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Hypoxia-Inducible mir-210 Regulates Normoxic Gene Expression Involved in Tumor Initiation

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SUMMARY

Previous studies have suggested that the HIF transcription factors can both activate and inhibit gene expression. Here we show that HIF1 regulates the expression of mir-210 in a variety of tumor types through a hypoxia-responsive element. Expression analysis in primary head and neck tumor samples indicates that mir-210 may serve as an in vivo marker for tumor hypoxia. By Argonaute protein immunoprecipitation, we identified 50 potential mir-210 targets and validated randomly selected ones. The majority of these 50 genes are not classical hypoxia-inducible genes, suggesting mir-210 represses genes expressed under normoxia that are no longer necessary to adapt and survive in a hypoxic environment. When human head and neck or pancreatic tumor cells ectopically expressing mir-210 were implanted into immunodeficient mice, mir-210 repressed initiation of tumor growth. Taken together, these data implicate an important role for mir-210 in regulating the hypoxic response of tumor cells and tumor growth.

INTRODUCTION

Hypoxia, the condition of insufficient oxygen supply to tissues, results from a reduction in oxygen availability, inadequate oxygen transport, or the inability of the tissues to utilize oxygen. In normal tissues, high altitude exposure, anemia, and drugs can induce hypoxia-responsive pathways in the body. However, hypoxia is also a hallmark of cancer, where cancer cells shift their energy production from the TCA cycle to glycolysis (Kim and Dang, 2006). The hypoxia-inducible factors (HIFs) are a family of transcription factors that have been identified as important regulators of the cellular response to hypoxia (Semenza, 1998). Under normoxic conditions, the α subunit of HIF1 is hydroxylated at prolines 402 and 564 by prolyl-4-hydroxylases (PHDs), targeting HIF1α for proteasome destruction mediated by the von Hippel-Lindau (VHL) protein, an E3 ubiquitin ligase (Chan et al., 2005; Ivan et al., 2001; Jaakkola et al., 2001). Under hypoxic conditions, the activity of PHDs decreases, HIF1α is stabilized and transcriptionally regulates a large number of target genes involved in adaptation and protection against low oxygen conditions (Chi et al., 2006).

HIFs regulate an ever-increasing number of genes involved in glycolytic metabolism, angiogenesis, erythropoiesis, and metastasis (Chan and Giaccia, 2007; Semenza, 1998; Sullivan and Graham, 2007). Recently, microRNAs (miRNAs) have emerged as a new class of noncoding genes involved in regulating cell proliferation, differentiation, and viability (Bartel, 2004; Stefani and Slack, 2008). miRNAs are single-stranded small RNA molecules that are approximately 22 nucleotides in length. miRNAs primarily regulate gene expression through inhibition of RNA translation by base-pairing of their ''seed region,'' nucleotides 2–8, to their target genes' 3′UTR (Nilsen, 2007). miRNAs can also facilitate targeting of specific mRNAs for cleavage, resulting in the downregulation of target mRNAs (Jackson and Standart, 2007; Lim et al., 2005). It has been shown that miRNA expression is regulated by certain physiological stimuli (van Rooij et al., 2006). We hypothesized that some miRNAs are regulated by hypoxia given the critical roles oxygen homeostasis plays in cellular physiology and the broad spectrum of genes hypoxia regulates. Several recent studies have shown that mir-210 is induced by hypoxia and appears to be a HIF target gene, although no identification of functional HREs have been demonstrated (Camps et al., 2008; Giannakakis et al., 2008; Kulshreshtha et al., 2007).

In this report, we identified several hypoxia-regulated miRNAs through miRNA microarray analysis. We present evidence showing that one of the miRNAs, mir-210, is the predominant miRNA gene induced under hypoxic conditions in a broad spectrum of cancer types, and its transcriptional induction is HIF1α dependent. By immunoprecipitating the major functional component of the miRNA pathway, Argonaute 2 (AGO2), we identified a list of 50 genes as potential mir-210 targets, of which the majority are not known to be induced under hypoxia. The regulation of mir-210 by HIF can explain how HIF activation leads to inhibition of gene expression. Functionally, we show that ectopic expression of mir-210 represses tumor growth, linking HIF regulation to inhibition of tumor growth through mir-210 regulation.

