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The role of microRNA alterations in post-ischemic neovascularization

Kwast, R.V.C.T. van der

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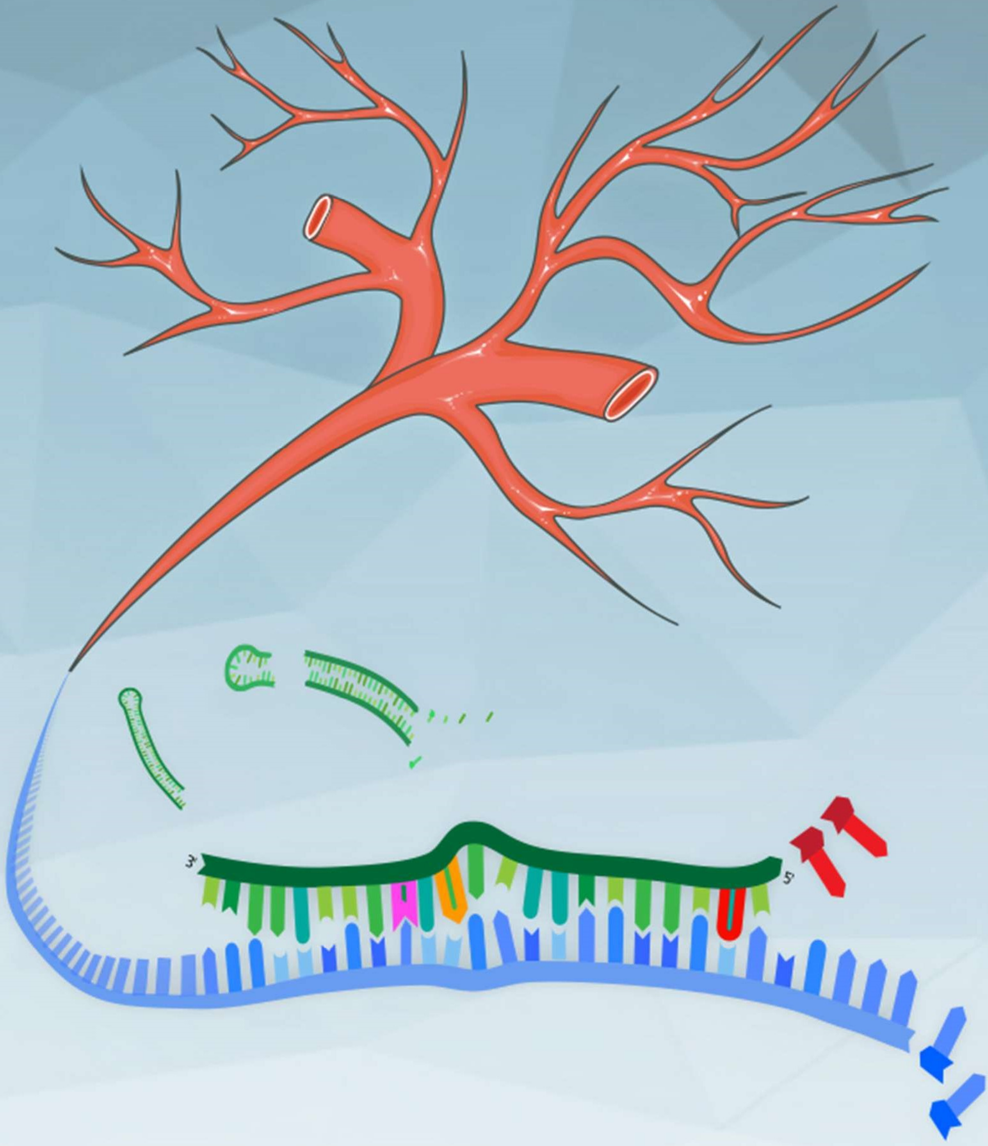

CHAPTER 6

Hypoxia induces of N6-methyladenosine modifications in vasoactive microRNAs

Reginald V.C.T. van der Kwast

Daphne A.L. van den Homberg

Paul H.A. Quax

A. Yaël Nossent

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ABSTRACT

N-6-methyladenosine (m6A) is an abundant RNA modification which was shown to regulate mRNA expression and splicing during ischemia and hypoxia caused by cardiovascular disease. MicroRNAs play an important role in regulating protein synthesis and m6A methylation of microRNAs can alter their functionality. However, the prevalence of microRNA m6A vascular cells and whether microRNA m6A is be dynamically regulated, remains unknown.

In this study, we studied vasoactive microRNA m6A methylation in vascular fibroblasts and examined the effect of hypoxia on microRNAs methylation. Of the 19 microRNAs investigated, a least 16 were highly enriched after immunoprecipitation of m6A containing RNAs from primary human arterial fibroblasts and a human fibroblast cell-line, suggesting vasoactive microRNAs are commonly m6A methylated in fibroblasts. Interestingly, at least 7 of the 19 microRNAs had the opposite methylation status in fibroblasts when compared to a previous study using HEK293 cells, suggesting that, like microRNA expression itself, microRNA m6A methylation can be cell-type specific. More importantly, we found that mature microRNA m6A levels increased upon subjecting cells to hypoxia. The hypoxia-induced increase of m6A did not appear to be due to changes in transcription of m6A-writer METTL3, cofactor WTAP or eraser ALKBH5, since, METTL3 and WTAP expression did not change and ALKBH5 mRNA expression even increased under hypoxia. Furthermore, knockdown of these m6A-associated proteins affected the expression of only a few mature or precursor microRNAs, suggesting m6A plays a more microRNA-specific role in microRNA biogenesis than previously thought.

In conclusion, our findings demonstrate that microRNA m6A methylation can be dynamically regulated under pathological conditions, such as hypoxia. While the mechanisms that mediate dynamic regulation of microRNA m6A require further investigation, changes in microRNA m6A could potentially impact microRNA functionality.

