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miR-200b downregulates Kruppel Like Factor 2 (KLF2) during acute hypoxia in human endothelial cells

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Abstract

The role of microRNAs in controlling angiogenesis is recognized as a promising therapeutic target in both cancer and cardiovascular disorders. However, understanding a miRNA's pleiotropic effects on angiogenesis is a limiting factor for these types of therapeutic approaches. Using genome-wide next-generation sequencing, we examined the role of an antiangiogenic miRNA, miR-200b, in primary human endothelial cells. The results indicate that miR-200b has complex effects on hypoxia-induced angiogenesis in human endothelia and importantly, that many of the reported miR-200b effects using miRNA overexpression may not be representative of the physiological role of this miRNA. We also identified the antiangiogenic *KLF2* gene as a novel target of miR-200b. Our studies indicate that the physiological changes in miR-200b levels during acute hypoxia may actually have a proangiogenic effect through Klf2 downregulation and subsequent stabilization of HIF-1 signaling. Moreover, we provide a viable approach for differentiating direct from indirect miRNA effects in order to untangle the complexity of individual miRNA networks.

Keywords

micro-RNA 200b; *KLF2*; HUVEC; HIF-1; HIF-2; hsa-miR-200b-3p

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1. Introduction

Angiogenesis promotes new blood vessel development from pre-existing vasculature and is a critical process in wound healing, the menstrual cycle, cancer, and various ischemic and inflammatory diseases. The process of angiogenesis provides cells with a controlled supply of oxygen and requires a complex control system with proangiogenic and antiangiogenic factors. Angiogenesis changes (Bergers and Benjamin, 2003) often accompany cardiovascular disorders, as well as with the development, progression, and metastasis of various human cancers. Hence, the molecular mechanisms that mediate angiogenesis have become promising therapeutic targets and biomarkers for both human cardiovascular diseases and cancer.

Recently, miRNAs that endogenously regulate gene expression via the RNA interference (RNAi) pathway have been shown to play a critical role in angiogenesis (Greco et al., 2014; Greco and Martelli, 2014; Madanecki et al., 2013). However, due to the complexity of the potential miRNA - mRNA interactions, their role in maintaining the angiogenic balance remains unclear. Often conflicting results from different groups have shown that the same miRNAs may have different mRNA targets and thus the effects on angiogenesis may be cell-type specific (Madanecki et al., 2013). Furthermore, for numerous miRNAs, their potential mRNA targets are based on correlative studies in cancer cell lines or by only following the effects of miRNA overexpression, which may be caused through indirect effects by targeting, for example, an upstream regulator or transcription factor.

miR-200b (miRBase id. MIMAT0000318 (Kozomara and Griffiths-Jones, 2014) is a miR-200 family member that is clustered with miR-200a and miR-429 on chromosome 1p36 (Chan et al., 2011). This miRNA is expressed in a variety of endothelial, stem and cancer cells (Brabletz and Brabletz, 2010; Choi et al., 2011), and modulates a wide range of cellular functions including proliferation, motility, apoptosis, and stemness (Brabletz and Brabletz, 2010). Alterations of miR-200b are well described in the context of the progression of epithelial cancers (Zhang et al., 2013b), and have been linked to the acquisition of a migratory, mesenchymal phenotype since miR-200b targets the transcription factors ZEB1 and ZEB2, two master regulators of the epithelial to mesenchymal transition (EMT) (Brabletz and Brabletz, 2010; Zhang et al., 2013b). However, miR-200b function in endothelial cells is less clear. To date, numerous studies have shown that miR-200b overexpression in human endothelial cells has potent antiangiogenic effects and inhibits VEGFA signaling (Chan et al., 2012; Chang et al., 2013; Li et al., 2017; Sinha et al., 2015). Furthermore, a large number of proangiogenic and anti-angiogenic mRNA targets have been proposed for miR-200b in human endothelia (Chan et al., 2011; Chan et al., 2012; Chang et al., 2013; Choi et al., 2011; Li et al., 2017; Sinha et al., 2015). The majority of these studies, however, have focused primarily on one mRNA target for miR-200b and have therefore overlooked the complexity of the angiogenic response. Additionally, many miR-200b overexpression studies often did not consider the physiological alterations of miR-200b levels in human endothelia during hypoxia as well as the wide range of other potential miR-200b target mRNAs that are not directly related to angiogenesis. The complexity of the miRNA networks and angiogenesis suggests that future developments in cancer therapies

that are based on miR-200b's anti-angiogenic properties will require a complete understanding of this miRNA's physiological role during hypoxic in human endothelium.

To examine miR-200b's functional role during angiogenesis, we followed its upregulation during hypoxia in primary human umbilical vein endothelial cells (HUVECs). To determine the extent of miR-200b's regulatory role in these cells, we followed its effects on the transcriptome during miR-200b depletion as well as during overexpression using genome-wide next-generation mRNA sequencing of the transfected HUVECs. Validation of the identified miR-200b network indicated that miR-200b has a pleiotropic effect on hypoxia-induced angiogenesis in human endothelia and that many of the known miR-200b effects using miRNA overexpression may not be representative of the physiological role of this miRNA. Furthermore in primary endothelial cells, we identified antiangiogenic Sp/Kruppel-like factor 2 (*KLF2*) as a novel miR-200b direct target and provide a viable approach for differentiating direct from indirect miRNA effects in order to untangle the complexity of miRNA networks.

2. Material and methods

2.1. Cell lines and culture conditions

Primary HUVECs (passage 2–6 were used only) pooled from 10 independent donors were obtained from Cellworks (UK, division of Caltag Medsystems Ltd), as well as ATCC (American Type Culture Collection) and maintained until passage five in EGM-2 BulletKit™ medium (Lonza). Cells were split either into 6-well plates or 10 cm dishes and allowed to grow to 70–80% confluence prior to the start of the experiments.

