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MiR-210 Links Hypoxia With Cell Proliferation Regulation in Human Laryngocarcinoma Cancer

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ABSTRACT

The microRNA hsa-miR-210 (miR-210) is associated with hypoxia; however its function has not fully identified. In the present study, we aim to detect its role concerning proliferation in Laryngocarcinoma. We found that miR-210 was highly expressed in hypoxia, which inhibited proliferation by inducing cell cycle arrest in G1/G0 as well as apoptosis. We further identified that miR-210 targeted fibroblast growth factor receptor-like 1 (FGFRL1). Down regulation of FGFRL1 decreased cell proliferation by promoting proportion of cells in G1/G0 phase and decreasing in S and G2/M phases. Moreover, overexpression of FGFRL1 effectively released the miR-210-induced suppression of SCC10A cell proliferation. Expression of miR-210 repressed tumor xenograft growth in vivo as well. Together, our findings reveal a new mechanism of adaptation to hypoxia that miR-210 inhibits the proliferation via inducing cell cycle arrest and apoptosis by the targeting of FGFRL1. J. Cell. Biochem. 116: 1039–1049, 2015.

KEY WORDS: MiR-210; HYPOXIA; CELL PROLIFERATION; LARYNGOCARCINOMA CANCER; FGFRL1

Human head and neck cancer is the eighth most common cancer in the US and the sixth most common one worldwide, representing 10–15% of all malignancies and causing 4–5% of all cancer-related deaths [American Cancer Society, 2012; Bădulescu et al., 2013]. Annually, there are approximately 650,000 patients diagnosed with head and neck cancers and 350,000 deaths in the world [Bădulescu et al., 2013]. Although intensive research and improvements in multimodality treatment, such as surgery, radiation, and chemotherapy, the 5-year survival still remains at 50–60% [Pulte and Brenner, 2010]. Early laryngeal cancer, one of the most common types of HNSCC, can usually be successfully treated with either radiotherapy or surgery. However, many advanced stage laryngeal carcinomas with limited treatment options are generally associated with considerable impairment to quality of life [Masuda et al., 2013]. Therefore, it is necessary to understand the underlying mechanism of tumor progression, as well as highlight the impact of genetic and molecular modifications in the early detection, the prediction of prognosis and therapeutic response to treatment of laryngeal carcinomas.

Hypoxia often occurs in tumors and tissue inflammation [Jordanovski et al., 2013]. Tumor hypoxia has been shown to be a

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Conflicts of interest: None.

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negative prognostic factor for head and neck cancers, associated with reduced therapeutic effect and decreased overall survival [Kikuchi et al., 2011; Rajendran et al., 2006], and maintaining tumor microenvironment. Therefore, hypoxia has recently been a focus of research. Hypoxia also regulates the expression of some genes that are sensitive to oxygen pressure. Hypoxia-inducible factor-1 (HIF1), as one of the most studied genes that is induced by low oxygen pressure, can subsequently influence the expression of a number of genes. This dysregulation can lead to many oncogenic phenotypes, such as tumor cell transformation, disruption of cellular metabolic processes, the invasive growth program, angiogenesis, and resistance to chemotherapy and radiation therapy [Shen et al., 2013].

MicroRNAs (miRNAs) are short, endogenous, non-coding RNA molecules (19–22 nucleotides) that bind to target mRNAs and cause RNA interference through translational repression or mRNA degradation [Ambros, 2008]. miR-210 is a main downstream effector gene of HIF-1, which is upregulated in most solid tumors including head and neck cancer [Gee et al., 2010; Wang et al., 2014], renal cell carcinoma, breast cancer [Rothe et al., 2011; Hong et al., 2012], prostate cancer [Porkka et al., 2007]. Its high levels have been linked to a negative clinical outcome and adverse prognosis [Porkka et al., 2007; Rothe et al., 2011; Hong et al., 2012; Wang et al., 2014]. miR-210 has been involved in tumor angiogenesis; this not only provides nutrients and oxygen, evacuates metabolic wastes and carbon dioxide to sustain cancer cells, but also facilitates metastasis [Hanahan and Weinberg, 2011]. Most of the evidence supports that miR-210 have significant effect on apoptosis [Fasanaro et al., 2008; Kim et al., 2009; Nie et al., 2011; Mutharasan et al., 2011]. miR-210's role in repressing cellular DNA damage response is supported by a recent study in which the expression of miR-210 results in double-stranded DNA breaks [Bindra et al., 2007]. Research has shown that miR-210 may have a much broader effect on cell cycle regulation by the downregulation of a group of mitosis-related genes, such as Cdc25B, Cyclin F, and Fam83D [He et al., 2013]. Overexpression of miR-210 promotes migration and invasion of breast cancer cell [Kosaka et al., 2013]. Together, miR-210 is involved in a wide variety of normal and pathological cellular processes including cell development, cell death, angiogenesis, metabolism, and oncogenesis [Chan et al., 2009; Huang et al., 2009; Gee et al., 2010; Shen et al., 2013]. However, the molecular mechanism of miR-210 in malignant diseases has not been completely understood. Therefore, an increased knowledge about miR-210's functions is necessary, which may finally lead to novel diagnostic and therapeutic approaches in cancer in future.