INTRODUCTION

Cardiovascular diseases are the leading cause of death worldwide.[1] Cardiovascular diseases have diverse and complex pathologies. In general, however, they all result in local shortages in the blood supply, known as ischemia, due to an arterial occlusion for example. Ischemia causes the affected tissues to become starved of oxygen and nutrients, which lead to tissue death if left unresolved.[1,2] The cellular response to oxygen starvation, or hypoxia, stimulates the growth of blood vessels towards the ischemic tissue, referred to as angiogenesis, which can help restore blood flow to ischemic tissues.[3]

During the last decade, microRNAs were shown to play an important role in cardiovascular disease and the cellular response to hypoxia.[4] MicroRNAs are a class of short (~22 nucleotides) non-coding RNA molecules, which inhibit translation of mRNAs into proteins. Like all RNAs, microRNAs are subject to post-transcriptional modifications.[5] One of the most abundant RNA modifications in eukaryotic cells is the conversion of adenosine into N^6-methyladenosine (m6A). m6A is a reversible chemical modification which is regulated by methylating and demethylating enzymes.[6] The m6A 'writer' complex, composed of METTL3 and METTL14 (Methyltransferase Like 3 and 14), methylates nuclear RNAs in mammalian cells.[7] WTAP (Wilms' tumor 1-associating protein) is one of several cofactors which can interact with the writer complex to guide the complex to specific RNA-targets.[8] m6A sites can also be demethylated by 'erasers' ALKBH5 (alkB homolog 5) and FTO (fat mass and obesity-associated protein), which allows for more dynamic regulation of m6A compared to most other RNA modifications.[9,10]

Most of the m6A research has been focused on methylation of mRNAs and other RNAs much longer than microRNAs. These studies have shown that m6A has important biological functions and is important for maintaining cardiovascular homeostasis.[11] Knockdown of m6A was shown to result in maladaptive cardiac remodelling and signs of heart failure.[12] However, in failing mammalian hearts and in hypoxic cardiomyocytes m6A is increased, which leads to cardiac hyperthrophy.[13] These findings indicate that m6A plays an important role for m6A in cardiovascular disease and the response to hypoxia.

Recently, several studies have demonstrated that m6A is important for microRNA biogenesis. Alarcon *et al* found that the primary transcript from a microRNA gene, the pri-miR, often contains METTL3-dependent m6A.[14,15] Knockdown of METTL3 lead to a global reduction of mature miRNA expression and concomitant accumulation of unprocessed pri-miRs, whereas overexpression of METTL3 lead to an increase in miRNA maturation. Therefore, m6A appears to promote the maturation of pri-miRs to mature microRNAs.

Berulava *et al* recently used m6A-specific immunoprecipitation to demonstrate that hundreds of mature microRNAs actually contain m6A in HEK293 cells, a human embryonic kidney cell line.[16] Such m6A methylation of mature microRNAs can have functional implications, since studies have shown that m6A methylated mature microRNAs can have decreased mRNA silencing activity because m6A blocks noncanonical A:G base pairing.[17-19] Interestingly, several microRNAs with a known vasoactive function were found to be methylated in HEK293 cells by Berulava *et al*.[16] However, whether these mature vasoactive microRNAs are also subject to m6A methylation in other cell types is unknown. Furthermore, it is also unknown if microRNA m6A methylation is dynamically regulated in response to hypoxic conditions, similar to mRNA m6A methylation.

Therefore, in this study we selected a number of vasoactive microRNAs which were found to be m6A methylated in HEK293 cells and examined if they are also methylated in primary vascular fibroblasts and in a fibroblast cell line, which are known to have high microRNA expression levels.[20] Furthermore, we determined whether vasoactive microRNA m6A methylation is regulated in response to hypoxia. Finally, we investigated if vasoactive microRNA m6A methylation affects their biogenesis using knockdown studies of the m6A writer METTL3 and its cofactor WTAP and eraser ALKBH5.

MATERIALS & METHODS

Cell culture

Human Umbilical Arterial Fibroblasts (HUAFs) were isolated and cultured in DMEM GlutaMAX (Invitrogen) at 37°C under 5% CO_2, as described before.[21] Human skin fibroblasts BJ (ATCC, CRL-2522) were obtained from American Type Culture

Collection (ATCC) and cultured at 37°C under 5% CO2 using Minimum Essential Medium Eagle (Sigma-Aldrich), supplemented with stable L-Glutamine (Invitrogen), 10% fetal bovine serum (PAA) and 100U penicillin & 100ug streptomycin per mL (Lonza). Medium was refreshed every 2-3 days. Cells were passed at 80-90% confluency using trypsin-EDTA. For hypoxic conditions, HUAFs and BJ cells were first seeded and cultured at 37°C with 20% O_2 for 24h, and subsequently cultured with 1% O_2 for the remainder of the experiment.

rt/qPCR

Total RNA was isolated using Trizol reagent (Life Technologies) following the manufacturer's protocol. RNA concentration and purity were measured using Nanodrop (Nanodrop® Technologies). A High Capacity cDNA kit (Applied Biosystems) was used for the reverse transcription reaction to synthesize cDNA of the extracted RNA. rt/qPCR of mRNAs and pre-microRNAs was performed using Quantitect SYBR green (Qiagen) and the reactions were run on a ViiA7 system (Applied Biosystems, for primer sequences, see **Supplemental Table I**). TaqMan MicroRNA assays (ThermoFisher) were used for rt/qPCR of microRNAs, consisting of a reverse transcription reaction using a specific microRNA primer and a qPCR reaction with TaqMan probes. For normalization of the measurements, we used the housekeeping genes RPLP0 and RPL13a for mRNA reactions and SNORD44 for the pre-miR and mature microRNA reactions. To obtain the fold change of RNA expression, $2^{\Delta\Delta Ct}$ values were calculated using the Ct values obtained with rt/qPCR. Results are displayed as relative expression in experimental conditions relative to expression in control conditions.

m6A Immunoprecipitation

The High Pure miRNA Isolation Kit (Sigma-Aldrich) was used to isolate separate fractions of small (<100 nucleotides) and large RNAs (>100 nucleotides) according to the manufacturer's instructions. Immunoprecipitation of the small RNA fraction was performed using the EZMagna RIP kit (Millipore) as described previously.[22] To immunoprecipitate RNA containing m6A, 5 μg of affinity purified anti-m6A polyclonal antibody (Synaptic Systems, 202 003) or negative control anti-rabbit IgG (Millipore PP64B) were coupled to magnetic beads and small RNAs were incubated with these

antibodies in immunoprecipitation (IP) buffer overnight at 4 °C. Before immunoprecipitation, 10% of RNA was taken out to serve as input reference. After overnight incubation, RNA bound to the magnetic beads were immobilized using magnets and unbound RNA was washed away. Then, bound RNA was eluted with proteinase K buffer, consisting of RIP wash buffer, 10% SDS and Proteinase K as supplied by the manufacturer. Immunoprecipitated RNA was isolated according to standard protocol using Trizol LS reagent (Life Technologies).

cDNA synthesis for pre-microRNA quantification was performed using SuperScript™ IV VILO™ Master Mix (Invitrogen) and subjected to rt/qPCR using primers against a selection of pre-microRNAs (for primer sequences, see **Supplemental Table I**). MicroRNA quantification was performed using TaqMan microRNA assays, as described above.