2.2. Induction of hypoxia

Hypoxia was induced in a CO_2/O_2 incubator for hypoxia research (Tri-gas Binder CB150). Briefly, cells were cultured in 2 cm dishes at 0.9% O_2 for the time periods specified. Control cells were maintained in normoxic conditions in the same incubator and harvested at the specified times.

2.3. Isolation of RNA and microRNA

Total RNA containing the microRNA fraction was isolated using miRNeasy kit (Qiagen). RNA concentrations were calculated based on the absorbance at 260 nm. RNA samples were stored at −70°C until use.

2.4. Next generation RNA sequencing analyses

HUVECs (passage 3) were used for the RNA isolation and analyses. Following rRNA (ribosomal RNA) depletion, the remaining RNA fraction was used for library construction and subjected to 100bp paired-end sequencing on an Illumina HiSeq 2000 instrument. Sequencing reads were aligned to the human reference genome assembly (hg19) using TopHat (Trapnell et al., 2009). Transcript assembly and estimation of the relative abundances were carried out with Cufflinks (Trapnell et al., 2010).

2.5. Bioinformatic analysis of potential miRNA effects

GeneAnalytics™ (geneanalytics.genecards.org), is a comprehensive gene set analysis tool for rapid contextualization of expression patterns and functional signatures embedded in the postgenomics Big Data domains, such as Next Generation Sequencing (NGS), RNAseq, and microarray experiments (Ben-Ari Fuchs et al., 2016). The webserver was used to place NGS results into physiological context using built in biological pathway algorithm. In GeneAnalytics, matched SuperPaths appear with their matching score and link to the relevant webcard in PathCards, as well as the list of matched genes and total number of genes associated with each SuperPath. The scoring algorithm in the pathways category is based on the algorithm used by the GeneDecks Set Distiller tool (Stelzer et al., 2009). Briefly, all genes in each SuperPath are given a similar weight in the analysis, and the matching score is based on the cumulative binomial distribution, which is used to test the null hypothesis that the queried genes are not over-represented within any SuperPath pathway unification is employed on all of the sources found in GeneCards. The results were independently confirmed with Quiagen Ingenuity® Pathway Analysis (IPA®).

2.6. Measurement of mRNA and miRNA levels using quantitative Real Time PCR (qRT-PCR)

We used TaqManOne-Step RT-PCR Master MixReagents (Applied Biosystems) as described previously (Bartoszewska et al., 2013; Bartoszewski et al., 2011; Bartoszewski et al., 2014) using the manufacturer's protocol. The relative expressions were calculated using the comparative relative standard curve method (Larionov et al., 2005). We used *18S* rRNA as the relative control for our studies. We also validated this relative control against another housekeeping gene, TATA-binding protein (*TBP*). As relative controls for miRNA quantification, we validated and used *RNU44* and *RNU48*. TaqMan probes ids used were: *18S* - Hs99999901_s1; *TBP* - Hs4332659_m1; *HIF1A* - Hs00153153_m1; EPAS1 - Hs01026149_m1; KLF2 - Hs00360439_g1; *RNU44* - 001094; *RNU48* - 001006; miR-200b – 002251. Complete list of TaqMan assays used is provided in Supporting Materials (Supporting Table S3).

2.7. miRNA analogs and target protector transfections

miR-200b mimic (id MC10492) and antagomiR (id MH10492) were purchased from Ambion. HUVECs were transfected using the Lipofectamine RNAiMax according to manufacturer's protocol. miR-200b mimic and antagomiR were used at final concentrations of 10 nM and 20 nM, respectively. The transfected cells were cultured for 2 days prior to further analysis. The degree of miRNA over-expression or knockdown was determined by qRT-PCR. Target protector (TP) were designed with Qiagen and purchased from Qiagen and directed against the *KDR* (5'-CATTTTGATCTTCTATTTGGTCCGTTACATTCACAAGCTC-3') and *KLF2* (5'-GGACCCAGAGAACCGGGCCGGGCACAGCTG-3') mRNAs. TP were used at final concentration of 600 nM. cel-miR-67 was used as a control (Ambion assay id MC22484). As an additional control, Ambion siRNA Negative Control 1 (no. 4390843), Ambion mimic control (no. 4464060) and Ambion antagomiR control (no. 4464076) were used as well.

2.8. Western Blots

Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris- HCl, pH 8.0) supplemented with protease Inhibitor Complete Mini (Roche) on ice for 15 min. The cell lysates were rotated at 4°C for 30 min and the insoluble material was removed by centrifugation at 15,000 g for 15 min. Protein concentrations were determined by BioRad™ Protein Assay using bovine serum albumin (BSA) as a standard. Following the normalization of protein concentrations, lysates were mixed with an equal volume of 2× Laemmli sample buffer and incubated for 5 min at 95°C prior to separation by SDS PAGE on stain-free TGX gradient gels (BioRad). Following SDS-PAGE, the proteins were transferred to polyvinylidene fluoride membranes (300 mA for 90 min at 4°C). The membranes were then blocked with BSA (Sigma-Aldrich) proteins dissolved in PBS/ Tween-20 (3% BSA, 0.5% Tween-20 for 1–2 hours), followed by immunoblotting with the primary antibody specified for each experiment Klf2 (Sigma SAB1403063 diluted at 1:500), Hif-1α (Abcam ab16066, diluted at 1:1000); VEGFA (Abcam ab51745, diluted at 1:250), HIF-2α (Abcam ab199, diluted at 1:800), beta Actin (Abcam ab1801, diluted at 1:1000), VEGFR2 (Abcam ab39256, diluted at 1:500). After the washing steps, the membranes were incubated with goat anti-rabbit IgG (H+L) or with goat anti-mouse IgG (H+L) HRP-conjugated secondary antibodies (BioRad) and detected using ECL (Amresco). Densitometry was performed using Image Lab software v. 4.1 (BioRad).

