Published OnlineFirst September 7, 2012; DOI: 10.1158/0008-5472.CAN-12-1383

Microenvironment and Immunology

Cancer Research

Hypoxia-Inducible miR-210 Regulates the Susceptibility of Tumor Cells to Lysis by Cytotoxic T Cells

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Abstract

Hypoxia in the tumor microenvironment plays a central role in the evolution of immune escape mechanisms by tumor cells. In this study, we report the definition of miR-210 as a miRNA regulated by hypoxia in lung cancer and melanoma, documenting its involvement in blunting the susceptibility of tumor cells to lysis by antigen-specific cytotoxic T lymphocytes (CTL). miR-210 was induced in hypoxic zones of human tumor tissues. Its attenuation in hypoxic cells significantly restored susceptibility to autologous CTL-mediated lysis, independent of tumor cell recognition and CTL reactivity. A comprehensive approach using transcriptome analysis, argonaute protein immunoprecipitation, and luciferase reporter assay revealed that the genes PTPN1, HOXA1, and TP53I11 were miR-210 target genes regulated in hypoxic cells. In support of their primary importance in mediating the immunosuppressive effects of miR-210, coordinate silencing of PTPN1, HOXA1, and TP53I11 dramatically decreased tumor cell susceptibility to CTL-mediated lysis. Our findings show how miR-210 induction links hypoxia to immune escape from CTL-mediated lysis, by providing a mechanistic understanding of how this miRNA mediates immunosuppression in oxygen-deprived regions of tumors where cancer stem-like cells and metastatic cellular behaviors are known to evolve. *Cancer Res; 72(18); 4629–41. ©2012 AACR.*

Introduction

Cytotoxic T lymphocytes (CTL) are important effector cells in tumor rejection and play a crucial role in host defense against malignancies in both mouse and human (1, 2). Currently, most cancer immunotherapy approaches involve the generation of CTLs against tumor-associated antigens (TAA) through vaccination strategies that induce or optimize TAA-specific immune responses (3). However, tumor rejection does not always follow successful induction of tumor-specific immune responses (4). Numerous studies have shown a paradoxical coexistence of cancer cells with TAA-specific T cells in immune-competent hosts. Moreover, tumor cells themselves play a crucial role in controlling the antitumor immune response (5), allowing them to maintain their functional disorder and evade destruction by CTLs. In this regard, it has been suggested that tumor cell growth *in vivo* is not only influenced by CTL-tumor cell recognition (6) and tumor susceptibility to cell-mediated death, but also by the complex and highly dynamic tumor microenvironment, providing very important clues to tumor development and progression (7).

Hypoxia, a common feature of solid tumors and one of the hallmarks of the tumor microenvironment, is known to favor tumor survival and progression (8, 9). Although hypoxia has been reported to play a major role in the acquisition of tumor resistance to cell death (10, 11), the molecular mechanisms that enable the survival of hypoxic cancer cells have not been fully elucidated. Recently, attention has been focused on the mechanisms by which hypoxic tumor cells alter their transcriptional profiles to modulate glycolysis, proliferation, survival, and invasion, allowing them to persist under the conditions of hypoxic stress (12, 13).

Emerging evidence has shown that aberrantly expressed miRNAs are highly associated with tumor development, progression, and specific clinical phenotypes such as disease progression or recurrence (14). Recently, a set of hypoxia-regulated miRNAs (HRM) were identified that suggest a link between a tumor-specific stress factor and control of gene expression (15, 16). One particular miRNA, miR-210, has been frequently reported as the master regulator of tumor hypoxic response (17); however, a significant number of additional miRNAs have also been linked to the cellular response to hypoxia (18). Although the role of miR-210 in tumorigenesis, angiogenesis, mitochondrial metabolism, cell survival, and DNA repair has been well characterized (17), its role in the immune response remains unknown. Of particular interest is its role in the regulation of tumor susceptibility to antigen-specific killer cells.

We have previously reported that hypoxic induction of HIF1α and pSTAT3 (19) and autophagy (20) modulates tumor

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doi: 10.1158/0008-5472.CAN-12-1383

Published OnlineFirst September 7, 2012; DOI: 10.1158/0008-5472.CAN-12-1383

cell susceptibility to CTL-mediated lysis. In this study, we conducted miRNA profiling to improve our understanding of tumor cell resistance to CTL-mediated cell lysis under hypoxic stress. We provide evidence that hypoxia-induced miR-210 regulates tumor cell susceptibility to CTL-mediated cell lysis by a mechanism involving its downstream targets PTPN1, HOXA1, and TP53I11.

Materials and Methods

Culture of tumor cells and CTLs

The human IGR-Heu NSCLC (non–small cell lung carcinoma) cell line and its autologous TIL clone Heu171 were derived and maintained in culture as described (21). The human melanoma cell line NA-8 and CTL clones R11, R18P1, and R2C9 were provided by Dr. Pedro Romero (Ludwig Center for Cancer Research, Lausanne, Switzerland). Human NSCLC tissues were obtained from Dr. Fathia Mami-Chouaib (INSERM U753, IGR, France).

Reagents and antibodies

SDS was obtained from Sigma. Mouse anti-HIF-1α from BD Transduction Laboratories; rabbit anti-PTPN1, anti-pSTAT3, anti-PIM1, and mouse anti-STAT3 from Cell signaling; and rabbit anti-TP53I11, mouse anti-Actin-HRP, and goat-anti-HOXA1 from Abcam.

Hypoxic conditioning of tumor cells

Hypoxic treatment was conducted in a hypoxia chamber as previously described (19).

^{51}Cr cytotoxicity assay

The cytotoxic activity of the CTL clone (Heu171) was measured by a conventional 4-hour ^{51}Cr release assay (22).

Tumor necrosis factor and interferon production assay

TNF-β and IFN-γ production by the CTL clones Heu 171 (cocultured with IGR-Heu) and R2C9 (cocultured with NA-8) were measured as described (19).

Flow cytometry analysis

Flow cytometry analysis was conducted by using a FACS-Calibur flow cytometer (19).

Gene silencing by RNA interference

Predesigned siRNA (HIF-1α, PTPN1, HOXA1, and TP53I11) were obtained from Ambion and transfected as described earlier (19).

miR-210 blockade and overexpression

Transfections were conducted with anti-miR-210 (miR inhibitor, Ambion) and pre-miR-210 (miR precursor, Ambion) under hypoxic and normoxic conditions, respectively. siPORT NeoFx Transfection Agent (NeoFx; Ambion) was used for transfection according to manufacturer's instructions. Anti-miR-CT (miR negative control, Ambion) and pre-miR-CT (miR positive control, Ambion) were used as controls under hypoxic and normoxic conditions, respectively.

RNA isolation and SYBR-GREEN qRT-PCR

Total RNA was extracted from the samples with TRIzol solution (Invitrogen). DNase I-treated 1 μg of total RNA was converted into cDNA by using TaqMan Reverse Transcription Reagent (Applied Biosystems) and mRNA levels were quantified by SYBR-GREEN qPCR method (Applied Biosystems). Relative expression was calculated by using the comparative C_t method (2-ΔC_t). Primer sequences are available upon request.

MicroRNA (miR) isolation and detection and microRNA microarray experiment

For extraction of miRs, TRIzol (Invitrogen) was used. DNase I-treated total RNA (8 ng) was subjected to qRT-PCR analysis using TaqMan miR Reverse Transcription Kit (Applied Biosystems). The miR-210 was detected and quantified by using specific miRNA primers from Ambion. Expression levels of mature miRNAs were evaluated using comparative C_t method (2-ΔC_t). Transcript levels of RNU44 were used as endogenous control. miRNA microarray analysis was conducted using Agilent human miRNA microarray. Rosetta resolver software was used for analysis. The microarray data related to this paper have been submitted to the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-1157.

Western blot analysis

Western blotting was conducted as previously reported (20).