DRACH motif analysis

Transcriptome wide studies, which used m6A immunoprecipitation on fragments of Total RNA, showed that m6A was most prevalent in a certain sequence motif, which can be described as [AGU][AG]AC[ACU] or with iupac code DRACH.[23,24] To analyse our precursor and microRNA sequences for this motif, we used the sequences of our vasoactive microRNAs from miRBase (http://www.miRbase.com) and analysed these sequences for [AGU][AG]AC[ACU] in R Studio (RStudio Desktop 1.2.5001).

siRNA-mediated knockdown of METTL3, WTAP and ALKBH5

BJ cells were seeded in 12 wells flat bottomed plates and cultured overnight at 37°C. At 65% confluency, the cells were transfected with an siRNA (small interfering RNA) against METTL3, WTAP or ALKBH5 by using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol. Predesigned siRNAs (WTAP (SASI_Hs01_00039282), ALKBH5 (SASI_Hs02_00350577), METTL3 (SASI_Hs01_00044317)) were purchased from Sigma Aldrich and used with a final concentration of 40nM or 75nM siRNA, respectively. A previously validated scrambled sequence siRNA (5′-UCUCUCACAACGGGCAU(dT)(dT)-3′) was used as a negative control.[25]

Cells were transfected for 48 hours, after which they were washed with PBS and lysed with Trizol for subsequent RNA isolation, as described above.

Statistical analysis

Statistical analysis was performed using Student's t-tests and graphs were built using GraphPad Prism software (v8; GraphPad). p-values below 0.05 were considered statistically significant.

RESULTS

The m6A modification is present on vasoactive microRNAs in primary arterial fibroblasts

First, we selected a subset of 19 vasoactive microRNAs to study in detail in fibroblasts (**Table**). This was done by cross-referencing the findings of Alarcon *et al*[14] and Beruluva *et al*[16] on m6A methylation of microRNAs in HEK293 cells with 5 review studies, which comprehensively cover microRNAs that are relevant in cardiovascular biology and disease.[4,26-29] The cross-referencing yielded a selection of 14 vasoactive microRNAs that were m6A methylated in one or both studies. In order to examine whether microRNA-m6A is consistent between HEK293 cells and vascular cells, we also included 5 additional vasoactive microRNAs that were not found to be methylated in HEK293: miR-155, known as a typical multifunctional microRNA which is involved in immunity, inflammation, cancer, as well as cardiovascular diseases,[30] as well as 4 other vasoactive microRNAs (miR-329, miR-433, miR-487b and miR-494) that we have studied previously.[20-22,31]

Then we investigated whether these 19 vasoactive microRNAs were methylated in primary human umbilical artery fibroblasts (HUAFs) by comparing their enrichment in the m6A immunoprecipitated fraction versus negative control IgG fraction using rt/qPCR. We found that 16 of 19 mature microRNAs displayed a more than 25-fold enrichment in the m6A pulldown compared to the IgG pulldown (**Table**), indicating m6A methylation of microRNAs is abundant in vascular fibroblasts. Minimal or no enrichment m6A enrichment was found in miR-223 and miR-136, which were identified to be methylated in HEK293 by Beruluva *et al*. Furthermore, every selected vasoactive microRNA which was not methylated in HEK293 cells was highly enriched in the methylated fraction of HUAFs. These findings suggest that, like microRNA expression itself, microRNA m6A methylation is cell type specific.

We also examined whether the pre-miRs of the vasoactive microRNAs were enriched in the m6A immunoprecipitated fraction. We could only detect 5 pre-miRs of

the 19 selected vasoactive microRNAs (**Table**), which is consistent with previous findings that pre-miRs are rapidly processed and thus rather transient.[32,33] However, each of the detected pre-miRs was enriched in the m6A fraction, suggesting m6A methylation is indeed already present in the pre-miRs.

Table. Enrichment of selected in vasoactive microRNAs in m6A immunoprecipitation fraction of primary human arterial fibroblasts

#	Selected microRNAs	Previous findings using HEK293 cells		m6A immunoprecipitation using HUAFs (m6A IP/IgG IP)	
		m6A affects microRNA biogenesis[14]	Mature microRNA m6A methylated[16]	Mature microRNA fold enrichment	Pre-miR fold enrichment
1	**hsa-miR-10b**	✓	✓	67078	*n.d.*
2	**hsa-miR-103a**	✓	✓	46306	165
3	**hsa-miR-485**	✓	✓	30523	*n.d.*
4	**hsa-miR-423**	✓	✓	18363	108
5	**hsa-miR-30d**	✓	✓	12245	*n.d.*
6	**hsa-miR-329**			3627	146
7	**hsa-miR-126**	✓		3159	307
8	**hsa-miR-155**			3117	*n.d.*
9	**hsa-miR-16**	✓	✓	2783	*n.d.*
10	**hsa-miR-494**			1807	*n.d.*
11	**hsa-miR-487b**			1457	*n.d.*
12	**hsa-miR-381**	✓		487	*n.d.*
13	**hsa-miR-191**	✓	✓	295	*n.d.*
14	**hsa-miR-370**		✓	271	*n.d.*
15	**hsa-miR-410**		✓	138	28.4
16	**hsa-miR-433**			45.6	160
17	**hsa-miR-539**		✓	5.93	*n.d.*
18	**hsa-miR-223**		✓	3.34	*n.d.*
19	**hsa-miR-136**		✓	0.018	*n.d.*

MicroRNA enrichment in the m6A immunoprecipitated fraction (m6A IP) relative to the negative control fraction (IgG IP). The fold enrichment was calculated using the averages of each fraction after measuring them in triplicate. HUAF, human umbilical arterial fibroblasts; n.d., not detected.

Hypoxia induces an increase of m6A-methylation in microRNAs

To be able to detect changes in m6A under stress conditions like hypoxia, cells with minimal variability should be used. Therefore, these experiments were conducted with a well characterized human fibroblast cell-line (BJ cells)[34,35], rather than in primary arterial fibroblasts. Similar to the primary fibroblasts, the microRNAs with the lowest enrichment after m6A immunoprecipitation in BJ cells were also miR-136 and miR-223 (**Figure 1a**), suggesting these cells provide a suitable alternative. The 17 other selected vasoactive microRNAs were all enriched in the m6A immunoprecipitated fraction of BJ cells.