In this study, we aimed to investigate the mechanism of miR-210 inhibited tumor cell growth in laryngocarcinoma cancer . We found that high expression of miR-210 inhibited cell proliferation by down-regulating the expression of FGFRL1.

MATERIALS AND METHODS

MATERIALS

The following antibodies were used: HIF1α(BD company), a-tubulin (Sigma), Caspase (Santa Cruz Biotechnology), FGFRL1(GeneTex), GLUT1 (Epitomics). Anti-mouse and anti-rabbit secondary antibodies, conjugated to horseradish peroxidase for western blotting, were obtained from Vector Laboratories. FGFRL1 siRNA and scrambled siRNA come from Dharmacon.

CELL CULTURE AND TRANSFECTION

Head and neck squamous cell carcinoma (HNSCC) cell line SCC10A was derived from the primary lesion of a larynx carcinoma, and has been extensively characterized by its phenotypes in vitro and in vivo [Ballo et al., 1999; Zuo et al., 2011]. Hep2 was another larynx carcinoma cell, which was kindly provided by Prof. Guan in Cancer Instititute of Central South University. HEK293 (human embryonic kidney cell line) cells were purchased from Shanghai BIOLEAF Biotechnology Co., Ltd. (Shanghai, China). Cells were normally maintained at low passage in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen).

Briefly, for constitutive expression of miR-210, SCC10A, and Herp2 cells were transfected with miR-210 in a GV217 vector. The miR-210 vector GV217 was constructed in our lab by the primers: 5′-TCCGCTCGAGGGGTCGGGCTGGGC AGG-3′ and 5′-ATGGGG-TACCCCCCTCCCCACGGTATCC-3′. The stable transfected cells were selected with G418 to yield the miR-210 cell lines. The cell lines, which were not subsequently cloned, exhibited elevated levels of ectopically expressed miR-210, as confirmed by northen blot analysis and real-time PCR. Cloned cells lines were not used in the current study.

In addition, FGFRL1 without the 3′-UTR region expression vector GV219 was constructed in our lab and was transfected into SCC10A cells with Lipofectamine 2000 reagent (Invitrogen). Full-length human FGFRL1 without the 3′-UTR region gene sequence was amplified using the following primers: 5′- TCC GCTCGAGAT-GACGCCGAGCCCCCTGT -3′ and 5′-ATGGGGTACCCTAGCACT GA-TAGTGGATGTGCTG-3′. An FGFRL1-overexpressing plasmid GV219- FGFRL1 was constructed by inserting the FGFRL1 cDNA into an expression vector GV219/FGFRL1. The fragment containing the 3′-UTR of FGFRL1 fragment was cloned into pGL3 vector.

For RNA interference analysis, siRNA targeting FGFRL1 was delivered into SCC10A cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After 48 h of transfection, cells were subjected to proliferation assays as described below or cell lysates were collected and analyzed by Western blot.

RNA EXTRACTION AND QUANTITATIVE REVERSE TRANSCRIPTASE- PCR

Total RNA was extracted by using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. qRT-PCR was used to confirm the expression level of miRNAs. qRT-PCR was performed with a miR-specific primer on the ABI PRISM 7500 real-time PCR system (Applied Biosciences), compared with normalization control U6.

IN VIVO EXPERIMENTS

Balb/c nude mice were subcutaneously injected SCC10A cells (2×10^6 cells in 200 μl matrixgel/mouse). Tumors were measured every 3 days. Ten mice of each group were utilized for the in vivo tumor growth assay.The size of the tumors were determined by firstly

measuring length (L) and width (W) and then calculating the volume ($V = W^2 \times L \times 0.5$) [Zhang et al., 2013; Meiling et al., 2013].

ASSAY OF CELL PROLIFERATION

About cell proliferation assay, in brief, cells were plated at 3×10^3cells/well in 96-well plates with triplicate wells. Cell proliferation was performed using the MTT assay. OD was assessed by measuring the absorbance at 490 nm using a microtiter plate reader. MTT was purchased from Sigma.

CELL CYCLE ANALYSIS

Cell cycle analysis was performed using propidium iodide staining. Briefly, cells were washed in phosphatebuffered saline (PBS) and fixed in 70% ethanol. Fixed cells were then washed twice in PBS and stained in propidium iodide (50 μg/ml) in the presence of 50 μg/ml RNase A (Sigma–Aldrich), and then incubated for 1 h at room temperature in the dark. Flow cytometry analysis was done by using a FACScan (Beckman Coulter, Fullerton, CA). The data were analyzed by using WinMDI 2.8 software.