2.9. Statistical analysis

Results were expressed as means ± standard deviations (SD). Statistical significance among means was determined using the Student's t-test (two samples, paired and unpaired). The correlation coefficients and p values were calculated according to Spearman Rank-Order Correlation (Spearman, 1904).

3. Results

To understand the role of miR-200b in hypoxia-mediated angiogenesis, we first performed a time-course study and monitored miR-200b regulation in primary human endothelial cells. Primary HUVECs (pooled from 10 independent donors) were exposed to hypoxia (0.9% O2) for up to 48 h and miR-200b levels were measured at the specified time points. As shown on Figure 1A, miR-200b expression was induced at 2 h and stayed elevated during the entire 48 h time course. Our initial *in silico* analysis identified hundreds of potential human genes that could be controlled by miR-200b. The MiRanda algorithm predicts over 7000 mRNAs as having potential miR-200b target sites (Betel et al., 2010), whereas miRDB predicts 757 potential mRNA targets (Wong and Wang, 2015). Given that miR-200b was significantly (from ~2.5 to 4 fold) and continuously upregulated during hypoxia (Figure 1A), a large number of angiogenesis-related mRNA targets could be regulated either directly or indirectly through miR-200b's actions. To distinguish between those two possibilities, we analyzed how miR-200b mimic overexpression and antagomiR depletion had on the mRNA profiles using genome-wide transcriptome sequencing (RNA-seq) analysis on primary HUVECs under normoxic conditions. Our goal was to determine the effects of this particular miRNA without the complicating effects of hypoxia and hypoxia inducible factor (HIF) expression.

Reduction of miR-200b levels with the antagomiR resulted in the significant (two-fold $\log_2$ change) upregulation of 221 mRNAs, and a reduction of 109 mRNAs (Supportive Table 1). Overexpression of miR-200b with mimic caused a downregulation of 230 genes, and upregulation of 252 mRNAs (Supportive Table 1). However, among 221 mRNAs that were upregulated after the antagomiR, 102 were significantly induced with mimic as well. Similarly, among 230 genes downregulated upon miR-200b overexpression, 56 were significantly reduced by antagomiR (Supportive Figure 1). Surprisingly, we did not identify a gene using RNAseq that was either induced or reduced after transfection with antagomiR or mimic, respectively, using our selection criteria. We did, however, identify the control genes (*ZEB1* (Chen et al., 2011b) and *KDR* (Choi et al., 2011)) that showed this reciprocal pattern as expected, but the magnitude of the expression changes was below our selection cut-off criteria. This suggested that modulation of miR-200b levels results in a very wide network of secondary interactions that could mask the miRNA's direct effects. As an alternative, we lowered our selection criteria to allow for the selection of genes that were either induced by antagomiR and in parallel, reduced or were not affected by mimic, or reduced by mimic and induced or not affected by antagomir. This strategy resulted in the selection of 377 genes that were potentially regulated by miR-200b (Supportive Table 2). Further, using Gene Ontology Software, we narrowed our focus to 16 mRNAs from the RNAseq data that were postulated to be involved in the cellular response to hypoxia and angiogenesis, and the putative angiogenesis-related miR-200b targets.

These mRNAs were further tested with miRNA target sites predicting algorithms for potential miR-200b binding sites (miRanda (Betel et al., 2010), Targetscan7.1 (Agarwal et al., 2015) and RNAhybrid (Kruger and Rehmsmeier, 2006)) and the effects of miR-200b modulation were verified with qPCR both in normoxic and hypoxic conditions (Table 1, Supportive Figure 2). Since mimic transfection caused miRNA overexpression, much higher (> 100-fold) than physiological levels of miR-200b (Figure 1B **left panel**) and antagomir dramatically inhibited miR-200b expression (Figure 1B **right panel**), we only considered potential target mRNAs that were reduced on mimic treatment and induced by antagomir treatment, preferably during both normoxia and hypoxia.

Although miRNA overexpression (mRNA) negatively affected all of the selected mRNAs, the antagomir only upregulated only 1 of the mRNAs, Krupple-like factor 2 (*KLF2*), and thus this represented a potential direct target of miR-200b. *KDR*, which encodes the VEGFR2, and already reported as miR-200b target (Choi et al., 2011), fulfilled our criteria during normoxia, but not during hypoxia (Supportive Figure 2). *KLF2* is a novel target that encodes for a transcription factor that is antiangiogenic (Kawanami et al., 2009).

During acute hypoxia, Klf2 protein increases (Figure 2A) as has previously been shown (Bhattacharya et al., 2005; Kawanami et al., 2009) and goes up approximately 2-fold after 16 h. In parallel, Hif-1α and Hif-2α are increased during this same time with Hif-1α maximal at 8 h and Hif-2α continuing to increase at 16 h (Figure 2A). The mRNA changes for *KLF2* increase 2.5-fold during hypoxia with significant elevations at 2 and 4 h (Figure 2B), and this agrees with a previous study suggesting that *KLF2* is induced during hypoxia (Bhattacharya et al., 2005; Kawanami et al., 2009).

Given that *KLF2* mRNA and miR-200b expression profiles during hypoxia were positively correlated (correlation coefficient 0.6 and p value 0.002, Spearman rank order test n=18), and that KLF2 mRNA has a potential miR-200b target site, we next examined effects of miR-200b overexpression (mimic) and depletion (antagomiR) on *KLF2* mRNA levels during normoxia and after 4 hours of hypoxia. As shown in Figure 3A, *KLF2* mRNA was significantly reduced along with increased miR-200b levels and accumulated upon miR-200b depletion, both during normoxia and hypoxia. These miR-200b-induced changes were preserved on Klf2 protein levels as well. As shown in Figure 3B, reduction of miR-200b expression with antagomiR significantly induced Klf2 protein expression under both conditions. However, miR-200b overexpression didn't decrease the Klf2 protein, suggesting that the proteins levels were stable during the 4-hour time course.