Next, we investigated whether the level of vasoactive microRNA m6A methylation in BJ cells is regulated in response to hypoxia. We found that mature microRNA m6A enrichment levels generally increased in vasoactive microRNAs after hypoxia, since the overall average enrichment level was significantly increased by 1.6 fold ($P > 0.0001$) (**Figure 1a&b**). Individual microRNA m6A methylation levels of five microRNAs (miR-10b, miR-155, miR-485, miR-487b and miR-423) were significantly increased in the m6A immunoprecipitates of hypoxic cells compared to that of control cells (**Figure 1c**). A similar trend ($P > 0.075$) was observed for five other microRNAs (miR-539, miR-410, miR-329, miR-433 and miR-30d). The overall average microRNA expression also increased slightly by 1.3-fold under hypoxia ($P > 0.01$), however no direct correlation could be observed between the relative increase in microRNA methylation and the relative increase in microRNA expression (**Figure 1c**).

The effect of hypoxia on mRNA levels of METTL3, WTAP and ALKBH5

Next, we measured changes in expression of m6A writer or eraser proteins under hypoxia. BJ cells were cultured and exposed to hypoxia for 0, 12, 24, 36 or 48 hours. We validated that hypoxia was successful by the increased expression of vascular endothelial growth factor α (VEGFα) (**Figure 2a**), a gene induced by hypoxia signalling.[36] Hypoxia did not change the mRNA levels of m6A writer complex proteins METTL3 and WTAP in BJ cells (**Figure 2c&d**). However, ALKBH5 expression was significantly increased after hypoxia at all timepoints, peaking at 24h (**Figure 2b**). These findings suggest the hypoxia-induced increase in mature microRNA m6A

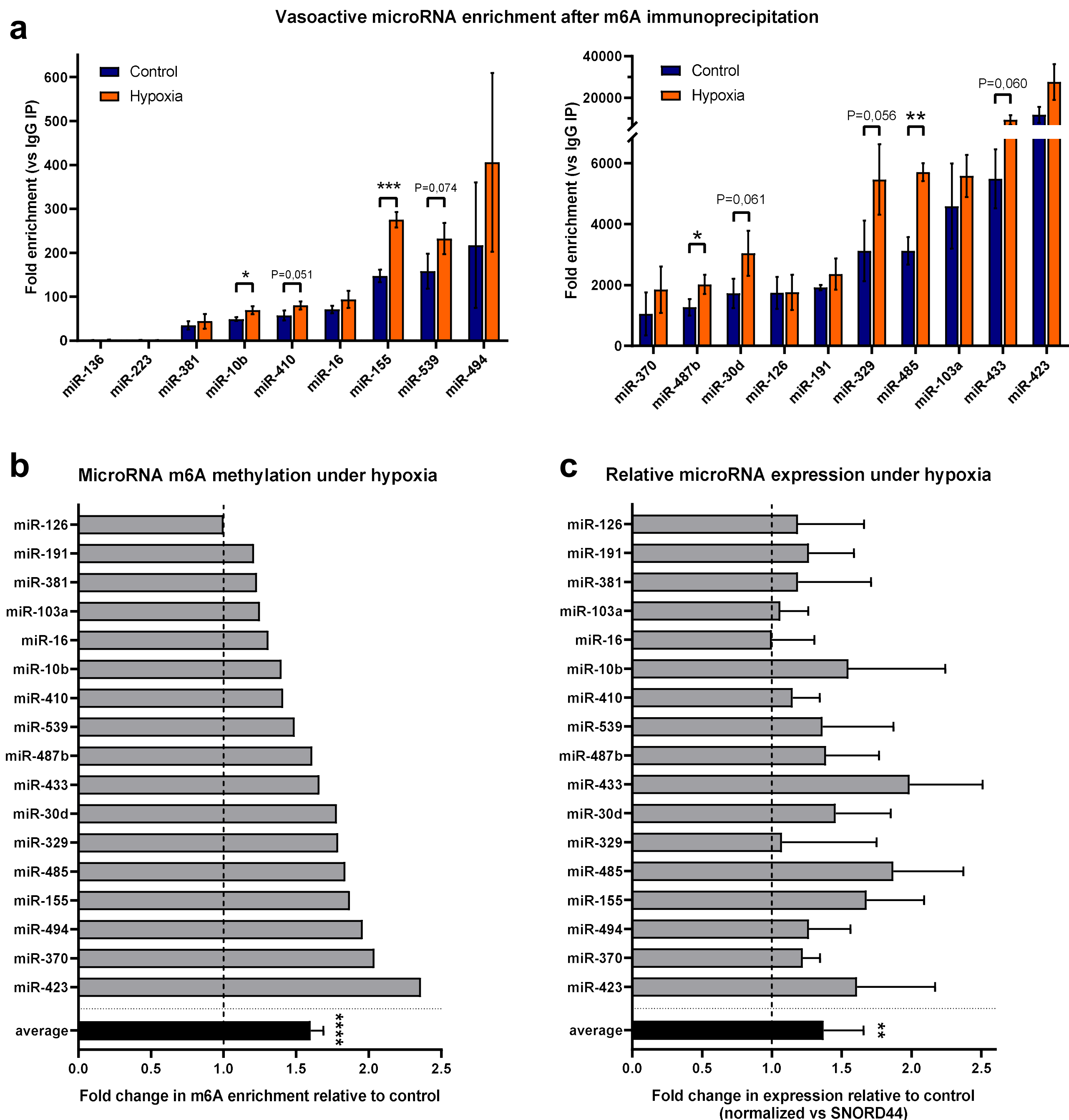


Figure 1. Effect of hypoxia on m6A methylation of selected vasoactive microRNAs. (**a**) Enrichment of vasoactive microRNA after m6A immunoprecipitation on small RNAs from BJ cells cultured under hypoxic conditions for 24h or control conditions, relative to the expression in the negative control immunoprecipitation (IgG IP). (**b**) Fold change in m6A enrichment under hypoxia relative to control conditions. Gray bars represent the average value measured per microRNA, whereas the black bar represents the average of all measured microRNAs. (**c**) Hypoxia-induced fold change in expression of mature vasoactive microRNAs, relative to control conditions. MicroRNA expression was normalized to SNORD44. Statistically significant differences are indicated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Results represent three independent experiments.

