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Short communication

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.

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

Abbreviations: 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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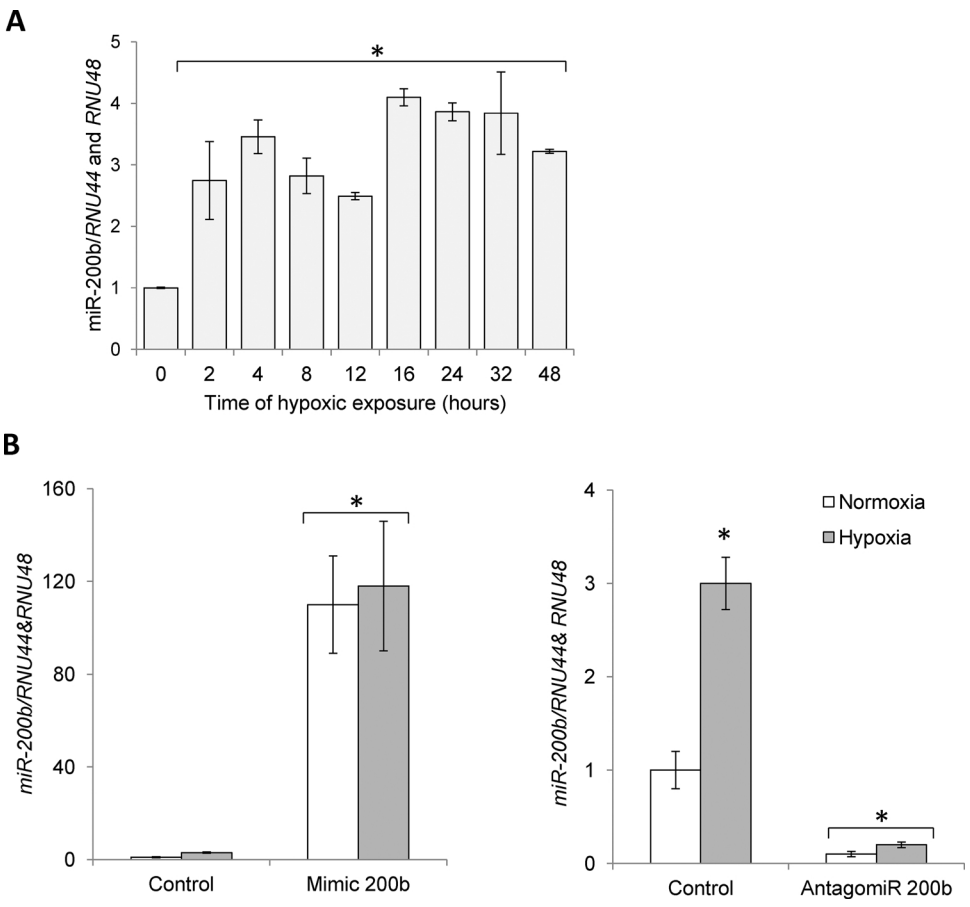

Fig. 1. Regulation of miR-200b during hypoxia in primary HUVECs. (A) The miR-200b levels were monitored in qRT-PCR experiments. The results from 3 independent experiments (n = 12) are plotted normalized to RNU44 and RNU48 RNA levels and expressed as a fold-change over the normoxic control. (B) HUVECs 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.

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, 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.