followed a hyperbolic curve (Figure 16B). Based on our studies, we propose a model for modulation of CARE-binding miRNA activity by hnRNP L (Figure 17). In this model under normoxic conditions VEGFA is being repressed by CARE-binding miRNAs to maintain its physiological expression level. Upon stress such as hypoxia, the demand for VEGFA increases and the repressive effects of the CARE-binding miRNAs are removed by enhanced cytoplasmic concentration of hnRNP L which competes with the miRNA/RISC in order to bind to the CARE resulting to the increased level of VEGFA.

**P. Modulation of CARE-binding miRNA activity in hypoxic human HEK 293T cells**

In this project, we studied human macrophage-like monocytic U937 cells because of the critical role of VEGFA regulation by TAMs in the hypoxic microenvironment of solid tumors. We were interested to test the global effect of hnRNP L on modulation of CARE-binding miRNA activity during hypoxia. We transfected CARE-binding miRNAs in human HEK293T cells and after 24 hr of incubation in hypoxia or normoxia, we measured the level of VEGFA in total cell lysates (Figure 18). Interestingly, hypoxia was able to modulate the activity of the miRNAs that target hnRNP L binding site the same as human macrophages. We conclude that the regulation of VEGFA expression by modulation of miRNA activity is not limited to macrophages and that many other cell types can also take advantage of this type of VEGFA regulation.
A

Relative luciferase level

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IB: anti-c-Myc antibody

GAPDH

IB: anti-GAPDH antibody
Figure 16. Competition assay. (A) HEK cells were transfected with miR-299, FLuc reporter, RLuc as internal transfection control and different concentrations of vector expressing hnRNP L (top panel). The relative levels of luciferase activity were measured in each treatment (second panel from the top). The amount of hnRNP L expression was detected by immunoblotting using anti-c-Myc antibody. GAPDH was used as loading control (bottom panels). (B) The hnRNP L derepression follows a hyperbolic curve. The relative level of luciferase activity was plotted against the concentrations of vectors expressing hnRNP L from the above experiment.
Figure 17. Schematic showing effects of CARE-binding miRNAs and hnRNP L on VEGFA expression. In normoxia, translation of VEGFA mRNA is negatively regulated by endogenous miRNA/RISC complexes binding to CARE (left). In hypoxia, hnRNP L is translocated to cytoplasm where it binds CARE, prevents miRNA/RISC activity and increases VEGFA expression (right) [145].
Figure 18. Hypoxia prevents miRNA mediated inhibition of VEGFA expression in HEK 293T cells. Cells were incubated with miRNAs under normoxic or hypoxic conditions for 24 hr. Cell lysates were subjected for immunoblot analysis with anti-VEGFA (top) and anti-GAPDH (middle) antibodies. VEGFA was expressed as percent of normoxic control (bottom). Results are expressed as mean sd for three independent experiments. An asterisk * indicates a significant difference P<0.05 two-tailed t-test [145].
Q. Conclusions

Modulation of miRNA activity by RBPs is a newly established mechanism of gene regulation. Several recent reports introduce miRNAs and the RBPs responsible for the modulation of miRNA activity and the phenotypic effects of these modulations. Unfortunately, in none of these reports is the mechanism of modulation established. In the work presented here, we proposed and established a defined mechanism for the modulation of miRNA activity by RBP. We showed for the first time that the regulatory protein binding elements of 3’-UTRs can be a hot spot for being targeted by miRISCs and a competition mechanism driven by the law of mass action determines gene expression levels. In our example, the regulatory CARE element on the 3’-UTR of VEGFA, a critical regulator of angiogenesis, is targeted by both hnRNP L, an RBP involved in VEGFA mRNA stabilization and miRISCs including miR-297, -299, -567 and -609.