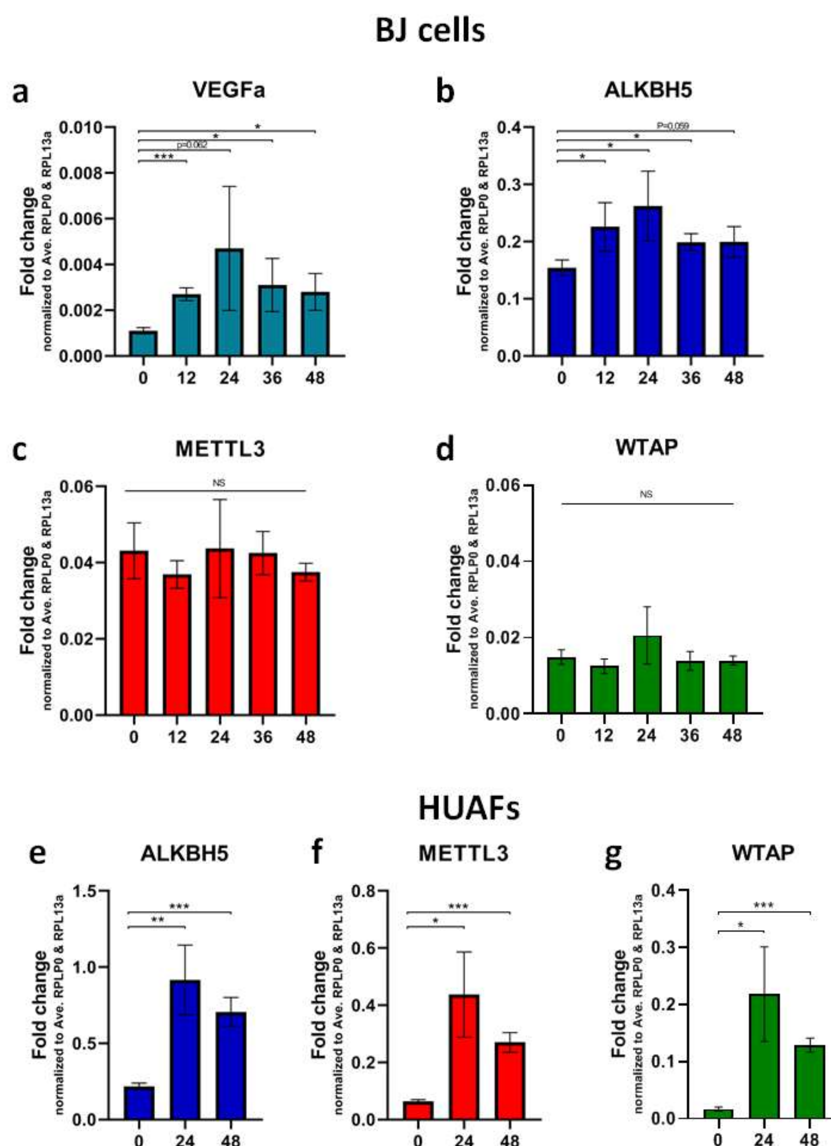


Figure 2. Expression of ALKBH5, METTL3 and WTAP under hypoxic conditions. (**a-d**) Relative mRNA expression levels of VEGFα (**a**), ALKBH5 (**b**), METTL3 (**c**) and WTAP (**d**) in BJ cells cultured under 0, 12, 24, 36 or 48 hours of hypoxic conditions. (**e-g**) Relative mRNA expression levels of ALKBH5 (**e**), METTL3 (**f**) and WTAP (**g**) in HUAFs cultured under 0, 24 or 48 hours of hypoxic conditions. All mRNA levels were normalized to RPLP0 and RPL13a. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Results represent three independent experiments.

methylation is not directly due to changes in transcription of m6A writer or eraser proteins.

To examine whether transcription of m6A writer or eraser genes in HUAFs is similarly affected by hypoxia, we subjected HUAFs to 24h and 48h of hypoxia. In contrast to transcription regulation in BJ cells, we found that transcription of the METTL3, WTAP and ALKBH5 genes was significantly increased after hypoxia in

HUAFs (**Figure 2e-g**). Both 24h and 48h of hypoxia exposure showed increased mRNA levels, but, similar to ALKBH5 expression in BJ cells, levels appeared to peak at 24h of hypoxia. These findings indicate that hypoxia-induced m6A methylation in general might be more dynamic in primary vascular fibroblasts than in the cell line.

microRNA expression after knockdown of ALKBH5, WTAP or METTL3

To examine the effect of different m6A modulating proteins on microRNA biogenesis, we knocked down ALKBH5, METTL3 or WTAP using siRNAs and measured the effect on mature microRNA expression as well as the expression of microRNA precursors (pre-miRs).

Knockdown of ALKBH5 expression by 88% (**Figure 3a**) did not change expression of most of the precursor and mature microRNAs (**Figure 3b-c**). Only the expression of mature miR-16 and pre-miR-377 were significantly increased compared to the negative control after ALKBH5 knockdown. A few more trends were observed, with mature miR-410 and pre-miR-103a1 expression levels tended towards an increase, while expression of mature miR-223, which was not methylated, tended towards a decrease after ALKBH5 knockdown.

Knockdown of METTL3 was 67% efficient, but only caused a significant change in miR-329 expression, which decreased by 2-fold (**Figure 3d-f**). The siRNA against WTAP decreased WTAP transcript expression by 77% (**Figure 3g**). However, mature microRNA expression levels also remained largely unaffected by knockdown of WTAP (**Figure 3h&i**). The expression of some pre-miRs appeared to decrease after WTAP knockdown, which was significant for pre-miR-126 and pre-miR-221.