Immunoprecipitation of FLAG/HA Ago2-containing RISC—argonaute 2 ribonucleoprotein immunoprecipitation

IGR-Heu cells were transfected with pIRESneo-FLAG/HA Ago2 (Addgene plasmid 10821; ref. 23) by using Lipofectamine 2000 Transfection Reagent (Invitrogen). Immunoprecipitation was conducted as described (24). B2M, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and 18S (shown to be RISC-associated, but not miR-210 targets) were used for normalization.

Luciferase reporter assay for miR-210 target gene validation

The 3′ UTRs of PTPN1, HOXA1, and TP53I11 were cloned into a pSI-CHECK-2 vector by PCR amplification of genomic DNA. Primer sequences are available upon request. IGR-Heu cells were cotransfected with 800 ng pSI-CHECK-2 and 10 nmol/L of pre-miR in 24-well plates with Lipofectamine 2000 (Invitrogen) in OPTIMEM (Invitrogen) medium. After 48 hours, firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega).

Immunohistochemical staining for CA-IX expression

Hypoxic zones were detected by CA-IX staining on human NSCLC sections. Immunohistochemistry was conducted as previously described (25).

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Locked nucleic acid-based *in situ* detection of miR-210 in human NSCLC tissues

Human NSCLC sections were fixed in 4% paraformaldehyde. miRNA ISH Optimization Kit 2 (FFPE; Exiqon) was used for miR-210 staining as per manufacturer's protocol.

Statistics

Data were analyzed with GraphPad Prism. Student *t* test was used for single comparisons. Data were considered statistically significant when *P* was less than 0.005.

Results

miR-210 is the miRNA predominantly induced by hypoxia in NSLCC and melanoma cells

To identify the miRs that are induced by hypoxia in non–small cell lung cancer (NSCLC) and melanoma, we conducted miRNA expression profiling using human NSCLC (IGR-Heu) and human melanoma (NA-8) cell lines following incubation of these cells under conditions of normoxia (21% O_2) or hypoxia (1% O_2) for 6, 16, or 24 hours (Fig. 1A–D). Several miRs that exhibited more than a 2-fold change under hypoxia were identified in both cell lines. Twenty-six miRs were upregulated in IGR-Heu cells and 9 miRs were upregulated in NA-8 cells at one or more time points under hypoxia as compared with normoxia (Supplementary data S1). Among these putative hypoxia-induced miRs, miR-210 was highly upregulated in IGR-Heu cells (Fig. 1A) and NA-8 cells (Fig. 1C). Volcano plot analysis further confirmed that miR-210 was the most highly (more than 10-fold change) and significantly (adjusted *P* value < 0.05) upregulated miR under hypoxia in IGR-Heu cells (Fig. 1B) and NA-8 cells (Fig. 1D).

Expression of miR-210 in hypoxic zones of human NSCLC tissues

To determine whether miR-210 is selectively expressed in the hypoxic zones of human NSCLC tissues, we first selected human NSCLC tissues on the basis of a well-established hypoxia marker (CA-IX; carbonic anhydrase-9; ref. 13) expression by immunohistochemistry (data not shown). We identified 12 CA-IX–positive tissues out of 31 NSCLC specimens. We next conducted miR-210 staining by *in situ* hybridization on serial sections. As shown in Fig. 2A and B, miR-210–positive staining was observed in the hypoxic zones (CA-IX–positive staining) of tumor areas. While 23% of the tumor areas were positively stained for CA-IX, 19% of the tumor areas were positive for miR-210. As depicted in Fig. 2C, an overlap of CA-IX and miR-210 staining was observed: 68% of the CA-IX–positive cells were also positive for miR-210. Moreover, using ImageJ JACoP analysis, a positive Pearson correlation coefficient between miR-210 expression ($r = 0.685$) and CA-IX positivity was found. These findings clearly indicate *in vivo* miR-210 expression in the hypoxic zones of NSCLC tissues.

miR-210 upregulation in melanoma and NSCLC under hypoxia is HIF-1α–dependent

We next measured the expression levels of miR-210 in hypoxic IGR-Heu and NA-8 tumor cells. As shown in Fig. 3A, hypoxia resulted in the significant induction of miR-210 after 6 hours (4-fold), 16 hours (6-fold), and 24 hours (10-fold) in IGR-Heu cells. Similarly, Fig. 3B shows that miR-210 was upregulated in NA-8 cells as early as 6 hours (2-fold), 16 hours (4-fold) and 24 hours (6-fold) of hypoxia induction. Similar results were obtained by using a panel of 5 human NSCLC and 5 human melanoma cell lines (Supplementary data S2). We further show that siRNA-mediated knockdown of HIF-1α with 3 different siRNAs efficiently silenced HIF-1α and completely inhibited miR-210 induction in IGR-Heu cells under hypoxia (Fig. 3C). Same results were obtained using NA-8 cells (Fig. 3D). Collectively, these results indicate that hypoxia-induced miR-210 is HIF-1α–dependent.

Hypoxia-induced miR-210 regulates tumor cell susceptibility to CTL-mediated lysis

To investigate the potential role of hypoxia-induced miR-210 in regulating the hypoxic tumor cell susceptibility to CTL-mediated lysis, IGR-Heu and NA-8 tumor cells were transfected with anti-miR-210 (miR-210 inhibitor) or anti-miR-CT (miR-control inhibitor) and cultured under hypoxia. Transfection of these cells with anti-miR-210 resulted in the abrogation of miR-210 expression in both IGR-Heu (Fig. 3E) and NA-8 (Fig. 3F) cell lines as compared with anti-miR-CT (miR-control inhibitor).

We next examined the susceptibility of IGR-Heu and NA-8 tumor cells in which miR-210 expression had been abrogated to CTL-mediated lysis under hypoxic conditions. Interestingly, as shown in Fig. 3G, there was a significant and remarkable restoration of tumor cell susceptibility to CTL-mediated lysis in hypoxic IGR-Heu cells transfected with anti-miR-210 as compared with anti-miR-CT. More interestingly, this restoration of susceptibility to CTL-induced killing in miR-210 abrogated IGR-Heu tumor cells (anti-miR-210–transfected cells) was observed at all effector:target ratios (E:T) tested in comparison with control cells. Similarly, data depicted in Fig. 3H clearly show that hypoxic NA-8 cells transfected with anti-miR-210 were more susceptible to CTL-mediated lysis as compared with cells transfected with anti-miR-CT. Similar results were obtained for NA-8 cells with 2 different CTL clones (Supplementary data S3). These data strongly indicate that hypoxia-induced miR-210 plays an important role in the acquisition of tumor cell resistance to CTL-mediated lysis.

miR-210 in hypoxic cells does not alter tumor cell recognition and CTL reactivity

We next examined the influence of miR-210 on MHC class I molecule expression. As shown in Table 1, no difference in staining with HLA class I-specific antibody and HLA-A2–specific antibody was observed in hypoxic IGR-Heu cells transfected with miR-210 inhibitor (anti-miR-210) as compared with cells transfected with anti-miR-CT. Similarly, no difference was observed in normoxic IGR-Heu cells transfected with miR-210 precursor (pre-miR-210) as compared with miR-control precursor (pre-miR-CT). Similar results were obtained for NA-8 cells (Table 1). Next, we analyzed the reactivity of the