Our studies exhibit several important findings regarding VEGFA regulation. First, we added a number of new miRNAs to the small list of anti-angiogenic miRNAs. Second, we established a new role for hnRNP L as a modulator of miRNA activity that controls VEGFA expression. Third, we discovered a new mechanism of VEGFA regulation established by an interplay between miRNAs and RBP. Last, the modulation of miRNA activity occurs only during hypoxic stress, the feature of neoplastic microenvironment, where the amount of cytoplasmic hnRNP L in human macrophages that play a critical role in tumor progression and metastasis increases and competes away the repressive effect of miRNAs targeting the hnRNP L-binding site. VEGFA produced by these cells cannot be compensated by VEGFA from other cell sources within the tumor.
The discovery of CARE-binding miRNAs that target and repress VEGFA and the mechanism of their modulation of activity by hnRNP L can contribute to provide miRNA-based anticancer therapeutic tools. Over-expression of CARE-binding miRNAs strongly inhibits VEGFA expression. VEGFA is the principle agonist of angiogenesis and cancer cells hijack VEGFA to promote tumor vascularization and growth. Reduction of VEGFA level or action by VEGFA or VEGFA receptor antagonists has been successfully applied in cancer therapy. These conventional anti-VEGFA therapies target either circulating protein or cell surface receptors. In contrast, therapies using chemically stabilized miRNAs target VEGFA at the level of intracellular synthesis. This approach can be combined with cell-type specific targeting strategies to minimize the adverse systemic consequences of VEGFA reduction including vascular disturbance and regression of blood vessels. Since modulation of the activity of CARE-binding miRNAs by hnRNP L follows the rule of mass action which is determined by the concentration of the reagents, we propose that the target specific administration of these miRNAs can compete away hnRNP L binding to VEGFA in macrophages in hypoxic area of solid tumors. These miRNAs can be more effective miRNA candidates to be used for cancer therapies. In the hypoxic microenvironment of solid tumors, tumor associated macrophages induce VEGFA expression leading to tumor progression and metastasis. By binding to CARE of VEGFA 3’-UTR, hnRNP L is one of the critical proteins involved in VEGFA induction. Administration of CARE-binding miRNAs combined with cell-type specific targeting strategies that target TAMs can reduce the amount of VEGFA very effectively in these critical cells that play a major role in tumor progression and metastasis. The overall effect of VEGFA reduction by these miRNAs is much higher
than other miRNAs targeting VEGFA because not only will they repress the VEGFA protein level but also they prohibit binding of hnRNP L to the VEGFA mRNA.

Also, our studies revealed some new facts regarding hnRNP L expression and shuttling during stress. We showed that hnRNP L expression does not upregulate in stress, rather its cellular localization changes. In normoxia, hnRNP L is highly localized in nucleus and is interacting with VEGFA pre-mRNA only, indicating that it is involved in the VEGFA splicing process. The concentration of hnRNP L in the cytoplasm is low in normoxia. Upon hypoxic stress, hnRNP L, independent of VEGFA mRNA, shuttles highly to the cytoplasm and its cytoplasmic concentration increases by 3 to 4 fold.

In the following chapter, I will present evidence regarding the biological relevance of CARE-binding miRNAs.
IV. Characteristics and Physiological significance of CARE-binding miRNAs

*A part of this chapter has been published in the EMBO Journal [145].*

As mentioned above, overexpression of CARE-binding miRNAs downregulates the endogenous expression of VEGFA in human monocytes. This effect was inhibited in stress condition of hypoxia because of hnRNP L mobilization into the cytoplasmic fractions and hnRNP L association with VEGFA mRNA. The previous chapter focused on the stress-dependent modulation of CARE-binding miRNA activity and the involved mechanism. This chapter focuses on the characteristics and physiological relevance of CARE-binding miRNAs.

*A. Mechanism of VEGFA repression by CARE-binding miRNAs*

miRNAs can downregulate gene expression by either of two post-transcriptional mechanisms: mRNA cleavage or translational repression [10]. We examined the mechanism by which CARE-binding miRNAs downregulate the expression of their target. The effect of CARE-binding miRNAs on the endogenous mRNA level of VEGFA in U937 cells was investigated by quantitative real-time RT-PCR. We hypothesized that if any of the CARE-binding miRNAs downregulates VEGFA expression through mRNA cleavage, we would expect to measure a decrease in VEGFA mRNA level after 24 hr of miRNA transfection when compared to the control. Our data revealed that the mRNA level of VEGFA did not change after 24 hr of miRNA transfection while the protein level was reduced remarkably (Figure 19). These results are in line with recent studies.
Figure 19. CARE-binding miRNAs do not inhibit $VEGFA$ mRNA expression. After transfection with miRNAs for 24 hr, $VEGFA$ mRNA amount was measured by real-time, quantitative RT-PCR and normalized to $GAPDH$ mRNA. Relative $VEGFA$ mRNA levels of the above samples were measured after total RNA extraction with real time quantitative RT-PCR using $2^{-\Delta CT}$ method in which $\Delta C_T = C_T (VEGFA) - C_T (GAPDH)$ [145].
demonstrating that some targets of miRNAs are repressed without detectable changes in mRNA levels, while other targets show mostly mRNA destabilization [69]. To examine whether the CARE-binding miRNA inhibitory effect on its target is at the level of translation, a polysome profile analysis was performed. The association of luciferase mRNAs bearing a HSR 3’-UTR with polysomes after 24 hr of CARE-binding miRNA transfection was investigated in human HEK293T cells. Our results indicated that the repressive effect of CARE-binding miRNAs on the expression of luciferase reporter bearing VEGFA HSR is due to a translational repression mechanism. Briefly, after 24 hr of transfection, cell lysates were fractionated by sucrose gradient to separate large, rapidly translating polysome fractions from small, non-translating ribonucleoprotein fractions (Figure 20). RT-PCR analysis using FLuc specific primers showed that CARE-binding miRNA, miR-299, completely shifted the reporter from the rapidly translating to the non-translating fractions whereas miR-control did not have any effect. GAPDH mRNA was primarily in the translating fractions and was unaffected by miR-299. Altogether, our studies show that CARE-binding miRNAs have no or possibly only a minimal effect on mRNA decay and their repressive activities are mostly due to translational repression.