We further verified the miR-200b direct interaction with *KLF2* mRNA target sequence with use of specific Target Protector (TP) (Bartoszewska et al., 2015; Janaszak-Jasiecka et al., 2016; Summerton, 2007). TPs bind to specific complementary RNA sequences and block miRNA binding; however, they do not trigger the RNAi (Love et al., 2008). Target protectors increase mRNA target expression by a modest amount and only in cells where the mRNA is already expressed (Staton and Giraldez, 2011). As shown in Figure 4, both *KLF2* mRNA and protein were resistant to miR-200b overexpression in the presence of specific TP morpholino, confirming the direct interaction between miR-200b and this mRNA. Importantly, the KLF2 TP increased the physiological mRNA and protein levels of *KLF2*. This observation is consistent with our previous experiments where miR-200b overexpression had little effect on Klf2 protein. Thus physiological levels of miR-200b efficiently control the Klf2 protein levels both in normoxic and hypoxic conditions. *KDR* mRNA, being a previously validated target of miR-200b was used as a control for both mimic and anatgomiR as well as target protector experiments (Supportive Figure 3AB).

4. Discussion

The results of recent reports suggest that miR- 200b negatively regulates angiogenesis in human endothelial cells. Chan and coworkers showed in HMECs (human microvascular endothelial cells) that miR-200b is downregulated by hypoxia and thus, the levels of its target, the proangiogenic *ETS1* gene, are induced (Chan et al., 2011). Another study reported that in A549 cells, miR-200b targeted the predicted binding sites in the 3'UTRs of *VEGF, FLT1*, and *KDR* using miRNA overexpression-based luciferase reporter assays (Chan et al., 2011; Choi et al., 2011; Roybal et al., 2011).

In our present and previous studies (Bartoszewska et al., 2015) in primary HUVECs demonstrated that miR-200b levels are dynamically induced during a hypoxia time course and illustrated that HIF-1 and HIF-2 expression was biphasic with HIF-2 going up as HIF-1 went down. Here we demonstrate that the miR-200b expression profile during hypoxia in primary HUVECs strongly resembles both HIFs and proangiogenic signaling. However, these data do not support miR-200b's antiangiogenic function, at least in HUVECs. In HMECs, miR-200b was shown to be downregulated after 24h of hypoxia only, whereas its levels remained constant during 6 and 12 hours of hypoxia. Although HMECs and HUVECs

are both primary human endothelial cells, they are from different vascular beds and therefore have differential miR-200b expression profiles during hypoxia.

In the present studies, we have validated miR-200b expression profile during hypoxia in at least 6 independent experiments using primary cells from different batches and sources, and we have also observed that primary HUVECs exhibit the significant miR-200b induction in response to hypoxia until passage 6, whereas higher passages show no effect on miR-200b levels. Finally we have followed changes in *ETS1* levels not only during miR-200b overexpression, but also up on depletion of physiological levels of miR-200b during both in normoxia and under hypoxia. Our results indicated that miR-200b inhibition did not result in *ETS1* mRNA stabilization in HUVECs. Similar results were obtained for *FLT1* and *VEGFA*, which demonstrated that only miR-200b overexpression affected their mRNA levels during hypoxia (Supp. Figure 2).

To re-evaluate miR-200b role in regulation of hypoxia induced angiogenesis, we accessed the genome wide consequences of miR-200b modulation in primary HUVECs. Following the miR-200b inhibition and overexpression consequences on HUVECs transcriptome, we observed and although miR-200b levels modulation altered expression of large number of genes, none of these genes showed a pattern of opposite effects when transfected with antagomiR and mimics. After changing our selection criteria, we analyzed an extended list of miR-200b affected genes this resulted in the identification of 12 angiogenesis-related transcripts that could be potentially miR-200b targets, some of which had already been identified as miR-200b targets.

We have independently verified these targets with qPCR during hypoxia and normoxia in primary HUVECs. Importantly, although miR-200b overexpression resulted in downregulation of majority of the targets, the inhibition of physiological miR-200b levels resulted in induction of only 2 of them, both under normoxic and hypoxic conditions. One was *KDR*, while the other novel miR-200b target was *KLF2*. Finally using specific target protectors, we have confirmed the direct binding of miR-200b to these 2 mRNAs.

KDR mRNA expression was elevated on miR-200b inhibition suggesting that this gene is miR-200b target indeed. Interestingly, although miR-200b was specifically modulating *KDR* mRNA levels (Supportive Figure 3AB), the impact of this miRNA on VEGFR2 protein was less direct (Supportive Figure 3CDE). Despite the *KDR* mRNA levels being downregulated during the hypoxia time course (that could correspond to miR-200b induction), the VEGFR2 protein shown was transiently changed. Finally, despite miR-200b antagomiR rescuing VEGFR2 expression during hypoxia, the mimic treatment resulted in even more significant VEGFR2 accumulation, whereas no significant change in VEGFR2 expression was observed up on miR-200b modulation in normoxia. Although these observations require further studies, in HUVECs the miR-200b impact on VEGR2 protein levels is probably rather limited.

KLF2 is strongly expressed in endothelial cells and is necessary for normal vessel formation (Anderson et al., 1995; Kuo et al., 1997; SenBanerjee et al., 2004). Overexpression of this transcription factor inhibits Hif-1α and its target genes (Kawanami et al., 2009).