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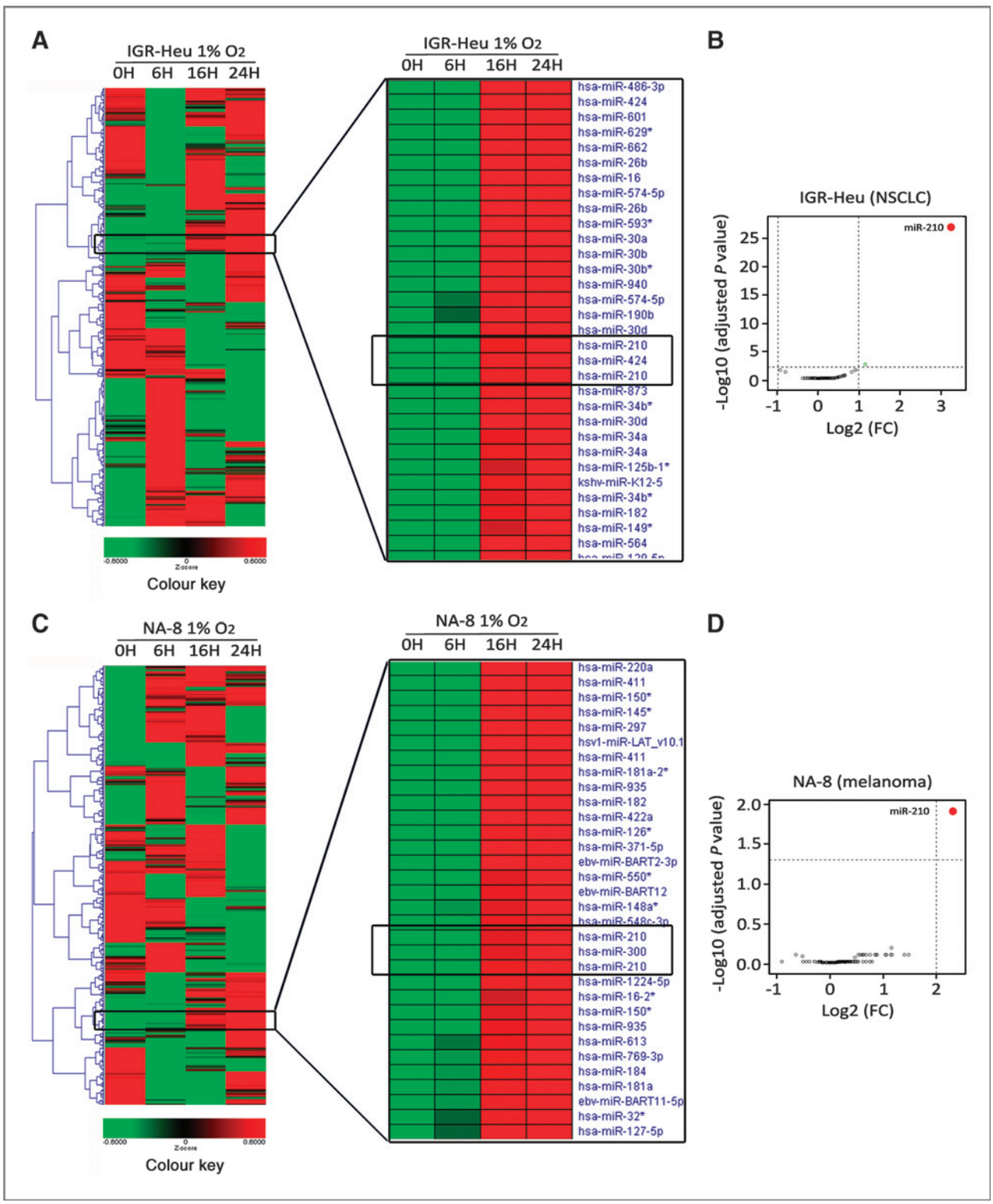

Figure 1. Hypoxic stress on tumor cells results in the upregulation of miRNA-210 (miR-210). A, heat map of the hypoxia regulated miRNAs in IGR-Heu cells. Red signifies upregulation, whereas green signifies downregulation. B, volcano plot of miRNA expression (Log_2 fold change) and adjusted *P* values for IGR-Heu under hypoxia 1% O_2. C, miR-210 is significantly upregulated in NA-8 tumor cells. Heat map of the hypoxia regulated miRNAs in NA-8 cells. Red signifies upregulation, whereas green signifies downregulation. D, volcano plot of miRNA expression (Log_2 fold change) and adjusted *P* values for NA-8 under hypoxia (1% O_2). Data shown are representative of 2 independent experiments.

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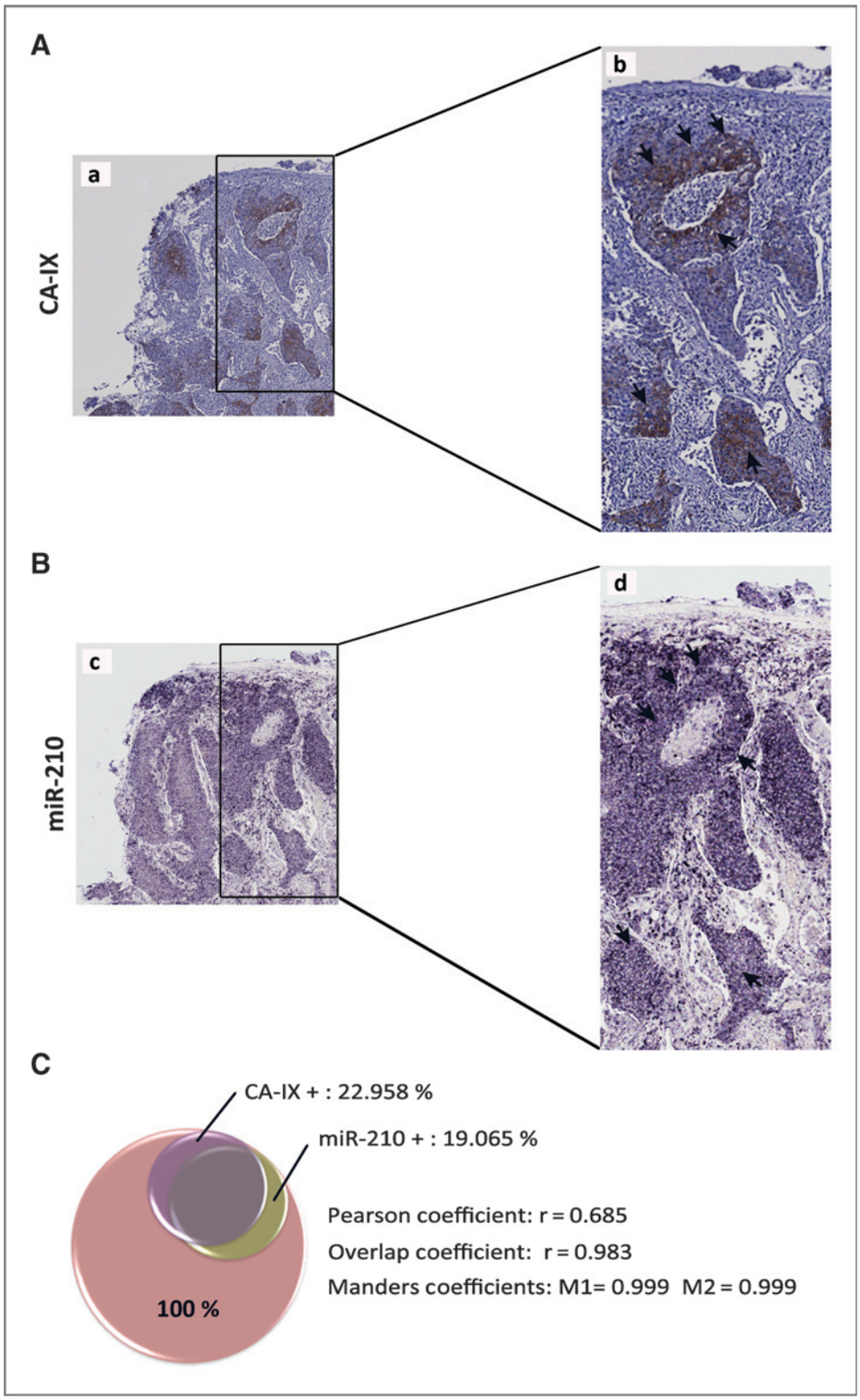

Figure 2. Hypoxia induced miR-210 staining in human NSCLC tissues colocalizes with CA-IX. Human NSCLC biopsies were stained for CA-IX expression by immunohistochemistry and miR-210 expression by *in situ* hybridization. A and B are representative images of CA-IX/miR-210 staining on serial sections of specimen (IGR-2220). C, Venn diagram representation of the staining analysis of CA-IX and miR-210 showing overlap on serial sections of specimen (IGR 2220). Colocalization analysis was conducted by using JACoP Plug-in in ImageJ software. Arrows, areas positively stained for CA-IX and miR-210. Magnification, ×50.