DISCUSSION

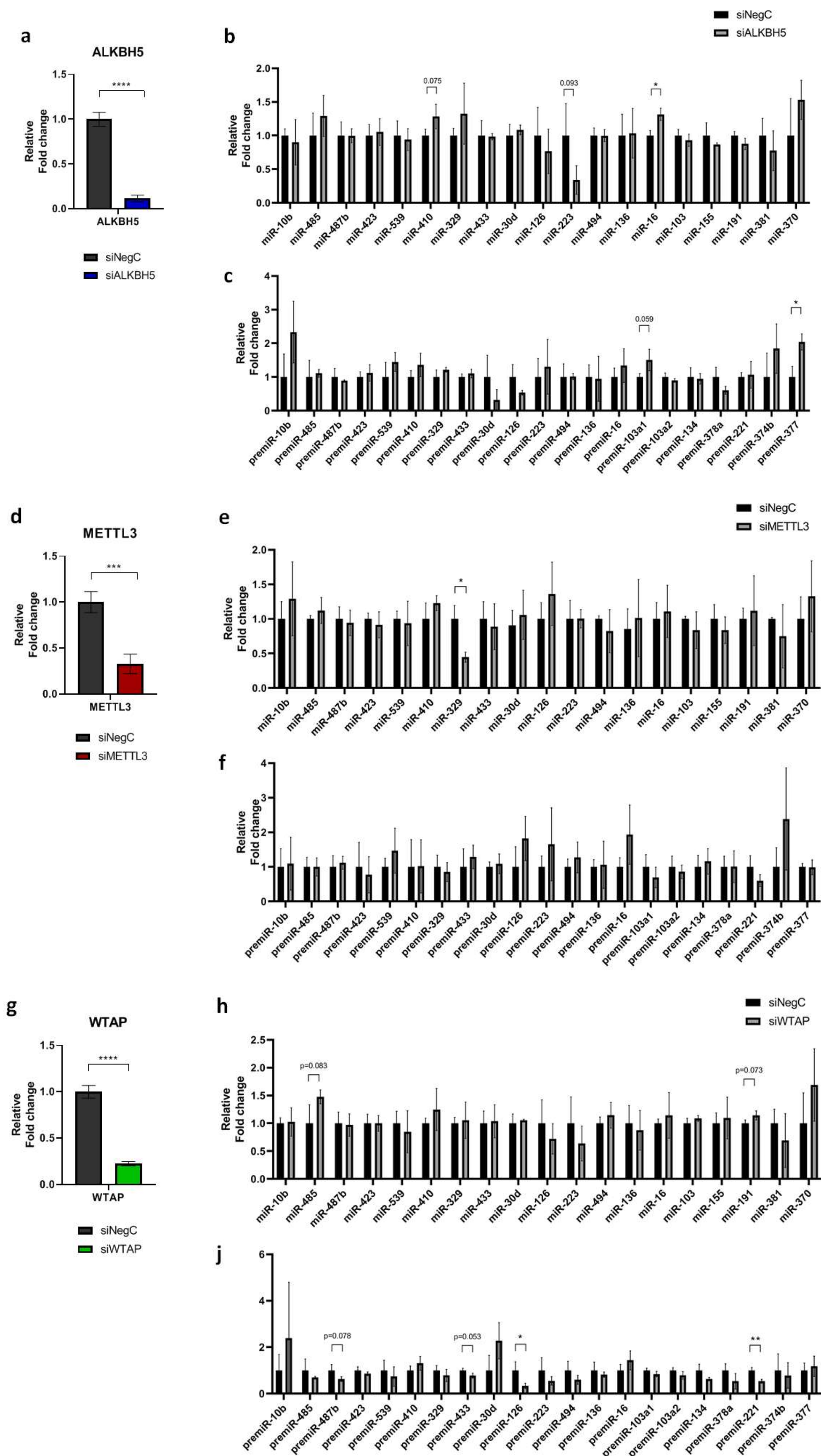
In this study, we used m6A immunoprecipitation to identify m6A methylation of selected vasoactive microRNAs in human fibroblasts, and to examine whether m6A levels are regulated under hypoxia. Of the 19 microRNAs investigated, a least 16 contained m6A in both primary human vascular fibroblasts and the human BJ fibroblast cell-line, indicating that vasoactive microRNAs are commonly m6A methylated in fibroblasts. Interestingly, the subset of microRNAs that contained m6A in fibroblasts was different compared to previous studies in HEK293 cells, suggesting

that, like microRNA expression itself, microRNA m6A methylation can be cell-type specific. More importantly, we demonstrated that mature microRNA m6A levels are increased under hypoxia. Hypoxia also caused an increase in m6A eraser ALKBH5 mRNA expression, but did not affect the expression of m6A-writer METTL3 and cofactor WTAP, indicating that the hypoxia-induced increase of microRNA-m6A was not caused by changes in transcription of m6A writers. Furthermore, knockdown of these m6A-associated proteins affected the expression of only a few mature or precursor microRNAs, suggesting that m6A plays a more microRNA-specific role in microRNA biogenesis than previously thought.

Our findings demonstrate for the first time that vascular microRNAs often contain m6A and that vasoactive microRNA methylation increases under hypoxic conditions. These findings are consistent with studies that have shown that global and mRNA m6A levels increase in response to hypoxia and ischemia,[12,13] and indicate that microRNA m6A levels are also actively and dynamically regulated.

While m6A immunoprecipitation helped us identify changes in microRNA methylation, it does not provide detailed information about the location of the m6A mark within the microRNA. The consensus DRACH motif has been identified for mRNA m6A methylation, and might thus provide a clue about potential microRNA methylation sites too.[23] Of the 17 mature microRNAs we found to be methylated, 12 microRNAs contained the DRACH in their pre-miR sequence and only 7 have the motif in their mature microRNA sequence. These observations suggest that this motif might not be as representative for microRNA methylation as it is for mRNA methylation. However, the methods to identify the exact location of m6A sites are currently not suitable for small RNAs like microRNAs, including the most recent technique called m6A-CLIP-seq.[37,38] Therefore, adapted or novel methodologies will be required to uncover the exact location of the m6A sites within mature microRNAs.

We postulated that changes in transcription of m6A-associated proteins could have caused the observed increase in microRNA m6A methylation under hypoxia, so we measured the expression of METTL3, WTAP and ALKBH5 mRNA under hypoxic conditions. However, no changes were observed in the expression of m6A-writer METTL3 or its cofactor WTAP. These findings suggest that the hypoxia-induced microRNA methylation is not facilitated by transcriptional regulation of writer


a
ALKBH5
Relative Fold change
ALKBH5
siNegC
siALKBH5
b
siNegC
siALKBH5
0.075
0.093
c
0.059
d
METTL3
METTL3
siNegC
siMETTL3
e
siNegC
siMETTL3
f
g
WTAP
WTAP
siNegC
siWTAP
h
siNegC
siWTAP
p=0.083
p=0.073
j
p=0.078
p=0.053

◄ **Figure 3. Pre-miR and microRNA expression levels after knockdown of ALKBH5, METTL3 and WTAP.** (**a**) Fold change in ALKBH5 mRNA expression after transfection with a ALKBH5-targeted siRNA compared to a nontargeting siRNA. (**b-c**) Relative fold change of pre-miR (**b**) and mature microRNA (**c**) levels after ALKBH5 knockdown compared to control treatment. (**d**) Fold change in METTL3 mRNA expression after transfection with a METTL3-targeted siRNA. (**e-f**) Relative fold change of pre-miR (**e**) and mature microRNA (**f**) levels after METTL3knockdown compared to control treatment. (**g**) Fold change in WTAP mRNA expression after transfection with a WTAP-targeted siRNA. (**h-i**) Relative fold change of pre-miR (**h**) and mature microRNA (**i**) levels after WTAP knockdown compared to control treatment. All microRNA expression levels are normalized to SNORD44. * P≤0.05, ** P ≤ 0.01, *** P ≤ 0.001. Results represent 3 independent experiments.

complex components. While we have not verified that protein expression mimics the observed mRNA levels, recent studies have revealed that changes in m6A levels can also be achieved without changes in m6A-writer complex abundance. For example, it was shown that the rate of m6A methylation can be regulated by modulating the activity of METTL3 through SUMOylation.[39] Furthermore, m6A-reader proteins HNRNPA2B1 and NF-κB associated protein (NKAP) were found to specifically promote processing of pri-miRs with m6A into mature microRNAs, suggesting the expression of these m6A-readers could ultimately dictate the levels of mature microRNAs with m6A. Therefore, the mechanisms that mediate dynamic regulation of m6A levels appear to be more complex than previously anticipated and remain to be defined.