Importantly, Klf2 was shown to selectively promote Hif-1α degradation during hypoxia in a von Hippel-Lindau-independent but proteasome-dependent manner through disruption of the interaction between Hif-1α and its chaperone Hsp90 (Bhattacharya et al., 2005; Kawanami et al., 2009). Interestingly, Klf2 has no effect on Hif-2α protein stability (Kawanami et al., 2009). Finally, Klf2 was shown to reduce VEGF receptor 2 (VEGFR2) expression, which directly affected VEGFA signaling (Bhattacharya et al., 2005; Kawanami et al., 2009). Thus Klf2, as a selective natural HIF-1 activity inhibitor, was proposed as a "molecular switch" modulating endothelial angiogenic balance during hypoxia (Feinberg et al., 2004; Kawanami et al., 2009; Lin et al., 2005; SenBanerjee et al., 2004). Here, we demonstrate that acute hypoxia-induced miR-200b expression modulates Klf2 mRNA levels directly (Figures 3 and 4), and thus contributes to the HIF-1/HIF-2 switch in human endothelia during hypoxia.

5. Conclusions

In summary, our studies suggest that the physiological changes in miR-200b levels during hypoxia have a proangiogenic effect through Klf2 downregulation and stabilization of HIF-1 signaling. Furthermore, we show that majority of known antiangiogenic effects of miR-200b could result from the artificial overexpression models used for both target selection and assessment of angiogenesis. Whereas, our studies indicate that they are not affected by miR-200b at physiological levels, and thus some of these gene targets are not functional targets as has been suggested in other studies. Furthermore, our data clearly point out that understanding the complexity of the potential single miRNA - mRNA interactions and related pleiotropic effects on cell signaling is a limiting factor in the development of novel miRNAbased therapies in a number of human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

KLF2	Kruppel Like Factor 2
HUVEC	human umbilical vascular endothelial cells
miRNA	microRNA
TP	target protectors
VEGFA	vascular endothelial growth factor A
VEGFR2	vascular endothelial growth factor A receptor 2
KDR	Kinase insert domain receptor

HIF hypoxia inducible factor

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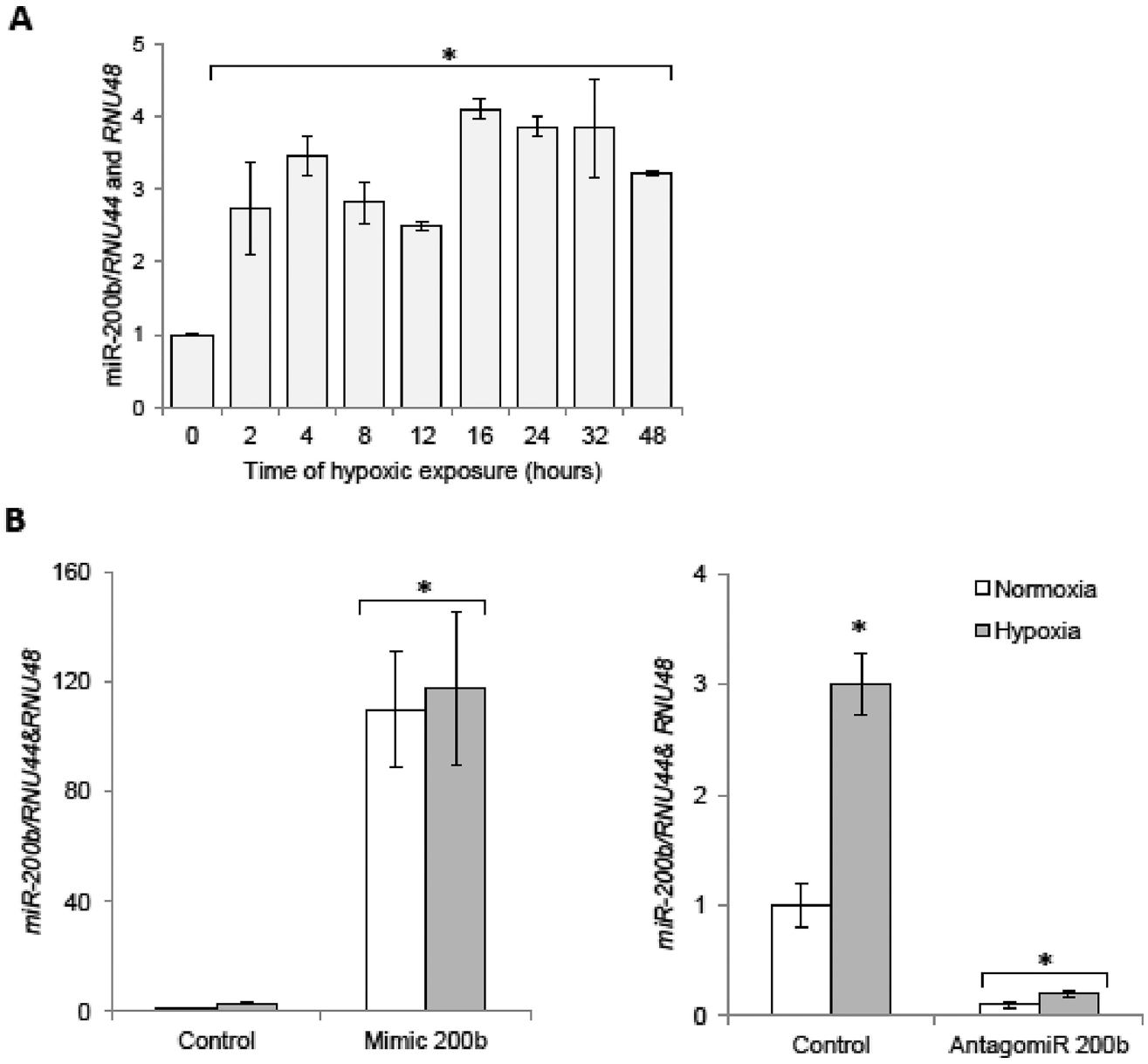

Figure 1.

