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miR-296 Regulates Growth Factor Receptor Overexpression in Angiogenic Endothelial Cells

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SUMMARY

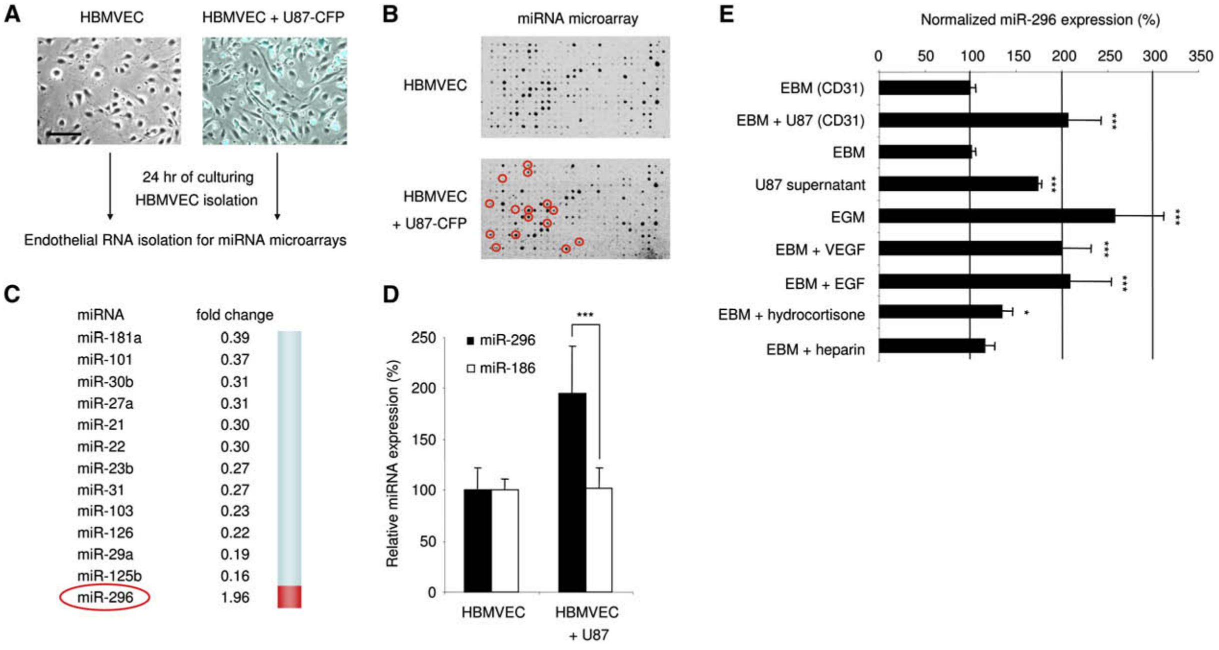
A key step in angiogenesis is the upregulation of growth factor receptors on endothelial cells. Here, we demonstrate that a small regulatory microRNA, miR-296, has a major role in this process. Glioma cells and angiogenic growth factors elevate the level of miR-296 in primary human brain microvascular endothelial cells in culture. The miR-296 level is also elevated in primary tumor endothelial cells isolated from human brain tumors compared to normal brain endothelial cells. Growth factor-induced miR-296 contributes significantly to angiogenesis by directly targeting the hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) mRNA, leading to decreased levels of HGS and thereby reducing HGS-mediated degradation of the growth factor receptors VEGFR2 and PDGFRβ. Furthermore, inhibition of miR-296 with antagomirs reduces angiogenesis in tumor xenografts in vivo.

INTRODUCTION

Angiogenesis is the formation of new blood vessels during growth and development as well as in disease-related processes like tumor growth, wound healing, and restoring blood flow to tissues after injury (Folkman, 2007). De novo angiogenesis is a critical factor in cancer. For instance, malignant brain tumors are characterized by a marked increase in blood vessel formation, with tumor vessels having abnormal morphology that serves as a key feature in tumor grading (Brem et al., 1972; Folkerth, 2000). Increasing awareness of the importance of the vasculature in tumors has led to a focus on this as a therapeutic target (Kerbel and Folkman, 2002). The state of angiogenesis is a balance between pro- and antiangiogenic molecules, with a bias toward the proangiogenic mode (Jain, 2005). A common feature of angiogenic blood vessels is the high expression of proangiogenic growth factor receptors, such as platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR), which are targets of antiangiogenic therapies (Batchelor et al., 2007; Shih and Holland, 2006). Further understanding of the orchestration of this angiogenic switch should help in the development of strategies to harness the dynamics of blood vessel formation in human health and disease. Recently, the discovery of microRNAs (miRNAs) has increased our knowledge regarding the complex control of gene expression. miRNAs comprise a large group of endogenous noncoding RNAs that can block mRNA translation and/or negatively regulate its stability (Ambros, 2004). At this time, over 500 different miRNAs have