CTL clone to hypoxic autologous targets with abrogated miR-210. We observed no difference in TNF-α and IFN-γ production by the autologous T-cell clone in response to stimulation with IGR-Heu cells (Fig. 4A and C) and NA-8 cells (Fig. 4B and D) with abrogated (anti-miR-210) or overexpressed miR-210 (pre-miR-210). These results clearly show that miR-210 does not alter tumor cell recognition and CTL priming in IGR-Heu and NA-8 tumor cells.

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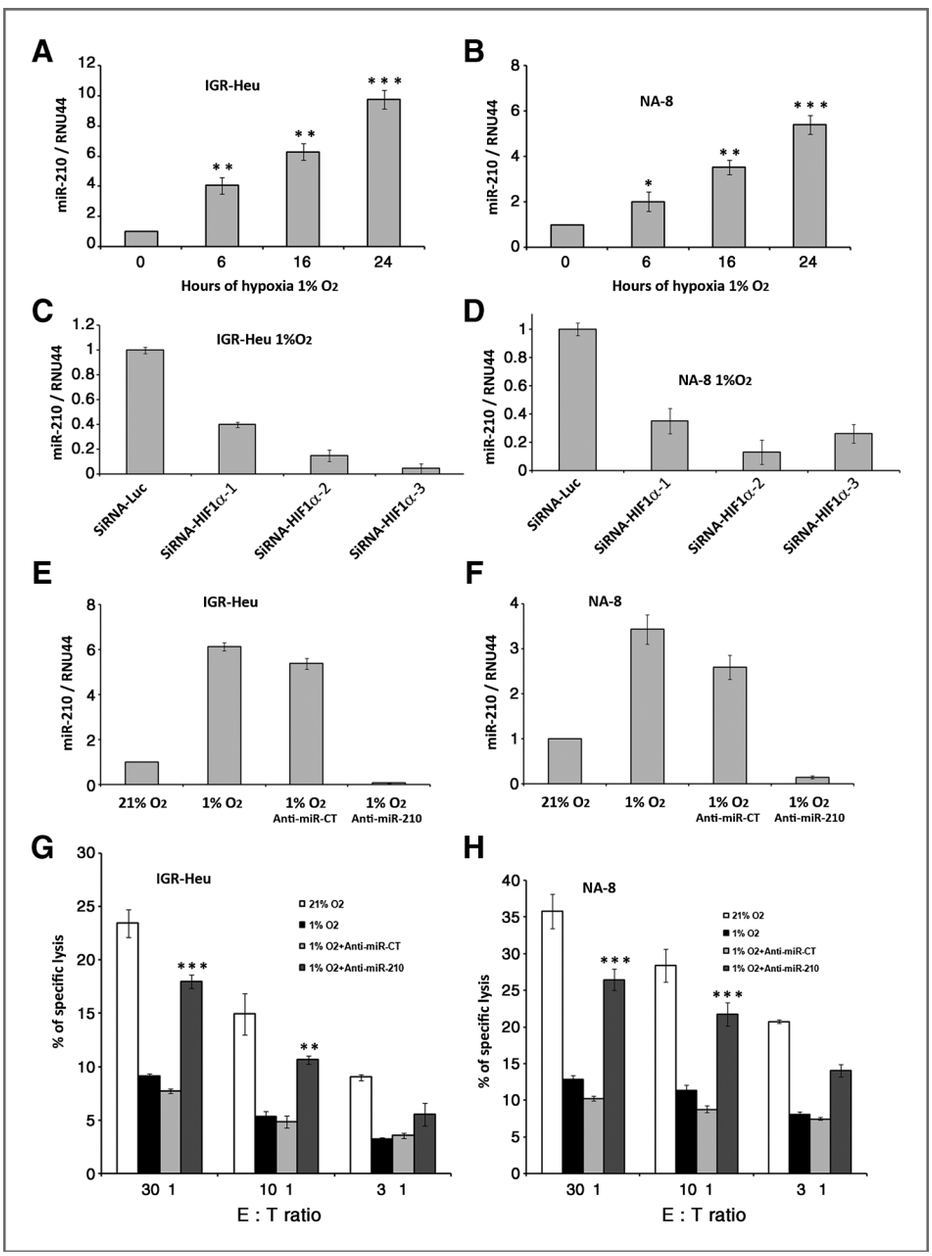

Figure 3. Hypoxia-induced miR-210 is HIF-1α–dependent in IGR-Heu cells and targeting miR-210 by anti-miR-210 under hypoxia resulted in the restoration of tumor cell susceptibility to CTL-mediated lysis. A, miR-210 expression was monitored by TaqMan qRT-PCR in IGR-Heu with or without exposure to 1% O_2 hypoxia at indicated times. B, miR-210 expression was monitored by TaqMan qRT-PCR in NA-8 with or without exposure to 1% O_2 hypoxia at indicated times. Expression levels of RNU44 were used as endogenous control. C, expression of miR-210 in IGR-Heu tumor cells transfected with different siRNAs targeting either HIF-1α or luciferase (Luc) and cultured under hypoxic conditions (1% O_2) for 24 hours. D, expression of miR-210 in NA-8 cells transfected with different siRNAs targeting either HIF-1α or luciferase (Luc) and cultured under hypoxic conditions (1% O_2) for 24 hours. E and F, TaqMan qRT-PCR showing successful knockdown of hypoxia-induced miR-210 in IGR-Heu tumor cells (E) and NA-8 cells (F). G, CTL-mediated lysis of IGR-Heu tumor cells at different E:T ratios. Heu 171 cells were used as effectors. Data represents 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$). H, CTL-mediated lysis of NA-8 cells at different E:T ratios. R2C9 TIL clone was used as effector.

Table 1. Anti-miR-210 and pre-miR-210 does not affect tumor cell recognition in IGR-Heu and NA-8 tumor cells

	(NSCLC) IGR-Heu				(Melanoma) NA-8			
	HLA Class I		HLA-A2		HLA Class I		HLA-A2	
Conditions	21% O_2	1% O_2	21% O_2	1% O_2	21% O_2	1% O_2	21% O_2	1% O_2
Medium	99.9% (8444)	99.8% (9083)	99.5% (6151)	99.8% (6150)	99.91% (635.62)	99.96% (590.09)	99.92% (113.19)	99.91% (84.71)
Pre-miR-CT	99.8% (8222)		99.4% (6121)		99.90% (625.25)		99.90% (109.85)	
Pre-miR-210	97.5% (7745)		99.5% (5609)		99.87% (580.02)		99.91% (105.78)	
Anti-miR-CT		99.9% (7560)		99.7% (5365)		99.97% (580.12)		99.89% (87.89)
Anti-miR-210		99.9% (7599)		99.3% (5988)		99.98% (595.56)		99.90% (90.55)

NOTE: Analysis of surface expression of MHC class-I in IGR-Heu and NA-8 tumor cells under different conditions. HLA class I and HLA-A2 surface expression was detected by using W632 and MA2.1 antibodies, respectively. Isotypic control mAb (IgG) was used.