In contrast to METTL3 and WTAP, we observed an upregulation of mRNA of the m6A eraser protein ALKBH5 under hypoxia. ALKBH5 was shown to be a target of HIF1α (Hypoxia Inducible Factor 1 Subunit Alpha), which provides a potential mechanism for its hypoxia-inducibility.[40] In a recent study in a breast cancer cell model, Zhang *et al* showed that the upregulation of ALKBH5 under hypoxia was critical for the demethylation and stabilisation of NANOG mRNA.[41] However, in our study we observed an increase in m6A methylation of microRNAs rather than a decrease, making it unlikely that ALKBH5 targets any of the examined microRNAs. This is supported by previous findings that murine ALKBH5 predominantly targets 3'UTRs of longer mRNAs, which have a very different characteristics compared to microRNA transcripts.[9,41] Unlike that of ALKBH5, FTO's ability to act as a m6A-eraser was contested until recently.[42] New studies have suggested that decreased expression of FTO is indeed responsible for increased mRNA m6A methylation under

ischemia.[12,43] These findings suggest that either FTO or other still unknown erasers, and not ALKBH5, could play a role in the hypoxia-induced microRNA methylation that we observe.

To investigate whether the m6A-associated proteins ALKBH5, WTAP and METTL3 can influence microRNA expression or processing, we knocked down their expression individually using siRNAs. We found that these knockdowns only sporadically affected the expression of the microRNAs examined. While this could be due to the fact that we only inhibited the m6A machinery, rather than completely silence it, this seems like an unlikely explanation since we reached knockdown efficiencies of up to 88%. ALKBH5 and WTAP are known to be involved in only a specific subset of m6A methylations, which therefore may not include microRNAs.[8-10] METTL3, on the other hand, has been established as the primary m6A methylase and was previously shown to broadly regulate microRNA expression levels in different cell types.[7] A potential explanation could be that microRNA m6A methylation tunes expression in a microRNA specific manner in particular cell types or settings, instead of acting like a universal broad-scale microRNA regulator.

In conclusion, we demonstrated for the first time that vascular microRNAs often contain m6A methylation and that microRNA m6A is increases under hypoxic conditions. While both the underlying mechanisms require further investigation, our research demonstrates that microRNA m6A methylation can be dynamically regulated under pathological conditions, such as ischemia. Studies have shown that changes in microRNA m6A methylation can directly affect the microRNA's silencing capacity.[17-19,44] Therefore, dynamic microRNA m6A can potentially have a significant impact on microRNA mediated transcriptional regulation.

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SUPPLEMENTARY TABLES

Supplementary table I. Primer sequences

Name	Primer type	Sequence
mRNA primers		
hsa-METTL3 F	Forward	ACACGTGGAGCTCTATCCAG
hsa-METTL3 R	Reverse	CGGAAGGTTGGAGACAATGC
hsa-WTAP F	Forward	CTCCCTTCACCTTTCCTCTCCTG
hsa-WTAP R	Reverse	TGAAGTCTGTTTCACTCAATCGAAC
hsa-ALKBH5 F	Forward	ACCACCCAGCTATGCTTCAG
hsa-ALKBH5 R	Reverse	GCCGGTTCTCTTCCTTGTCC
hsa-HIF1α F	Forward	ATTTTGGCAGCAACGACACAG
hsa-HIF1α R	Reverse	CGTTTCAGCGGTGGGTAATG
hsa-VEGFα F	Forward	ATCACCATGCAGATTATGCGG
hsa-VEGFα R	Reverse	CCCCTTTCCCTTTCCTCGAAC
hsa-RPLP0 F	Forward	TCCTCGTGGAAGTGACATCG
hsa-RPLP0 R	Reverse	TGTCTGCTCCCACAATGAAAC
hsa-RPL13a F	Forward	GCTCATGAGGCTACGGAAAC
hsa-RPL13a R	Reverse	CCGTACATTCCAGGGCAACA
Pre-microRNA primers		
hsa-premiR-329 F	Forward	AGGTTTTCTGGGTTTCTGTTTCTT
hsa-premiR-329 R	Reverse	AGGTTAACCAGGTGTGTTTCGT
hsa-premiR-410 F	Forward	AGGTTGTCTGTGATGAGTTCG
hsa-premiR-410 R	Reverse	AGGCCATCTGTGTTATATTCGT
hsa-premiR-103a1 F	Forward	ACAGTGCTGCCTTGTTGCATA
hsa-premiR-103a1 R	Reverse	CCTGTACAATGCTGCTTGATCC
hsa-premiR-103a2 F	Forward	ACAGTGCTGCCTTGTAGCAT
hsa-premiR-103a2 R	Reverse	ACAATGCTGCTTGACCTGAA
hsa-premiR-423 F	Forward	GGCAGAGAGCGAGACTTTTC
hsa-premiR-423 R	Reverse	CCTCAGACCGAGCTTTTGGAA
hsa-premiR-433 F	Forward	AGAGGCTAGATCCTCTGTGTTG
hsa-premiR-433 R	Reverse	GAGCCCATCATGATCCTTCTCA
hsa-premiR-126 F	Forward	TACTTTTGGTACGCGCTGTGA
hsa-premiR-126 R	Reverse	ACTCACGGTACGAGTTTGAAGT
hsa-premiR-134 F	Forward	TGTGACTGGTTGACCAGAGG
hsa-premiR-134 R	Reverse	GTGACTAGGTGGCCCACAG
hsa-premiR-487b F	Forward	TCCCTGTCCTGTTCGTTTTG
hsa-premiR-487b R	Reverse	AAGTGGATGACCCTGTACGAT
hsa-premiR-494 F	Forward	AGGTTGTCCGTGTTGTCTTC
hsa-premiR-494 R	Reverse	AGGTTTCCCGTGTATGTTTCATC
hsa-premiR-378a F	Forward	TCCAGGTCCTGTGTGTTACC
hsa-premiR-378a R	Reverse	CTGACTCCAAGTCCAGTGCTA
hsa-premiR-223 F	Forward	TGACAAGCTGAGTTGGACACT
hsa-premiR-223 R	Reverse	AACTGACACTCTACCACATGGA
hsa-premiR-146b F	Forward	CATAGGCTGTGAGCTCTAGCAA
hsa-premiR-146b R	Reverse	AAGTGGATGACCCTGTACGAT