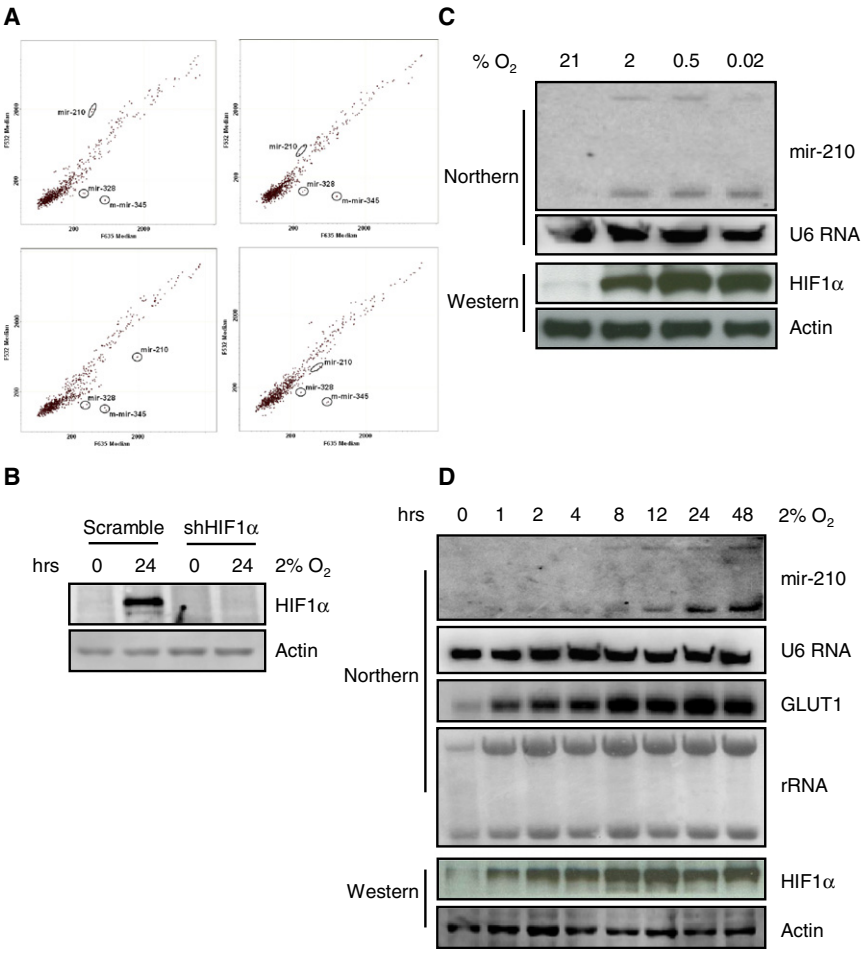

Figure 1. mir-210 Is the Predominant Hypoxia-Responsive miRNA

(A) Microarray analysis of miRNAs induced during the cellular response to hypoxia. SU86.86 (left panels) and SU86.86/shHIF1α cells (right panels) were split into two plates 24 hr before the hypoxic treatment, respectively. One plate stayed in normoxia, and the other was exposed to 2% O_2 for 16 hr. Then RNAs were harvested at the same time and used to conduct microarray analysis. Data are presented on a scatter plot showing log10-transformed signal intensities for each probe on both channels for the Cy3-labeled normoxic control and for the Cy5-labeled hypoxic sample (top panel). In the parallel dye swap experiment, normoxic sample was labeled with Cy5, and hypoxic sample was labeled with Cy3 (bottom panel). mir-210 is identified as the most robustly induced miRNA, and its induction is dependent on HIF1α.

(B) Western blot confirms efficient HIF1α knockdown by the shRNA construct in SU86.86 cells used in the microarray experiment.

(C) mir-210 is induced under different hypoxic stringencies by northern blot. SU86.86 cells were split into four plates 24 hr before treatment. Then each plate was exposed to normoxia, 2%, 0.5%, or <0.02% (anoxia) O_2 for 24 hr. Small nuclear RNA U6 was used as a loading control.

(D) The kinetics of mir-210 induction under 2% O_2 in SU86.86 cells. mir-210 expression reached the plateau after 24 hr of hypoxia and stayed high until 48 hr (top panel), which is consistent with the control, *GLUT1*, a classic HIF-regulated gene (middle panel). Western blot of HIF1α was shown to indicate hypoxia condition (bottom panel).

RESULTS

mir-210 Is the Predominant Hypoxia-Inducible MicroRNA

To identify miRNA genes regulated by hypoxia, we analyzed the expression profiles of the human pancreatic cancer cell lines SU86.86 and PANC1 using a miRNA microarray containing 314, 49, and 14 probes corresponding to mature forms of human, mouse, and rat miRNAs, respectively. After 16 hr under 2% oxygen, paired nomoxia/hypoxia miRNA samples were labeled with either Cy3 or Cy5. In order to detect nonspecific labeling and hybridization, dye-swapped experiments were performed in parallel. Several miRNAs appeared to be robustly induced by hypoxia (Figure 1A), such as hsa-mir-328, mmu-mir-345, and hsa-mir-210. However, in the dye swap experiment, only the expression of hsa-mir-210 changed reciprocally (Figure 1A). Because HIF1α is a major regulator of the cellular response to hypoxia, we established a SU86.86 cell line that expresses a small hairpin RNA (shRNA) that reduces HIF1α expression to less than 10% of the wild-type controls (Figure 1B). After stable knockdown of HIF1α, the induction of mir-210 under hypoxia was undetectable (Figure 1A), which suggests that mir-210 is a HIF1α-regulated gene. Subsequent miRNA northern verification of the miRNA microarrays indicated that several other miRNA genes were also induced by hypoxia, although their induction was not as pronounced as mir-210 (see Figure S1 available online). For these reasons, mir-210 was chosen for further analysis in the present study.

Since it has been known that certain genes/pathways are activated under specific oxygen tensions, such as the unfolded protein response pathway (UPR), we investigated the inducibility of mir-210 under more stringent hypoxic conditions. As shown in Figure 1C, mir-210 is induced by mild as well as stringent oxygen tensions. Furthermore, the kinetics of mir-210 induction under 2% oxygen is similar to a classic hypoxia regulated gene, *GLUT1* (Figure 1D). Taken together, our data suggest that mir-210 is highly responsive to changes in oxygen.

mir-210 Is Broadly Expressed and Is HIF1α Dependent

To investigate whether induction of mir-210 under hypoxia is tissue or cell-line specific, we examined the induction of mir-210 in pancreatic, breast, head and neck, lung, colon, and renal cell lines exposed to 2% oxygen for 24 hr by northern blotting. mir-210 was almost universally induced in all cell lines across different tissue origins (Figure 2A). Generally, mir-210 expression was low under normoxic conditions, and its expression was greatly induced after exposure to hypoxia.