Regulation of miR-200b during hypoxia in primary HUVECs. **(A)** The miR-200b levels were monitored in qRT-PCR experiments. The results from 3 independent experiments (n=12) are plotted normalized to RNU44 and RNU48 RNA levels and expressed as a fold-change over the normoxic control. **(B)** HUVECs were transfected with miR-200b mimic or antagomiR, and the miRNA levels were monitored in normoxic conditions and after 4 h of exposure to hypoxia. miRNA levels from 2 independent experiments (n=8) are plotted normalized to RNU44 and RNU48 RNA levels and expressed as a fold change over normoxic control. Error bars represent standard deviations. Significant changes ($p<0.05$) are marked with an asterisk.

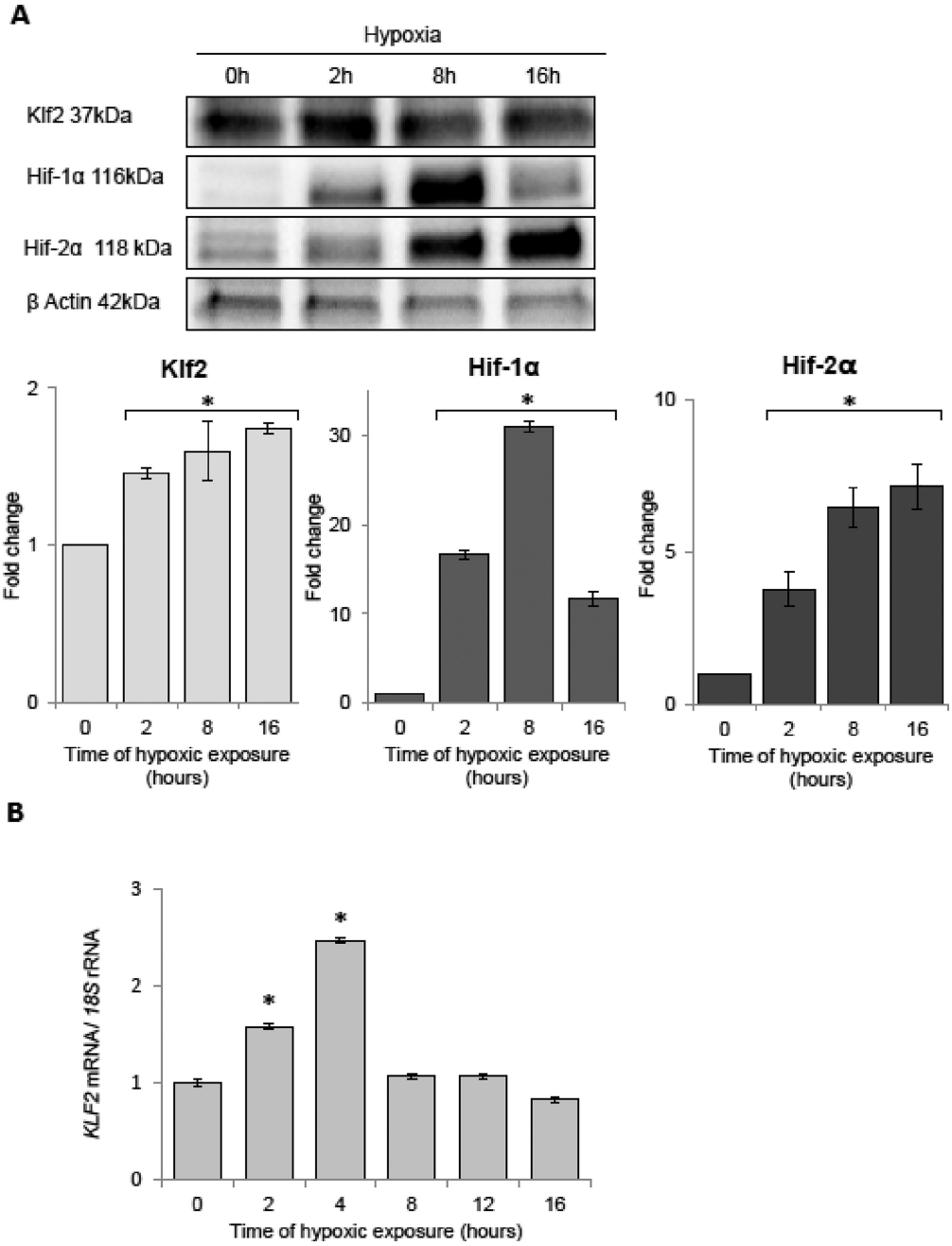

Figure 2.

Acute hypoxia induces dynamic changes in the protein expression profile of the Klf2 in HUVECs. **(A)** Hypoxia induces dynamic changes of protein levels of Klf2, Hif-1α, and Hif-2α. The protein levels of were detected with SDS-PAGE and Western Blot and related to total protein levels. 2 individual samples (4 μg of total protein per lane) were tested for each time point and the experiments were repeated twice. **(B)** The *KLF2* mRNA levels were monitored in qRT-PCR experiments. The results from 3 independent experiments (n=12) are plotted normalized to *18S* rRNA levels and expressed as a fold-change over the normoxic

control. Error bars represent standard deviations. Significant changes ($p<0.05$) are marked with an asterisk.