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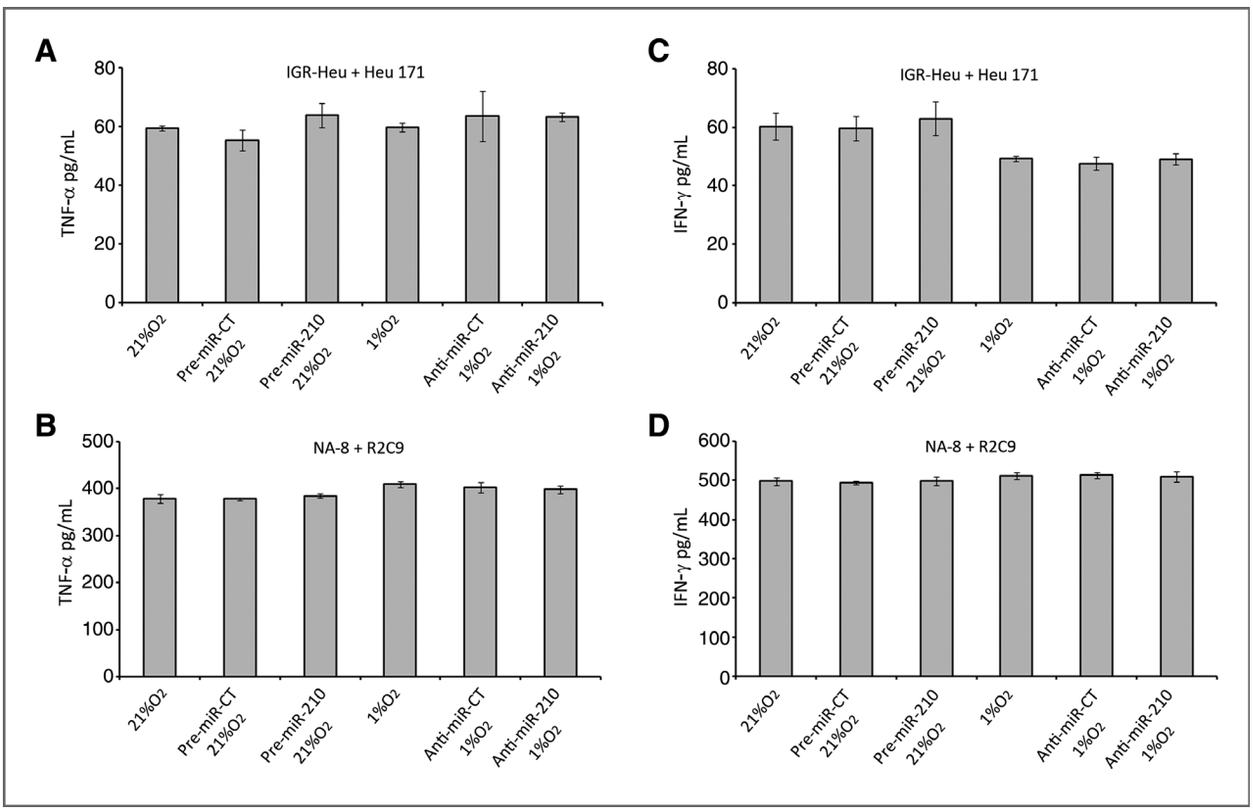

Figure 4. Anti-miR-210 and pre-miR-210 does not affect CTL priming in IGR-Heu and NA-8 tumor cells. A–D, TNF-α and IFN-γ production by the CTL clones Heu 171 (cocultured with IGR-Heu) and R2C9 (cocultured with NA-8) for 24 hours under different conditions. Data represents 3 independent experiments with SD.

miR-210 has no effect on hypoxia-induced transcription factors (HIF1α, HIF2α, and pSTAT3) in IGR-Heu cells

It has been well established that HIF1α, HIF2α, and pSTAT3 are the main mediators of hypoxia-induced responses in tumors (19, 20). We next wondered whether miR-210 is able to regulate these hypoxia-induced factors (HIF1α, HIF2α, and pSTAT3) in IGR-Heu cells. We observed that both HIF1α and pSTAT3 were increased under hypoxic conditions (6, 16, 24, 48, and 72 hours of 1% O_2). However, abrogation or overexpression of miR-210 (anti-miR-210 in hypoxic IGR-Heu cells and pre-miR-210 in normoxic cells) had no effect on HIF-1α and pSTAT3 protein levels. We also quantified, using the same experimental conditions, changes in mRNA expression levels of HIF1α, HIF2α, VEGF, and GLUT, no difference was observed (Supplementary data S4).

Identification of miR-210 candidate target genes in IGR-Heu tumor cells

We next conducted a comprehensive transcriptome analysis using RNA from IGR-Heu cells transfected with anti-miR-210 or anti-miR-CT under hypoxia. We selected a panel of 44 genes (validated miR-210 targets) from a miRNA database, miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/), which has the largest number of validated miRNA-target interactions (MTI).

We selected 11 genes that meet the following criteria:

1. Significantly downregulated under hypoxia as compared to normoxia.
2. Significantly upregulated when hypoxic IGR-Heu cells were transfected with anti-miR-210 as compared to transfected with anti-miR-CT.
3. Significantly downregulated when transfected with pre-miR-210 under normoxic conditions (Supplementary data S5).

As shown in Fig. 5A and B, the expression levels of ISCU, E2F3, FGFRL1, NPTX1, PTPN1, HOXA1, TP53I11, RAD52, and EFNA3 were, respectively, increased or downregulated following anti-miR-210 and pre-miR-210 transfection in IGR-Heu cells.

We selected 3 of these 11 identified genes (PTPN1, HOXA1, and TP53I11) based on their involvement in tumor cell death and apoptosis. These identified target genes were further validated by Western blot analysis. Figure 5C clearly shows that the protein level of PTPN1 was significantly decreased by pre-miR-210 as compared with pre-miR-CT under normoxia, and significantly increased when IGR-Heu cells were transfected by anti-miR-210 as compared with anti-miR-CT under hypoxia. Similar results were obtained for HOXA1 (Fig. 5D) and TP53I11 (Fig. 5E). PIM1 was used as control (Fig. 5F). Taken together, these results point to the regulation of PTPN1, HOXA1, and TP53I11 at both mRNA and protein levels by miR-210.

Validation of PTPN1, HOXA1, and TP53I11 as miR-210 target genes

To validate the identified targets, we inserted miR-210 binding sites sequence from the 3′ UTR of PTPN1, HOXA1, and TP53I11 mRNA (Fig. 6A) into the 3′ UTR of pSI-check2

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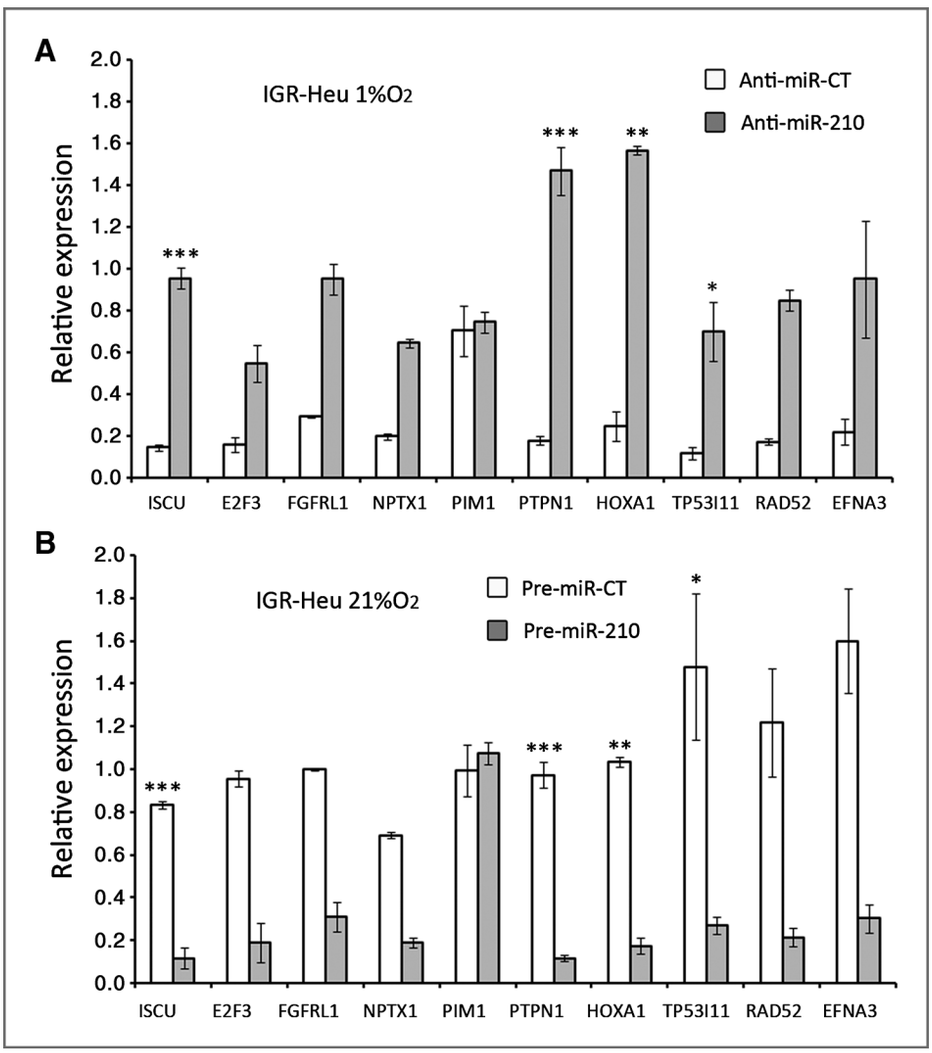