B. Endogenous expression of CARE-binding miRNAs in human monocyte-derived macrophages

In order to study the physiological role of CARE-binding miRNAs in human monocytes,
Figure 20. CARE-binding miRNA repression is mediated by translational repression.
Sucrose density gradient fractionation of lysates from HEK cells transfected with luciferase reporters bearing VEGFA HSR and either miR-299 (right panel) or miR-control (left panel) for 24 hr. Lysates were subjected to sucrose density gradient fractionation and RNA was monitored by absorption at 254 nm (top). RNA isolated from each fraction was subjected to RT-PCR to determine FLuc (middle) and GAPDH (bottom) mRNAs [145].
we first investigated whether any of these miRNAs are endogenously expressed in monocytes. The presence of CARE-binding miRNAs in three different monocytic cells, U937, THP1 and PBM were investigated by northern blot. miR-297 and -299 were detected in all three monocytic cell types (Figure 21) while miR-567 and -609 were not detected (data not shown).

C. The effect of hypoxic stress on the expression levels of CARE-binding miRNAs in human monocytes

We wanted to quantitatively measure the expression level of CARE-binding miRNAs in human monocytes and study if hypoxic stress has any effect on the level of these miRNAs. Since our previous studies revealed that the ectopically introduced CARE-binding miRNAs are not able to downregulate the VEGFA expression during hypoxia, we were also interested in studying whether the expression of these miRNAs is downregulated by hypoxia so that they are not able to have any effect on VEGFA expression. The relative endogenous levels of these miRNAs were measured by taqman microRNA quantitative real time RT-PCR and reported based on ΔC_T method [161] using miR-17-5p as the normalizer [141]. In order to study the effect of stress on the expression levels of these miRNAs, the relative expression levels were measured in both conditions of hypoxia and normoxia after 24 hr of treatments (Figure 22A). The data revealed that consistent with our previous Northern blot analysis, only miR-297 and -299 are expressed in monocytes and their expression levels are relatively low. Furthermore,
Figure 21. Presence of CARE-binding miRNAs in monocytes. miR-297 and -299 are endogenously expressed in monocyctic cells. Total small RNAs were isolated from human U937 cells, THP1 cells, and PBM under normoxic condition. RNA was subjected to Northern blot analysis using probes against miR-297 (top), miR-299, (middle), and U6 as control [145].
Figure 22. Hypoxia does not alter the expression levels of CARE-binding miRNAs. (A) miRNA amounts of 24 hr normoxia and hypoxia treated U937 cells (left) or PBM (right) were quantitatively determined by real-time quantitative RT-PCR after isolation of total small RNAs. miR-17-5p was used as normalizer. (B) U937 cells were incubated under conditions of normoxia (Nmx.) or hypoxia (Hpx.) for 24 hr and miR-297 and -299 were determined by Northern blot using LNA oligomer probes and U6 probe as loading control. All quantitative data are expressed as mean ± s.d. for n=3 independent experiments [145].
hypoxia did not alter the expression levels of these miRNAs. The hypoxic expression of these miRNAs was further confirmed using Northern blot (Figure 22B). The fact that miR-299 and -297 expression levels remain unchanged during stress indicates that the derepression of VEGFA in hypoxia is not due to downregulated CARE-binding miRNAs. The fact that these miRNAs are endogenously expressed and their expression levels are relatively low suggests that these miRNAs might be involved in tuning of VEGFA expression to maintain VEGFA at a required physiological level. Altogether, these data indicate that since miR-297 and -299 are endogenously expressed in human monocytes, they may have a physiological role in regulating VEGFA expression or other mRNAs. Our results also confirm that the observed derepression of VEGFA in hypoxia is not due to stress-dependent CARE-binding miRNA downregulation.