SIGNIFICANCE

The formation of new blood vessels by angiogenesis is essential for normal functions and is involved in many disease states, including cancer. Control of the angiogenic switch in endothelial cells involves changes in levels of pro- and antiangiogenic molecules acting in concert. Here, we show that glioma- or growth factor-mediated induction of miR-296 in endothelial cells results in increased levels of proangiogenic growth factor receptors. These results indicate that miR-296, belonging to the family of "angiomirs," is functionally linked to the angiogenic phenotype and therefore provide new insights into the role of miRNA regulation in neovascularization. Further, manipulation of miR-296 levels may prove therapeutic in the large number of diseases wherein angiogenesis is a critical component.



miRNA	fold change
miR-181a	0.39
miR-101	0.37
miR-30b	0.31
miR-27a	0.31
miR-21	0.30
miR-22	0.30
miR-23b	0.27
miR-31	0.27
miR-103	0.23
miR-126	0.22
miR-29a	0.19
miR-125b	0.16
miR-296	1.96

Figure 1. Glioma-Induced Dysregulation of miRNAs in Human Brain Endothelial Cells

(A) Primary human brain microvascular endothelial cells (HBMVECs) were cultured in the absence (left) or presence (right) of human U87-CFP glioma cells. Images were produced using a combination of light and fluorescence microscopy. Scale bar = 100 μm.

(B) Array hybridization analysis of miRNAs extracted from CD31$^+$ cells sorted from HBMVECs cultured without (upper array) or with (lower array) U87-CFP glioma cells for 24 hr. The density of the hybridization signals (black spots) reflects the relative expression level of particular miRNAs. Red circles indicate miRNAs that were significantly altered by coculture with glioma cells.

(C) A list of significantly decreased (fold change < 0.5) or increased (fold change > 1.9) miRNAs in HBMVECs exposed to U87-CFP glioma cells.

(D) Overexpression of miR-296 was confirmed by qRT-PCR analysis. RNA extracted from CD31$^+$ HBMVECs cultured in the absence or presence of U87 glioma cells was analyzed by qRT-PCR. The data were normalized to the level of GAPDH mRNA in each sample.

(E) HBMVECs were cultured in the presence or absence of U87 glioma cells, isolated using CD31 beads, and subjected to miR-296 qRT-PCR. Alternatively, HBMVECs were cultured in various culture media and subjected to miR-296 qRT-PCR. miR-296 levels were normalized to EBM (CD31).

Error bars indicate SD. *p < 0.05, ***p < 0.001 by t test.

been identified in human cells (Griffiths-Jones et al., 2006). Accumulating evidence indicates that regulation of miRNA levels is very important for proper growth and differentiation of many cell types and tissues (Bartel, 2004; Kloosterman and Plasterk, 2006; Krichevsky et al., 2003). It is also becoming clear that deregulated miRNA expression is a common feature of many human diseases, especially specific forms of cancer (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Ruvkun, 2006). Here, we aimed at identifying miRNAs that are critical to tumor angiogenesis.

RESULTS

Since glioma cells have a high capacity to induce angiogenesis (Brem et al., 1972; Folkerth, 2000), we used them as a means to stimulate this process in normal endothelial cells in a coculture system. Primary human microvascular endothelial cells isolated from normal human brain (HBMVECs; Cell Systems, ACBRI-376) were cultured in the presence or absence of human U87 glioma cells expressing cerulean fluorescent protein (CFP) in endothelial basal medium lacking additional angiogenic factors (EBM; Cambrex). Elongation of the endothelial cells was induced by the cancer cells as a first step in the activation of angiogenesis, as described previously (Khodarev et al., 2003) (Figure 1A). After 24 hr of either culturing the endothelial cells alone or coculturing them with human U87 glioma cells, the endothelial cells were isolated using CD31 magnetic beads (Dynal Biotech). The purity (>99%) of the endothelial cell preparation was confirmed by the absence of glioma cells expressing the CFP marker (data not shown). Total RNA was isolated from endothelial cells, and the small RNA fraction was hybridized to miRNA arrays containing probes for 407 mature miRNAs (as in Krichevsky et al., 2003) in order to identify differentially expressed miRNAs. Analysis of array hybridizations revealed 80 miRNAs expressed in HBMVECs at detectable levels (Figure 1B; see also Figure S1 available online) and confirmed the expression of a number of previously described miRNAs in endothelial cells (Kuehbacher et al., 2007; Poliseno et al., 2006; Suárez et al., 2007; Tuccoli et al., 2006). After exposure of HBMVECs to U87 glioma cells, the expression levels of a number of miRNAs changed significantly. This suggests that glioma cells can influence miRNA expression in endothelial blood vessel cells (Figures 1B and 1C). Most of the differentially expressed miRNAs were found to be downregulated. One miRNA, miR-296, was identified and further confirmed by quantitative RT-PCR (qRT-PCR) analysis to be upregulated. We used miR-186 as a control miRNA and GAPDH as a normalization control, both of which were uniformly expressed in endothelial cells in the presence or absence of tumor cells