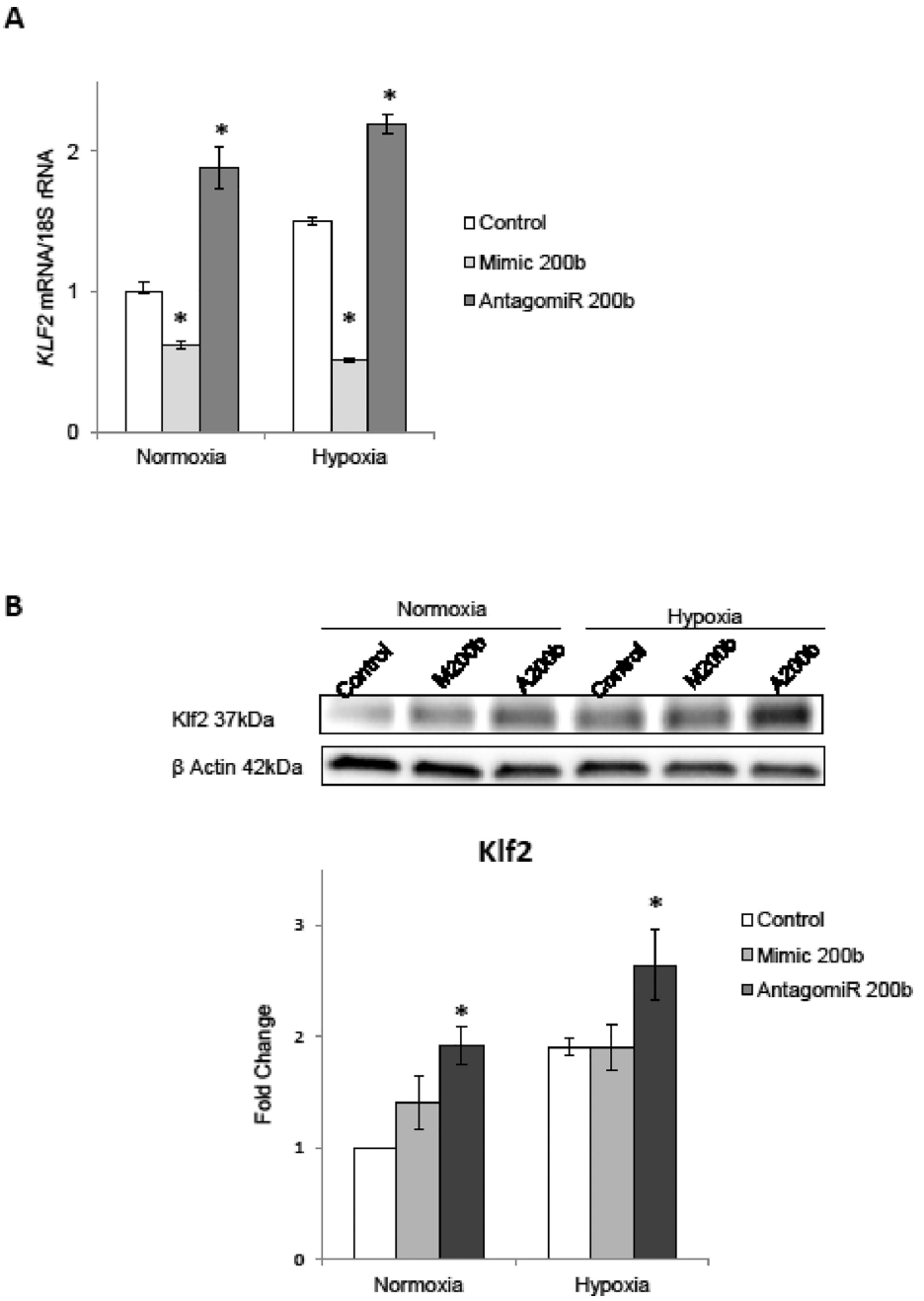

Figure 3.

miR-200b alters the expression of *KLF2*. **(A)** HUVECs were transfected with miR-200b mimic or antagomiR, and the mRNA levels were monitored in normoxic conditions and after 4 h exposure to hypoxia. *KLF2* mRNA levels from 3 independent experiments (n=12) are plotted normalized to *18S* rRNA or *TBP* mRNA levels and expressed as a fold change over the normoxic control. Significant changes ($p<0.05$) are marked with an asterisk. **(B)** The corresponding changes of Klf2 protein levels were detected with SDS-PAGE and Western Blot and normalized to the β-Actin and 2 individual samples (4 μg of total protein per lane) were tested for each treatment and the experiments were repeated twice.