D. Negative regulation of VEGFA by endogenous CARE-binding miRNAs

Given that CARE-binding miRNAs are expressed endogenously in human monocytes and are able to downregulate VEGFA when introduced into cells, it is of interest to investigate whether these endogenous miRNAs have any role in regulation of endogenous VEGFA in human monocytes. To determine the effect of U937 endogenous CARE-binding miRNAs on VEGFA expression, U937 cells were cotransfected with FLuc reporter RNA bearing the *VEGFA* HSR and chemically stabilized anti-miR-297 and -299. The inhibition of miRNAs was tested using Northern blot (data not shown). FLuc expression was nearly doubled by both anti-miRNAs, but not by the mutants and random control showing the specificity of the results and suggesting that these endogenous
miRNAs negatively regulate reporter expression (Figure 23A). To investigate the role of endogenous miRNAs on endogenous gene expression, anti-miR-297 and -299 were transfected into U937 cells, and VEGFA in cell lysates was measured by immunoblot after 24 hr. A modest 35-50% increase in VEGFA expression was observed after miRNA inhibition (Figure 23B). Cotransfection of both anti-miRNAs did not increase VEGFA level more than individual transfection of either miRNA. This may indicate that these two miRNAs do not have a cooperative function. To show the specificity of this observation, the increased level of VEGFA in the absence of these miRNAs was further measured in a set of treatments which we transfected U937 cells with the anti-miRNA mutants. A maximum 50% increase was observed in the absence of miR-299 and -297 but not in the mutant anti-miRNAs (Figure 23C). VEGFA is a secretory protein, and thus measurement in a cell lysate reflects the relative expression at a single point in time. To assess the time-integrated effect of microRNAs on VEGFA expression, we also measured the level released into the medium which is also the primary site of VEGFA function. Transfection with either anti-miR-297 or -299 or both induced nearly a 2-fold increase in VEGFA in the 24 hr conditioned medium but there was no change in the amount of secretory VEGFA from the cells treated with mutant anti-miRNAs (Figure 23D, E). These results reveal a physiological role of endogenous miR-297 and -299 in negative regulation of VEGFA in monocytic cells. This discovery has physiological and pathophysiological implications. VEGFA is the principal agonist of angiogenesis and is essential for blood vessel formation during development and tissue repair [162]. VEGFA also induces vessel permeability and leukocyte chemoattraction, events associated with chronic inflammation [162]. Cancer cells “hijack” VEGFA to promote tumor vascularization and growth.
Figure 23. Regulation of VEGFA by endogenous miR-297 and -299. (A) Endogenous miR-297 and -299 negatively regulate expression of HSR-bearing reporter. FLuc reporter upstream of the HSR was cotransfected with anti-miR-297, -299, control or mutant anti-miRNAs (mutations in the 2\textsuperscript{nd} and 3\textsuperscript{rd} nucleotides of the seed regions to test the specificity of the results) and with RLuc-expressing vector pRL-SV40 as control for transfection efficiency. After 24 hr, the relative Luc levels were determined, and expressed as fold change compared to random control [145]. (B) Endogenous miR-297 and -299 negatively regulate endogenous VEGFA expression. U937 cells were transfected with anti-miR-297, -299, or both. A random anti-miRNA used as control. Cell lysates were subjected to immunoblot analysis with anti-VEGFA (top) and -GAPDH (middle) antibodies. The amount of VEGFA was expressed as % of random control (bottom). (C) The same experiment as B but we also used two anti-miRNA mutants
(mutations in the 2\textsuperscript{nd} and 3\textsuperscript{rd} nucleotides of the seed regions) to test the specificity of the results [145]. (D) miR-297 and -299 negatively regulate VEGFA secretion. Cells were transfected as in B and conditioned medium concentrated by IP with polyclonal anti-VEGFA antibody, immunoblotted with monoclonal anti-VEGFA antibody (top) and quantitated by densitometry (bottom) using Image J. (E) The same experiment as (D) but we also measured the secretory VEGFA in mutant anti-miRNA treated cells [145]. All quantitative data are expressed as mean ± s.d. for \( n=3 \) independent experiments. An asterisk (*) indicates a significant difference \( P<0.05 \), two-tailed t-test.
Interestingly, recent studies point to the critical importance of VEGFA produced by tumor-associated macrophages for throwing the “angiogenic switch” that induces tumor angiogenesis during hypoxia [85, 163]. Overexpression of miR-297, -299, -567, and -609 all cause robust inhibition of VEGFA expression. However, the increase in VEGFA expression observed upon inhibition of endogenous miR-297 and -299 was more modest, and consistent with a "tuning" function of the miRNAs rather than an all-or-none response. Recent studies in vertebrates suggests that this type of regulation by miRNAs may be the norm rather than the exception [71]. Experimental criteria for a tuning relationship between a miRNA and its respective target have been suggested: the miRNA and the protein product of the target mRNA must both be present in the cell, and both up- and down-regulation of the respective target protein must be detrimental [71]. Certainly, the regulation of VEGFA by miR-297 and -299 fulfills these characteristics. Both of the miRNAs as well as VEGFA mRNA and protein are constitutive in monocytic cells. Accumulating evidence suggests that VEGFA expression must be regulated within a relatively narrow range. Deletion of a single VEGFA allele causes abnormal blood vessel formation and mid-gestational lethality in mice [164]. In contrast, modest overexpression induces aberrant vasculogenesis and heart development, and ultimately, embryonic lethality [165]. In adult human (and mice), elevated serum VEGFA is associated with a lethal hepatic syndrome [166]. Interestingly, although reduction of VEGFA level or action by VEGFA or VEGFA receptor antagonists has been successfully applied in cancer therapy, the same repression can cause vascular disturbance, regression of blood vessels, and an array of severe side effects that hamper its clinical applicability [167]. Moreover, deletion of macrophage-derived VEGFA results in accelerated tumor growth
These observations strongly indicate that VEGFA must be subject to tight bidirectional regulation. Negative regulation by miRNAs may contribute to management of VEGFA dosage within the optimal range by providing a negative regulatory effect that partially counterbalances positive transcriptional and post-transcriptional regulatory mechanisms.