hsa-premiR-485 F hsa-premiR-485 R	Forward Reverse	GAGGCTGGCCGTGATGAA GCCGTGTATGACTCGCTTTG
hsa-premiR-16-1 F hsa-premiR-16-1 R	Forward Reverse	AGCACGTAAATATTGGCGTTAAG CAGCAGCACAGTTAATACTGGA
hsa-premiR-377 F hsa-premiR-377 R	Forward Reverse	AGAGGTTGCCCTTGGTGAAT ACAAAAGTTGCCTTTGTGTGAT
hsa-premiR-374b+c F hsa-premiR-374b+c R	Forward Reverse	AGTGTCCTAGCACTTAGCAGG AGACACGGACAATGATAATACAACC
hsa-premiR-136 F hsa-premiR-136 R	Forward Reverse	ACTCCATTTGTTTTGATGATGGATTCT CATTTGAGACGATGATGGAGCA
hsa-premiR-221 F hsa-premiR-221 R	Forward Reverse	AGATTTCTGTGTTCGTTAGGCA AGCAGACAATGTAGCTGTTGC
hsa-premiR-30d F hsa-premiR-30d R	Forward Reverse	TGTAAACATCCCCGACTGGA AGCTGTGTCTTACAGCTTCC
hsa-premiR-10b F hsa-premiR-10b R	Forward Reverse	ACCCTGTAGAACCGAATTTGTG TCCCCTAGAATCGAATCTGTGACTA
hsa-premiR-539 F hsa-premiR-539 R	Forward Reverse	ATCCTTGGTGTGTTCGCTTT CTCAAAAAGAAATTGTCCTTGTATGATT
hsa-SNORD44 F hsa-SNORD44 R	Forward Reverse	AGCAAATGCTGACTGAACATGA AGTCAGTTAGCTAATTAGACCTTC
hsa-U6 F hsa-U6 R	Forward Reverse	AGAAGATTAGCATGGGCCCT ATTTGCGTGTCATCCTTGCG

MicroRNA primers

Name	**TaqMan microRNA Assay number**
hsa-miR-329	001101
hsa-miR-103a	000439
hsa-miR-191	002299
hsa-miR-10b	002218
hsa-miR-126	002228
hsa-miR-155	002623
hsa-miR-16	000391
hsa-miR-223	002295
hsa-miR-30d	000420
hsa-miR-423-5p	002340
hsa-miR-136	000592
hsa-miR-370	002275
hsa-miR-410	001274
hsa-miR-433	001028
hsa-miR-485-5p	001036
hsa-miR-487b	001285
hsa-miR-494	002365
hsa-miR-539	001286
U6 snRNA	001973
SNORD44	001094

Supplementary table II. Consensus DRACH motif in pre- and microRNA sequences

Name	microRNA sequence	Pre-microRNA sequence
hsa-miR-423	UGAGGGGCAGAGAGCG**AGACU**UU	UGAGGGGCAGAGAGCG**AGACU**UUUCUAUUUUCCA AAAGCU CGGUCUGAGGCCCCUCAGU
hsa-miR-30d	UGU**AAACA**UCCCCGACUGGAAG	UGU**AAACA**UCCCCGACUGGAAGCUGUA**AGACA**CA GCUAAGCU UUCAGUCAGAUGUUUGCUGC
hsa-miR-329	AACACACCUGGU**UAACC**UCUUU	GAGGUUUUCUGGGUUUCUGUUUCUUUAAUGAGG ACG**AAACA** CACCUGGU**UAACC**UCUUU
hsa-miR-126 (-5p)	CAUUAUUACUUUUGGUACGCG	CAUUAUUACUUUUGGUACGCGCUG**UGACA**CUUC**A AACU**CGU ACCGUGAGUAAUAAUGCG
hsa-miR-155	UUAAUGCUAAUCGUGAUAGGGGUU	UUAAUGCUAAUCGUGAUAGGGGUUUUUGCCUCCA AC**UGACU** CCUACAUAUUAGCAU**UAACA**
hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG	UAGCAGCACGUAAAUAUUGGCGUUAAGAUUCUAAA AUUAUC UCCAGUAU**UAACU**GUGCUGCUGA
hsa-miR-494	UG**AAACA**UACACGGG**AAACC**UC	AGGUUGUCCGUGUUGUCUUCUCUUUAUUUAUGAU G**AAACA** UACACGGG**AAACC**UC
hsa-miR-370	GCCUGCUGGGGUG**GAACC**UGGU	CAGGUCACGUCUCUGCAGUUACACAGCUCACGAGU GCCUGC UGGGGUG**GAACC**UGGU
hsa-miR-410	AAUA**UAACA**CAGAUGGCCUGU	AGGUUGUCUGUGAUGAGUUCGCUUUUAUUAAUGA CGAAUA **UAACA**CAGAUGGCCUGU
hsa-miR-381	UAUACAAGGGCAAGCUCUCUGU	AGCGAGGUUGCCCUUUGUAUAUUCGGUUUAU**UG ACA**UGGAA UAUACAAGGGCAAGCUCUCUGU
hsa-miR-10b	ACAGAUUCGAUUCUAGGGGAAU	UACCCUGUA**GAACC**GAAUUUGUGUGGUAUCCGUA UAGUCACA GAUUCGAUUCUAGGGGAAU
hsa-miR-539	AUCAUACAA**GGACA**AUUUCUUU	GGAGAAAUUAUCCUUGGUGUGUUCGCUUUAUUUA UGAUGA AUCAUACAA**GGACA**AUUUCUUU
hsa-miR-223	UGUCAGUUUGUCAAAUACCCCA *(not methylated)*	CGUGUAUU**UGACA**AGCUGAGUU**GGACA**CUCCAU GUGGUAG AGUGUCAGUUUGUCAAAUACCCCA

Prediction of m6A location on microRNA and premature microRNA sequences based on the consensus DRACH motif. We used the sequences of our vasoactive microRNAs from miRBase (http://www.miRbase.com) and analysed these sequences for [AGU][AG]AC[ACU]. The DRACH motifs are highlighted yellow and the predicted m6A location is marked by **A.**