Figure 5. Confirmation of a panel of mirtarbase (MTI-miRNA target identification) database-identified targets in IGR-Heu tumor cells. A, IGR-Heu tumor cells were transfected with anti-miR-210 or anti-miR-CT used as a control and cultured under hypoxia (1% O_2) for 24 hours. ISCU, E2F3, FGFRL1, NPTX1, PIM1, PTPN1, HOXA1, TP53I11, RAD52, and EFNA3 expression was monitored by SYBR-GREEN qRT-PCR. B, IGR-Heu tumor cells were transfected with pre-miR-210 or pre-miR-CT used as a control and cultured under normoxia (21% O_2) for 24 hours. ISCU, E2F3, FGFRL1, NPTX1, PIM1, PTPN1, HOXA1, TP53I11, RAD52, and EFNA3 expression was monitored by SYBR-GREEN RT-qPCR. Expression level of 18S was used as endogenous control.

vector and conducted luciferase reporter assays. We show that pre-miR-210 significantly reduced the luciferase activities of PTPN1, HOXA1, and TP53I11 reporters by 65% to 40% and 25%, respectively, as compared with pre-miR-CT (Fig. 6B). In contrast, pSI-check2 (empty vector control) and PIM1 reporter luciferase activities were not repressed by pre-miR-210, confirming that PTPN1, HOXA1, and TP53I11 target sites directly mediates repression of the luciferase activity through seed-specific binding. In addition, we used a biochemical assay based on the immunoprecipitation of RNA-induced silencing complexes (RISC) enriched for miR-210 and its targets. To accomplish this, IGR-Heu cells were stably transfected with pIRESneo-FLAG/HA Ago2 and cultured under normoxic or hypoxic conditions for 24 hours. miR-210 was selectively enriched in Ago2-HA immunoprecipitates under hypoxia as compared with normoxia, suggesting that the argonaute protein immunoprecipitation (miRNP-IP) worked efficiently (Supplementary data S6). To further validate this, IGR-Heu cells stably expressing pIRESneo-FLAG/HA Ago2 were transfected with pre-miR-210 or pre-miR-CT and cultured under normoxia. First, we showed that ISCU and EFNA3, 2 well-established miR-210 targets (24), were significantly enriched in normoxic pre-miR-210-Ago2-HA immunoprecipitates (Fig. 6C). Furthermore, as shown in Fig. 6C, PTPN1, HOXA1, and TP53I11 were selectively and significantly increased in the immunoprecipitates of the IGR-Heu cells transfected with pre-miR-210 as compared with pre-miR-CT. It is worth noting that B2M, GAPDH, and PIM1 remain unchanged (Fig. 6D). These results clearly show that PTPN1, HOXA1, and TP53I11 are validated targets of miR-210 in IGR-Heu cells.

miR-210 modulates IGR-Heu tumor target cell susceptibility to CTL-mediated lysis by targeting PTPN1, HOXA1, and TP53I11

We next asked whether miR-210 confers resistance in hypoxic tumor targets to CTL-mediated lysis by degrading its target genes (PTPN1, HOXA1, and TP53I11). Using a ^{51}Cr cytotoxicity assay and target gene silencing, we show that siRNA-mediated silencing of PTPN1 resulted in a significant decrease in the IGR-Heu tumor cell susceptibility to CTL-

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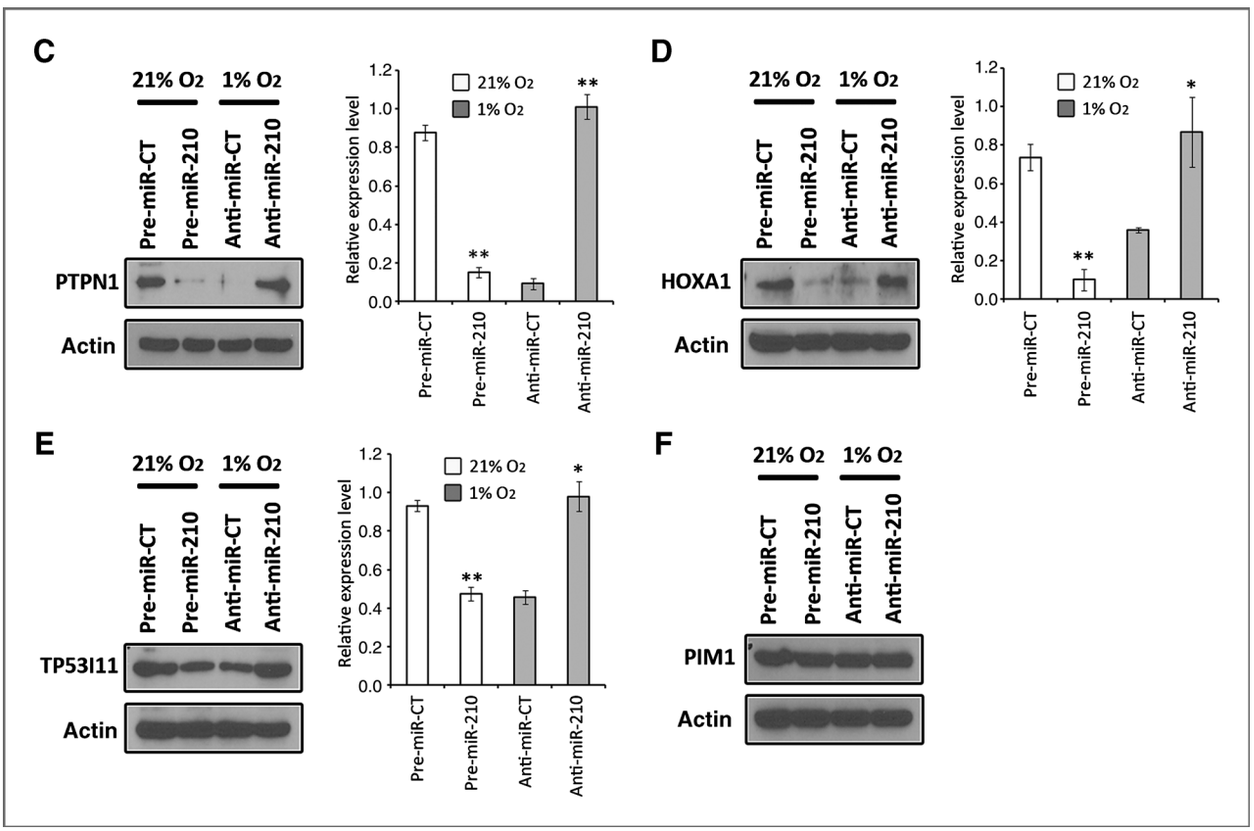