E. Endogenous expression of CARE-binding miRNAs in different cell types and the effect of hypoxia on the expression levels of these miRNAs

We know very little about CARE-binding miRNAs. miR-299 is upregulated in acute myeloid leukemia (AML), the most common acute leukemia in adults [169]. It is one of the miRNA signatures that predict the estrogen receptor (ER) status of breast cancer patients [170] and is highly expressed in fetal human lungs [171]. miR-297 is upregulated in CD4+ T cells [182] and in mammalian embryo when trophectoderm is specified [183]. miR-567 is downregulated in gastric cancer [184]. There is no report for miR-609.

In order to study the global role of CARE-binding miRNAs in VEGFA regulation, the endogenous level of these miRNAs in different cell types was investigated. In our study we have focused on human macrophage-like monocytes including U937, THP1 and PBM because of their important role in tumor progression and metastasis mediated by VEGFA regulation. Since VEGFA is a very important angiogenic factor and can be downregulated by endogenously expressed CARE-binding miRNAs, it is tempting to investigate whether these miRNAs are endogenously expressed in other cell types as well. The endogenous levels of CARE-binding miRNAs under both conditions of
normoxic and hypoxic stress were analyzed by quantitative real time RT-PCR taqman microRNA assay. Several human cell lines including HepG2 cells (human liver hepatocellular carcinoma cell line), HEK293T cells (human embryonic kidney 293 cells), A375 cells (human amelanotic melanoma cell line), HUVEC (human umbilical vein endothelial cells) were investigated after 24 hr of incubation in normoxia or hypoxia (Figure 24). The relative endogenous levels of miRNAs were reported based on $2^{-\Delta CT}$ [161] in which $\Delta C_T = C_T (\text{miRNA}) - C_T (\text{reference})$. We used miR-17-5p which ubiquitously expressed in all cell lines as the reference (normalizer) [143]. Our data indicated that miR-297 is expressed in all cell lines tested at a relatively high level, miR-567 is expressed in all cell lines tested at a relatively low level, miR-299 is only expressed at a relatively low level in HUVEC cells and miR-609 did not show any significant expression in any cell lines that I tested. The expression levels of these miRNAs did not change significantly in hypoxia when compared to normoxia (p< 0.05, two-tailed t-test).