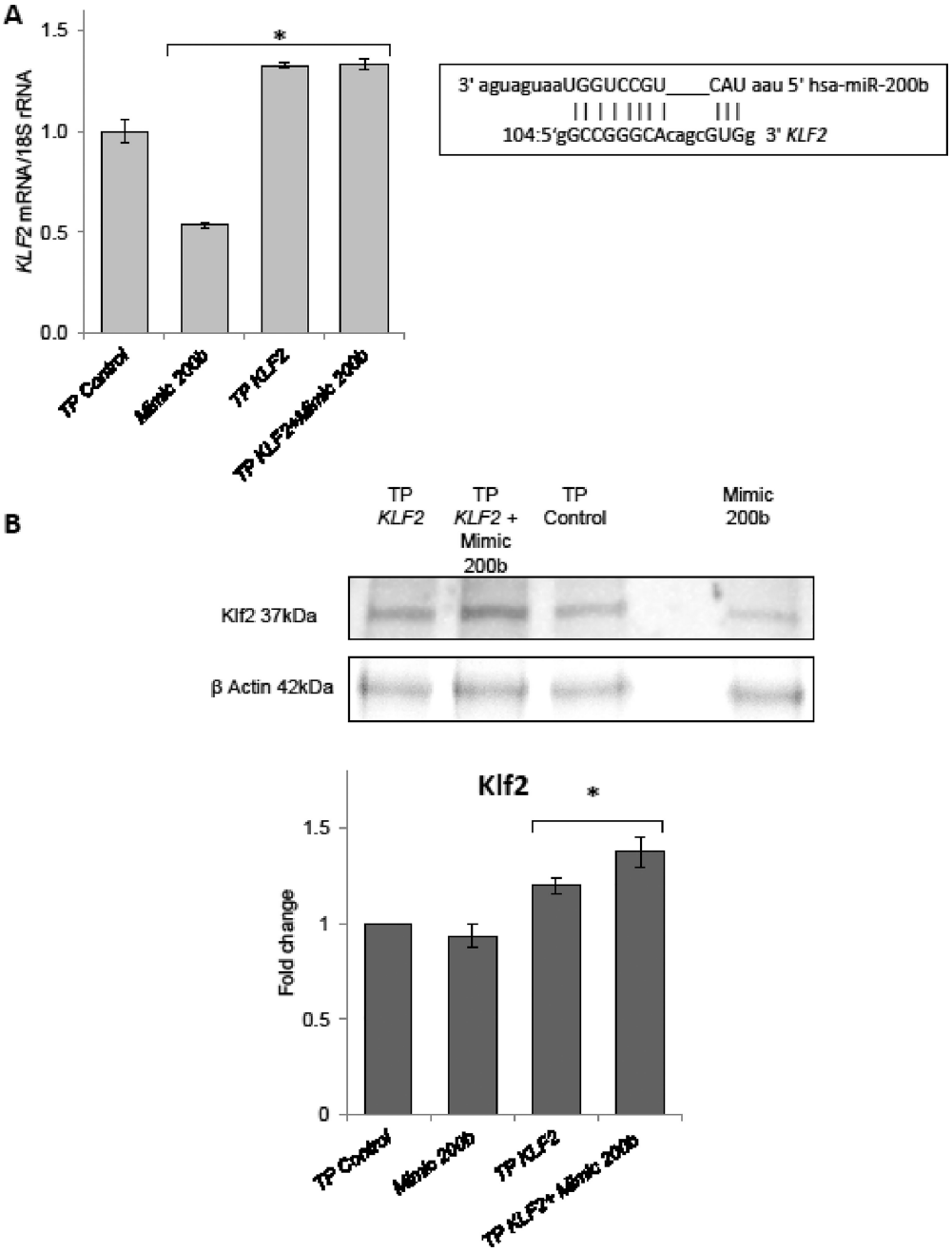

Figure 4.

miR-200b binds to predicted target sequence in the *KLF2* 3'UTR **(A)**.The RNAhybrid predicted miR-200b target site at 3' UTR of *KLF2* mRNA is presented at the top right. HUVECs were transfected with *KLF2* target sequence-specific target protector and/or the miR-200b analog (Mimic 200b) and exposed to hypoxia for 4 h. The *KLF2* mRNA levels were monitored in qRT-PCR experiments. The *KLF2* levels results from 2 independent experiments (n=8) are plotted normalized to *18S* rRNA levels and expressed as a fold change over the transfection control. Significant changes ($p<0.05$) are marked with an asterisk. **(B)**. The corresponding changes of Klf2 protein levels were detected with

SDSPAGE and Western Blot and normalized to the β-actin levels 2 μg of total protein per lane was loaded for each sample and the experiments were repeated twice.

Table 1

Genes related to the hypoxia-induced angiogenesis that were altered in HUVECs following miR-200b reduction/induction

The genes were preselected based on RNASeq experiments and subsequent Gene Ontology analysis. The presence of potential miR-200b target sequences was predicted in bioinformatics approach (Betel et al., 2008; Kruger and Rehmsmeier, 2006; Lewis et al., 2005). The changes in genes expression after miR-200b overexpression and inhibition were accessed by qPCR in normoxia and under hypoxia (Supportive Figure 2). The previously proposed miR-200b targets that are angiogenesis related were included as well and denoted with an asterisk. Opposite mRNA expression patterns after overexpression or inhibition of miR-200b are highlighted in grey.

Gene	miR-200b target site	miR-200b effect in normoxia	miR-200b effect under hypoxia	Role in angiogenesis	Ref.
KLF2	yes	Downregulated with mimic and upregulated with antagomiR	Downregulated with mimic and upregulated with antagomiR	antiangiogenic	(Bhattacharya et al., 2005; Kawanami et al., 2009)
**KDR*	yes	Downregulated with mimic and upregulated with antagomiR	Downregulated with mimic and not changed with antagomiR	proangiogenic	(Soldi et al., 1999)
**ETS1*	yes	Downregulated with mimic and antagomiR	Downregulated with mimic and antagomiR	proangiogenic	(Hashiya et al., 2004)
**VEGFA*	yes	Downregulated with mimic and antagomiR	Downregulated with mimic and antagomiR	proangiogenic	(Ferrara et al., 2003)
NOS3	yes	Downregulated with mimic only	Upregulated with antagomiR	proangiogenic	(Amano et al., 2003)
THSD7A	yes	Downregulated with mimic	No significant effects	antiangiogenic	(Wang et al., 2010)
ITGB3	yes	Downregulated with mimic	Downregulated with mimic	proangiogenic	(Brooks et al., 1994)
MYOF	yes	Downregulated with mimic	No significant effects	proangiogenic	(Yu et al., 2011)
NRP2	yes	Downregulated with mimic	Downregulated with mimic	proangiogenic	(Takashima et al., 2002)
FYN	yes	Downregulated with mimic and antagomiR	Downregulated with mimic	proangiogenic	(Sen and Johnson, 2011)
FZD4	yes	Downregulated with mimic	Downregulated with mimic	proangiogenic	(Chen et al., 2011a)
GRB10	yes	Downregulated with mimic	Downregulated with mimic	proangiogenic	(Soriano et al., 2004)
EP300	yes	Downregulated with mimic and antagomiR	Downregulated with mimic and antagomiR	proangiogenic	(Zhang et al., 2013a)
**FLT1*	yes	No effect	Downregulated with mimic and antagomiR	proangiogenic	(Birnbaum, 1995)
RGS5	yes	Downregulated with mimic	Downregulated with mimic	proangiogenic	(Mitchell et al., 2008)
HMOX1	yes	Downregulated with mimic and antagomiR	Downregulated with mimic and antagomiR	proangiogenic	(Malaguarnera et al., 2002)