Figure 5. (*Continued*) C–F, IGR-Heu tumor cells were transfected with either anti-miR-210 or anti-miR-CT and cultured under hypoxia (1% O_2) or transfected with pre-miR-210 or pre-miR-CT and cultured under normoxia (21% O_2) for 24 hours. Western blot analysis and densitometry analysis were conducted to show PTPN1 (C), HOXA1 (D), and TP53I11 (E) protein levels, which were significantly regulated by miR-210. F, PIM1 is used as a control. Data represent 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$, and ***, $P < 0.0005$).

mediated lysis (Fig. 7A). However, siRNA silencing of HOXA1 (Fig. 7B) or TP53I11 (Fig. 7C) had no effect on tumor cell susceptibility to CTL-mediated lysis. Surprisingly, as depicted in Fig. 7D, siRNA silencing of all 3 genes (PTPN1, HOXA1, and TP53I11) resulted in a dramatic decrease in target susceptibility to CTL-mediated lysis. Taken together, these results strongly show that miR-210 is able to confer resistance in hypoxic target cells by degrading PTPN1, HOXA1, and TP53I11.

Discussion

Tumors have evolved to use hypoxic stress to their own advantage by activating key biochemical and cellular pathways that are important in angiogenesis, cell-cycle regulation, cell survival, progression, survival, metastasis, and resistance to apoptosis (9–11, 19). Tumor resistance remains a crucial issue in immunotherapy, as it limits the effectiveness of cancer immunotherapy (5, 6). It has become clear that a hypoxic tumor microenvironment plays a determining role in neutralizing the immune system of the host and negatively impacting the outcome of therapy (26). We observed a significant miRNA dysregulation between normoxic and hypoxic melanoma and NSCLC cells (Supplementary data S1). This is in agreement with the concept that tumors may adapt to hypoxic stress in different ways depending on their genetic background and phenotypic characteristics (10).

To our knowledge, this is the first time we showed that miR-210 is expressed in the hypoxic zones of NSCLC tissues and in hypoxic melanoma tumor cells. Our results support the claim that induction of miR-210 is the most important hypoxia-induced miR in different cancer types (17, 27). miR-210 is also generally recognized as a robust HIF1 target (16, 28) and an *in vivo* marker of tumor hypoxia (29, 30). The increased expression of miR-210 correlates with a poor prognosis in patients with breast and pancreatic cancer (31, 32). Zhang and colleagues provided evidence indicating that miR-210 levels are high in tumors and correlate with their metastatic behavior, suggesting a potential oncogenic role for miR-210 (33, 34).

Although the role of miR-210 in tumorigenesis, angiogenesis, mitochondrial metabolism, cell survival, and DNA repair has been well characterized (17), its role in the immune response is less well understood, in particular, its role in the regulation of tumor cell susceptibility to antigen-specific killer T cells. We showed for the first time that expression of miR-210 under hypoxic conditions correlates with the alteration of tumor susceptibility to CTLs by a mechanism independent of target recognition and alteration of CTL reactivity. Mandelboim and colleagues have shown that several human miRNAs are able to

Published OnlineFirst September 7, 2012; DOI: 10.1158/0008-5472.CAN-12-1383

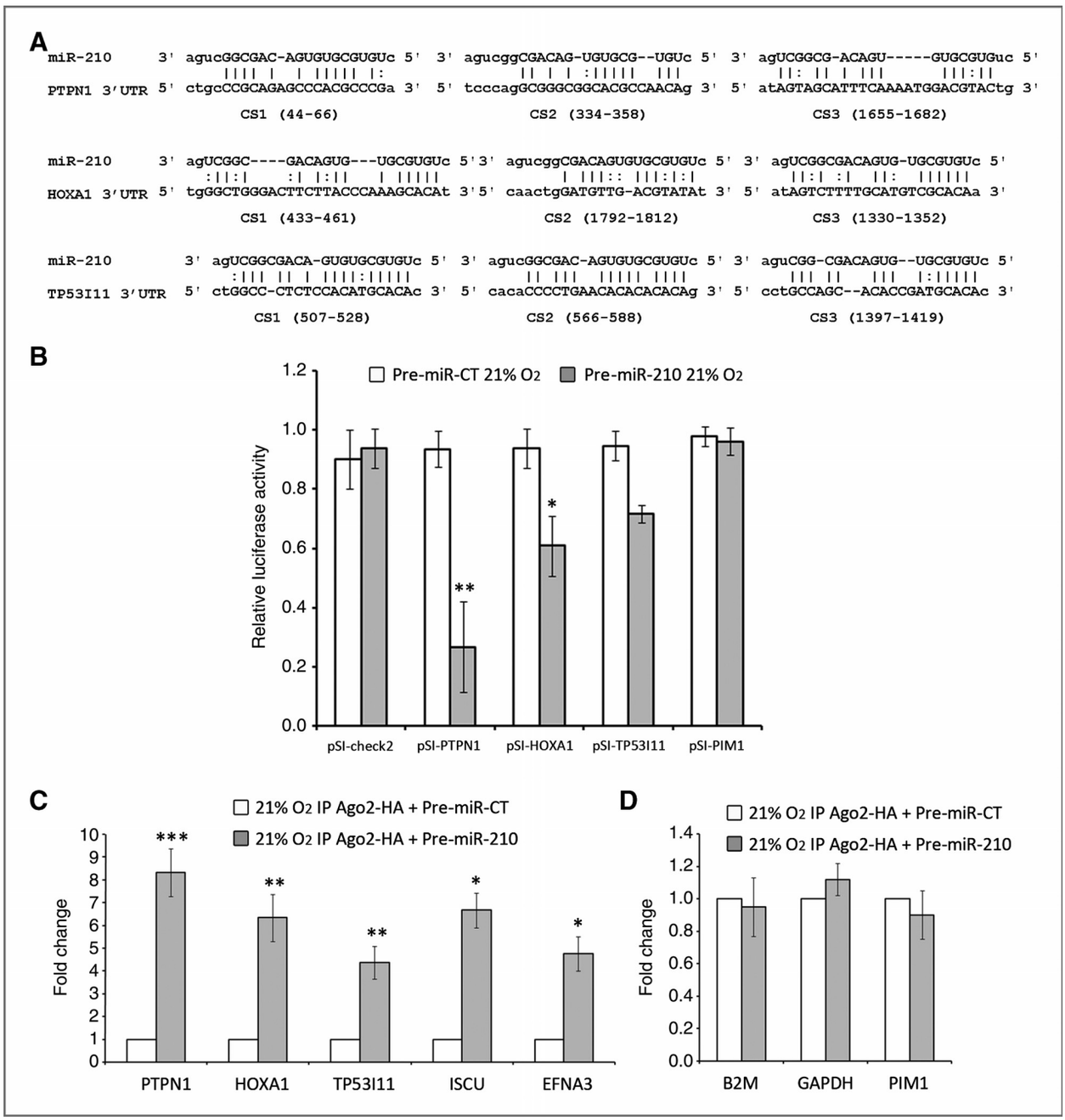

Figure 6. Confirmation of the identified miR-210 target genes. A, schematic view of miR-210 complementary sites (CS) in the 3′ UTR of PTPN1, HOXA1, and TP53I11 genes (predicted by miRanda). B, IGR-Heu cells were cotransfected with 10 nmol/L pre-miR-CT or pre-miR-210 and different pSI-check-2 constructs. After 48 hours, cells were harvested and luciferase activities analyzed. All *Renilla* luciferase activities were normalized to the firefly luciferase activity. pSI-PTPN1, pSI-HOXA1, pSI-TP53I11, and pSI-PIM1 correspond to distinct fragments of PTPN1, HOXA1, TP53I11, and PIM1 3′UTR containing miR-210 putative binding sites, respectively. pSI-check2 was used as an empty vector control. C, IGR-Heu cells stably expressing pIRESneo-FLAG/HA Ago2 were cotransfected with pre-miR-210 or pre-miR-CT and cultured under normoxia (21% O_2) for 24 hours. Anti-HA antibody was used to immunoprecipitate the miR-210/HA-Ago2–containing complexes. Anti-IgG antibody was used as IP-CT (immunoprecipitation control). RNA was purified and specific mRNAs were measured by SYBR GREEN qRT-PCR. D, B2M, GAPDH, and PIM1 did not display any significant differences and thus were used for normalization. Data represent 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$).