In conclusion, we showed that three of four CARE-binding miRNAs are expressed in a variety of cell types. The endogenous expression of CARE-binding miRNAs in several cell types indicates that these miRNAs may have a broad role in tuning of VEGFA regulation which maintains VEGFA level at the physiological range.

F. Identification of CARE-binding miRNA candidates that target other known hnRNP L-binding sites of specific mRNAs

Previous reports have identified several mRNAs whose stability can be regulated by hnRNP L. Here we employed three different software programs (TargetScan, MicroCosm
A

Relative expression

HepG2

- Nmx.
- Hpx.

miRNA

miR-297 miR-567 miR-299 miR-609

B

Relative expression

HEK 293T

- Nmx.
- Hpx.

miRNA

miR-297 miR-567 miR-299 miR-609
C

Relative expression

D

Relative expression

miRNA

miRNA

A375

Nmx.

Hpx.

HUVEC

Nmx.

Hpx.

miR-297  miR-567  miR-299  miR-609

miR-297  miR-567  miR-299  miR-609
Figure 24. CARE-binding miRNAs are expressed in different cell types and their expression levels remain unchanged in hypoxia. (A) HepG2, (B) HEK293T, (C) A375, (D) HUVEC were incubated in either hypoxia (Hpx.) or normoxia (Nmx.) for 24 hr and total small RNAs were isolated. 10 ng RNA was used for quantitative RT-PCR using taqman microRNA assay kit. miR-17-5p was used as normalizer. The relative expression level was measured using $2^{-\Delta C_T}$ in which $\Delta C_T = C_T$ of miRNA- $C_T$ of miR-17-5p. The error bars are SD of three independent experiments.
of the reported mRNAs. These data demonstrate that the hnRNP L-binding site of other mRNAs can also share the same binding site with miRNAs and suggest that modulation of miRNA activity by hnRNP L may occur in other specific mRNAs as well. Several reports have identified mRNAs with possible hnRNP L-binding sites including SLC2A1 (Glut-1)[172-173]; CD40 LG (CD154) [174]; BCL2 [175] and NOS2A (iNOS) [176]. Solute carrier family 2 (facilitated glucose transporter member 1) (SLC2A1) or Glut-1 encodes a major glucose transporter in the mammalian blood-brain barrier. Mutations in this gene have been found in a family with paroxysmal exertion-induced dyskinesia. Following stress such as hypoxia or hypoglycemia, Glut-1 expression increases which is largely mediated through increased mRNA stability [177]. It has been shown that Glut-1 has two major 3’-UTR RNA binding proteins including hnRNP A2 and hnRNP L that independently bind to this region [173]. The hnRNP L-binding site of human Glut-1 3’-UTR (nt 2192-2211) is highly homologous (70% identity) to the hnRNP L binding site of human VEGFA 3’-UTR which is involved in VEGFA mRNA stability [109]. We used three different software programs to identify miRNA candidates targeting the hnRNP L binding site of Glut-1 mRNA (Figure 25). Among these, TargetScan was able to detect three candidates including miR-140-5p and -876-3p (7mer-m8) that are broadly conserved among vertebrates and miR-1225-5p (7mer-1A) that is only conserved in human. CD154 or CD40 ligand (CD40LG) is expressed on the surface of T cells. It regulates B cell function by engaging CD40 on the B cell surface. A defect in this gene results in an inability to undergo immunoglobulin class switches and is associated with hyper-IgM syndrome. CD154 mRNA is unstable and its 3’-UTR contains dual cis-acting
Figure 25. miRNA candidates that target hnRNP L binding site of human Glut-1 3’-UTR. (A) 3’-UTR of Glut-1 consists of two independent protein binding sequences, hnRNP A2 (the light gray) and hnRNP L (the dark gray). Locations of the seed regions of the miRNAs that target hnRNP L binding site are depicted. (B) the sequence of miRNA candidates and sequence of their binding sites on Glut-1 3’-UTR as well as their exact positions are depicted as predicted by TargetScan.
elements [174]. Previous reports mapped the mRNA instability activity in the human CD154 3’- UTR to a 330-nt region containing a CU-rich element (CURE) and a CA-rich element (CARE) [178-179]. Further studies using the conserved mouse CD154 3’-UTR revealed that CURE binds to polypyrimidine tract binding protein (PTB) and functions as cytoplasmic mRNA instability element while CARE binds to hnRNP L and functions as an independent regulatory element that enhanced cytoplasmic mRNA stability [174].

miRanda detected four candidates including miR- 574-5p, -149, -760 and -345 that target the hnRNP L binding site of CD154 3’-UTR (Figure 26).

Nitric oxide synthase 2 (NOS2) or inducible iNOS is expressed in liver and is inducible by a combination of lipopolysaccharide and certain cytokines. Nitric oxide is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities. Murine iNOS mRNA degradation is prompted upon binding of hnRNP I and hnRNP L to a destabilizing region within its 3’-UTR. They bind at two distinct regions of the 3’-UTR. Inflammatory stimuli cause dissociation of the protein-mRNA complex and yield a more stable transcript resulting in sustained iNOS production. We used blast 2 software to find the identical region of the hnRNP L binding site in the human iNOS 3’-UTR. Nucleotides 419 to 442 of human iNOS 3’-UTR are 94% identical to the murine sequence. We then employed the above three software programs to identify miRNAs that target the hnRNP L binding site of human iNOS 3’-UTR. TargetScan predicted four miRNAs including miR-7 (7mer- m8) which is conserved among vertebrates, miR-485-3p (7mer-m8), miR-487a (7mer-m8) and miR-136 (7mer-1A) that are all conserved only in human (Figure 27).
Figure 26. CA154 3’-UTR protein binding elements and miRNAs that target hnRNP-L binding site. (A) CURE and CARE elements of CD154 3’-UTR are depicted and the miRNAs targeting CARE region were shown. (B) miRanda was able to predict four miRNA target sites for the CARE region. The sequence of miRNA candidates and sequence of their binding sites on CA154 3’-UTR as well as their exact positions are depicted.
Figure 27. iNOS 3’-UTR hnRNP L binding element and the miRNAs that target hnRNP L-binding site. (A) hnRNP L-binding site of human iNos 3’-UTR detected by blast 2 using the known murine hnRNP L binding site and the miRNAs targeting the element. (B) miRNAs predicted by TargetScan and MicroCosm Targets. The sequence of miRNAs and the sequence of their binding sites are depicted.
Microcosm targets also predicted miR-487a and miR-485-3p same as TargetScan as well as miR-154 (nt 412-433), miR-494 (nt 414-435) and miR-376a (nt 411-432).

BCL2 (B-Cell/lymphoma 2) encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes. Expression of BCL2 has been shown to confer cellular resistance to several anticancer drugs [180]. The CA repeats of BCL2 mRNA interact with hnRNP L and participate in destabilizing BCL2 mRNA [175]. TargetScan predicted three miRNAs including miR-1259 (7mer-1A), miR-147 (7mer-1A) and miR-569 (7mer-1A) that are conserved in human. miRanda predicted two miRNAs including miR-574(nt 139-161) and miR-7(nt 157-170) (Figure 28).

G. Conclusions

In chapter III, we showed that the activities of CARE-binding miRNAs are modulated by hnRNP L focusing on the effect of ectopic miRNAs. In chapter IV, we wanted to study if any of these miRNAs are endogenously expressed and if they can have a physiological impact on VEGFA regulation.

Previous reports showed the significance of VEGFA secretion by TAM-associated macrophages in the hypoxic microenvironment of a tumor. We showed that CARE-binding miRNAs, miR-297 and miR-299 are endogenously expressed in human peripheral blood monocytes and human monocyctic cell lines U937 and THP1 at relatively low levels and negatively regulate VEGFA expression. Their expression levels are not altered by stress. They are expressed in different cell lines and are not limited to monocytes so they may have a broad effect in negatively controlling VEGFA expression.
Figure 28. BCL2 3’-UTR hnRNP L binding element and the miRNAs that target hnRNP L-binding site. (A) hnRNP L-binding site of human BCL2 3’-UTR and the miRNAs targeting the element. (B) miRNAs predicted by targetScan and miRanda. The sequence of miRNAs and the sequence of their binding sites are depicted.
and maintaining VEGFA level at a proper physiological level. There might be more mRNAs that are regulated by an interplay between CARE-binding miRNAs and hnRNP L as predicted by computer prediction programs. CARE-binding miRNAs may be considered as important global regulators of gene expression where their activities can be modulated by hnRNP L.
V. Future directions

There are several questions that are raised by these studies. These questions can be divided into those that relate to physiological and pathological role of CARE-binding miRNAs in vivo and those that relate to investigating new transcripts that their expressions are influenced by interplay between hnRNP L and miRNAs and in a broad spectrum finding a transcriptome influenced by RBP-miRNA manipulations. Answering these questions will add to our knowledge of complex mechanisms of gene regulation and may permit development of novel treatments for human diseases.