regulate stress-induced immune responses mediated by the NKG2D receptor (35). It has been recently reported that frequent loss of miR-34a/miR-34c expression helps metastatic melanoma cells to escape the tumor immune surveillance by enhancing ULBP2 expression (36). Similarly, a report of Udea and colleagues indicate that dicer-regulated miR-222 and miR-

Published OnlineFirst September 7, 2012; DOI: 10.1158/0008-5472.CAN-12-1383

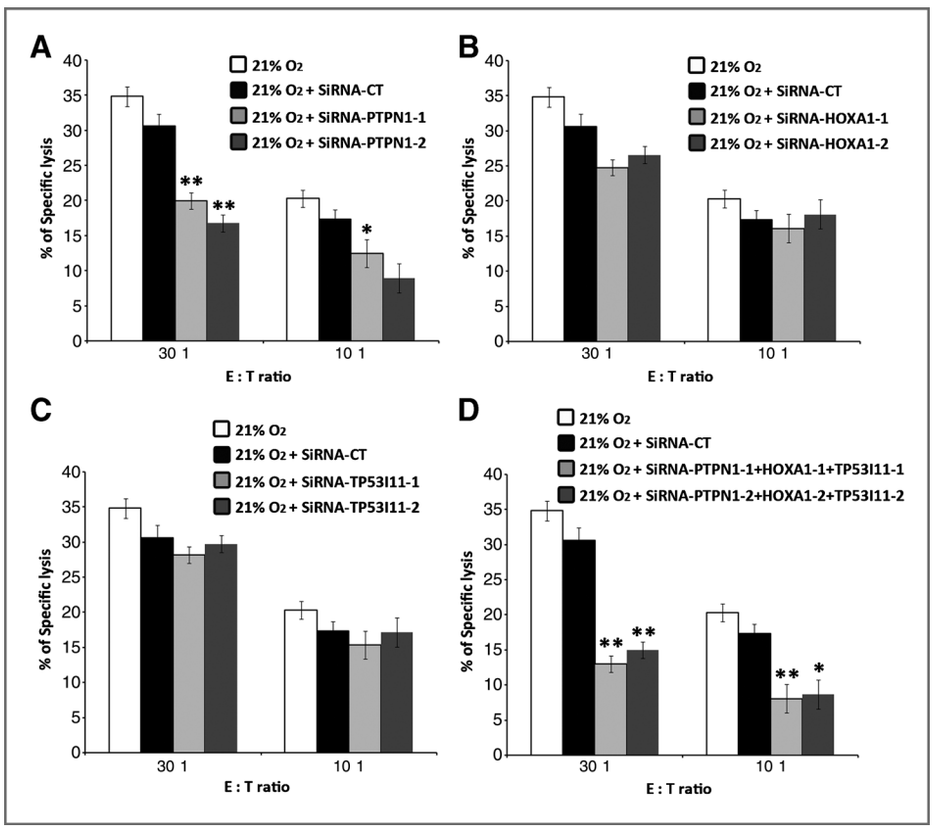

Figure 7. miR-210 targets PTPN1, HOXA1, and TP53I11 modulate IGR-Heu tumor target cell susceptibility to CTL-mediated lysis. IGR-Heu tumor cells were transfected with 2 different siRNA targeting either PTPN1 (A), HOXA1 (B), or TP53I11 (C), or siRNA targeting PTPN1 + HOXA1 + TP53I11 (D), or luciferase (Luc) used as a control and cultured under normoxic conditions (21% O_2) for 24 hours. Heu 171 cells were used as effectors. Data represent 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*, $P < 0.05$, **, $P < 0.005$; and ***, $P < 0.0005$).

339 promote resistance of cancer cells to CTLs by down regulating ICAM and subsequently affecting immune regulation (37).

To get more insight into how miR-210 confers resistance to hypoxic tumor targets to CTL-mediated lysis, we conducted a comprehensive transcriptome analysis to identify miR-210 target genes. Among these genes, E2F3, FGFRL1, and RAD52 have already been reported as validated targets of miR-210 involved in cell-cycle regulation and DNA repair (17, 38). However, we observed no effect on cell-cycle regulation when IGR-Heu cells were transfected with either anti-miR-210 or pre-miR-210 (data not shown). We showed that the expression levels of PTPN1, HOXA1, and TP53I11, were respectively upregulated or downregulated following anti-miR-210 or pre-miR-210 transfection in IGR-Heu cells. We also found that simultaneously silencing PTPN1, HOXA1, and TP53I11 resulted in a dramatic decrease in target susceptibility to CTL-mediated lysis. Among these 3 genes, PTPN1 has been shown to play a role in immune regulation. PTPN1 is a negative regulator of cytokine receptors and receptor tyrosine kinases in lymphohematopoietic cells (39). Knockdown of endogenous PTPN1 expression increases production of TNF-α, IL-6, and IFN-β in TLR-triggered macrophages (40). PTPN1 has been shown to function as a critical negative regulator of inflammatory responses (41). In addition, PTPN1 and TP53I11 have been reported to be involved in the regulation of cell survival and apoptosis (42–46). HOXA1 has been shown to be involved in cell proliferation and tumor initiation (30). It would be of major interest to determine how these genes interfere with CTL-induced hypoxic tumor cell death.

In conclusion, we provide evidence that hypoxia-induced miR-210 regulates tumor cell susceptibility to CTL-mediated lysis by a mechanism involving its downstream targets PTPN1, HOXA1, and TP53I11. Taken together, our studies show that miR-210 is mechanistically linked to the regulation of the antigen-specific tumor cell lysis and suggest that in addition to its potential as a prognostic biomarker, miR-210 may have therapeutic applications in the field of cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.Z. Noman, S. Buart, B. Janji, S. Chouaib
Development of methodology: M.Z. Noman, S. Buart, B. Janji, S. Chouaib
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.Z. Noman, P. Romero, S. Ketari, F. Mami-Chouaib, S. Chouaib
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.Z. Noman, S. Buart, S. Ketari, B. Mari, S. Chouaib
Writing, review, and/or revision of the manuscript: M.Z. Noman, S. Buart, P. Romero, B. Janji, F. Mami-Chouaib, S. Chouaib
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.Z. Noman, S. Chouaib
Study supervision: M.Z. Noman, B. Janji, F. Mami-Chouaib, S. Chouaib

Published OnlineFirst September 7, 2012; DOI: 10.1158/0008-5472.CAN-12-1383

Grant Support

This work was supported in part by grants from ARC (Association pour la Recherche sur le Cancer) N 1025, Ligue contre le Cancer (comité de Val de marne), INCA (Institut National du Cancer) Convention N 2009-009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 12, 2012; revised June 19, 2012; accepted July 2, 2012; published OnlineFirst September 7, 2012.

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Published OnlineFirst September 7, 2012; DOI: 10.1158/0008-5472.CAN-12-1383

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Published OnlineFirst September 7, 2012; DOI: 10.1158/0008-5472.CAN-12-1383

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Hypoxia-Inducible miR-210 Regulates the Susceptibility of Tumor Cells to Lysis by Cytotoxic T Cells

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Cancer Res 2012;72:4629-4641. Published OnlineFirst September 7, 2012.