A. What is the physiological role of CARE-binding miRNAs in vivo?

One question stemming from the cellular studies of endogenous levels of CARE-binding miRNAs and their effect on the endogenous level of VEGFA is the in vivo physiological importance of these miRNAs. The CARE element of VEGFA is conserved between human and mouse and the CARE-binding miRNAs, miR-297 and -299 target the mouse CARE element as well. These miRNAs are endogenously expressed in mouse monocytic Raw 264.7 cells (data not shown). Mouse knockouts are very important for studying the role of a gene in pathogenesis and studying the miR-297 or -299-knockout mice will provide us precious information regarding the physiological impact of a specific miRNA in tuning VEGFA expression which is one of the most important angiogenic proteins. Negative regulation by miRNAs may contribute to management of VEGFA dosage within the optimal range by providing a negative regulatory effect that partially counterbalances positive transcriptional and post-transcriptional regulatory mechanisms. These in vivo studies may provide a solution to the VEGFA dosage control problem.
B. What is the therapeutic potential of CARE-binding miRNAs?

Because of their negative regulatory effect on human VEGFA, CARE-binding miRNAs can be considered as tumor-suppressing-miRNAs or anti-angiogenic miRNAs and they may indeed have anti-cancer therapeutic potential. Recent studies indicate that miRNA replacement therapies hold great promise [181]. Since binding of these miRNAs and hnRNP L to the CARE element of VEGFA mRNA follows the law of mass action, one would expect that targeted delivery of these miRNAs in the hypoxic microenvironment of tumors could have powerful anti-tumorigenic activity. The targeted delivery of these miRNAs which results to localized high concentration of miRNAs, not only directly reduce VEGFA level by their canonical action but also by binding to hnRNP L-binding site, prohibit hnRNP L accessibility to its target which results in VEGFA mRNA destabilization. Future in vivo studies with systemic administration of these miRNAs using adeno-associated virus (AAV) [181] can reveal the significance of CARE-binding miRNAs as a powerful anti-cancer therapeutic tool.

C. What is the global role of hnRNP L in the modulation of miRNA activity?

Our preliminary studies revealed that hnRNP L-binding sites of other transcripts such as Glut-1 and CD154 mRNAs are also targeted by several predicted miRNA candidates. To broaden the novel role of hnRNP L in the modulation of miRNA activities and its global effect, our future plan will contain the target validation of these miRNAs using the luciferase reporter assay and gain of function studies and investigation of their activities.
modulated by hnRNP L. These studies will help us to establish a global role for hnRNP L as a modulator of the activity of miRNAs that target its binding site.

D. The global analysis of all transcripts that contain shared binding sites for RBPs and miRNAs

Our studies showed that hnRNP L-binding site is a hot target spot for miRNAs that can become a resistant miRNA target site due to cell stress. Because both RBPs and miRNAs that interact with mRNA 3’-UTRs are diverse and abundant, we can anticipate that many examples of this type of complex regulation are yet to be found. Our future goal is to globally identify the mRNA 3’-UTR target sites shared by both miRNAs and RBPs and to establish a transcriptome for all mRNAs regulated by a cross-reaction between miRISCs and RBPS targeting the same binding site. These studies will help to solve another layer of post-transcriptional gene regulatory complexities.

E. What are the roles of RNA recognition motifs of hnRNP L?

RBPs contain RNA recognition motifs (RRMs) that are involved in RNA-protein and protein-protein interactions. Previous studies revealed that hnRNP L has four RRMs. We will be studying the role of each RRM in the regulation of VEGFA expression. hnRNP L binds with high affinity to the CARE in the VEGFA-3’UTR and is one of the critical factors in VEGFA regulation under hypoxia. These studies will provide us the minimal
motifs required for hnRNP L functionality as a VEGFA mRNA stabilizer and as a modulator of miRNA activity.

F. Summary

There are still many things to learn about the modulation of miRNA activity by RBPs. In summary, our future directions will provide us precious information regarding the complex and meanwhile elegant newly established mechanism of gene regulation performed by cross-reactions between miRNAs and RBPs. We will test the potential of using miRNAs targeting RNA-binding site of a protein involved in cancer and suggest that these miRNAs may be more effective candidates for application of cancer therapeutic microRNA deliveries.
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