ANALYSIS OF CASPASE ACTIVITY

The caspase 3,7 fluorometric assay kit (Promega) was used to measure caspase 3,7 activity following the manufacturer's instructions.

WESTERN BLOT

Cells were extracted using lysis buffer [Zuo et al., 2010]. Protein concentration was measured with the bicinchoninic acid protein assay kit (Pierce) and processed for SDS-PAGE. After transferring ontonitrocellulose membranes (Millipore Corp.), proteins were probed with primary antibodies (FGFRL1 antibody, HIF1α, and actin from Santa Cruz Biotechnology) and secondary horseradish peroxidase coupled antibodies. Blots were developed by chemiluminescence using the enhanced chemiluminescence system.

LUCIFERASE REPORTER ASSAY

HEK293 and SCC10A cells were cultured in DMEM containing 10% fetal bovine serum in 5% CO_2 at 37°C. For transient transfection, briefly, 2×10^4 cells were plated in 96-well plates in DMEM plus 10% fetal bovine serum. After 12 h incubation, the cells were transfected with an 3′-untranslated region (3′-UTR) of FGFRL1 fragment using the Lipofectamine Plus (Invitrogen) transfection reagent. miR-210 was cotransfected with a plasmid expressing the the 3′-UTR of FGFRL1. As an internal control for transfection efficiency, 10 ng of pRL-TK were used. At 48 h after transfection, cells were harvested and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis of GLUT1 and FGFRL1 was carried out formalin-fixed and paraffin-embedded tissue sections using the standard immunohistochemical technique. Tissue sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and treated with an antigen retrieval solution (10 mmol/L sodium citrate buffer, pH 6.0). The sections were incubated with monoclonal mouse antihuman GLUT1 antibody (1:200 dilution), FGFRL1 antibody (1:200 dilution) overnight at 4°C and then were incubated with 1:1000 dilution of biotinylated secondary antibody followed by an avidin-biotin peroxidase complex (DAKO, Carpinteria, CA) according to the manufacturer's instructions. Finally, tissue sections were incubated with 3′,3′-diaminobenzidine (Sigma–Aldrich) until a brown color developed and counterstained with Harris' modified hematoxylin. Sections were blindly analyzed by two investigators in order to provide a consensus on results of IHC by light microscopy (Olympus). Evaluation of staining is according to Cheng report [Cheng et al., 2008]. Each case was rated according to a score that added a scale of intensity of staining to the area of staining. In brief, at least 10 high-power fields were randomly chosen, and >1,000 cells were counted for each section. The intensity of staining was analyzed by the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was graded as follows: 0, nostaining of cells; 1+, <30% of tissue stained positive; 2+, 30–60% positive staining; 3+, >60% positive staining. The minimum score when summed (extension + intensity) was therefore, 0, whereas the maximum score was 6. A combined staining score (extension + intensity) of ≤2 was considered to be a negative staining (low staining). A 3–4 score was a moderate staining, while a 5–6 score was evaluated to be a strong staining [Cheng et al., 2008].

LASER CAPTURE MICRODISSECTION

Laser Capture Microdissection (LCM) was applied to purify the tumor cells of xenograft from the mouse stromal cells, which was done as described [Cheng et al., 2008] with slight modifications. Briefly, 8 μl-thick frozen sections of xenograft tumor were placed on a membrane-coated glass slides (Leica), fixed in 75% alcohol for 30 s, and stained with 0.5% violet-free methyl green (Sigma). After washing in DEPC water for 30 s, the sections were air-dried for 1 min and microdissected by a Leica AS LMD system (Leica). The enrichment of cancer cells was store at −80°C until RNA extraction and verified by qRT-PCR for miR-210.

STATISTICS

Statistical Analysis Student's *t*-test was used for the statistical analysis of interval data, with $P < 0.05$ considered as significant. However, the statistical analysis of Goodman–Kruskal Gamma was applied in the analysis of correlation between GLUT1 and FGFRL1 expression level in LSC tissue.

RESULTS

MIR-210 RELATED WITH HYPOXIA INHIBITS THE CELL PROLIFERATION

As HIF1α is a major regulator of the cellular response to hypoxia, the kinetics of HIF1α (Top) induction under 2% oxygen were showed in head and neck cancer cell lines, SCC10A, and Hep2 cells (Fig. 1A). At the same time, the cell growth became much slower in hypoxia (Fig. 1B). To investigate the mechanism of hypoxia inhibiting the cell proliferation, we stably expressed miR-210 in SCC10A cells and Hep2 cells by transfecting with miR-210 in a GV217 vector firstly. Then we examined the expression of miR-210 in SCC10A and Hep2 cell lines by real-time PCR. The miR-210 cell lines represented stable

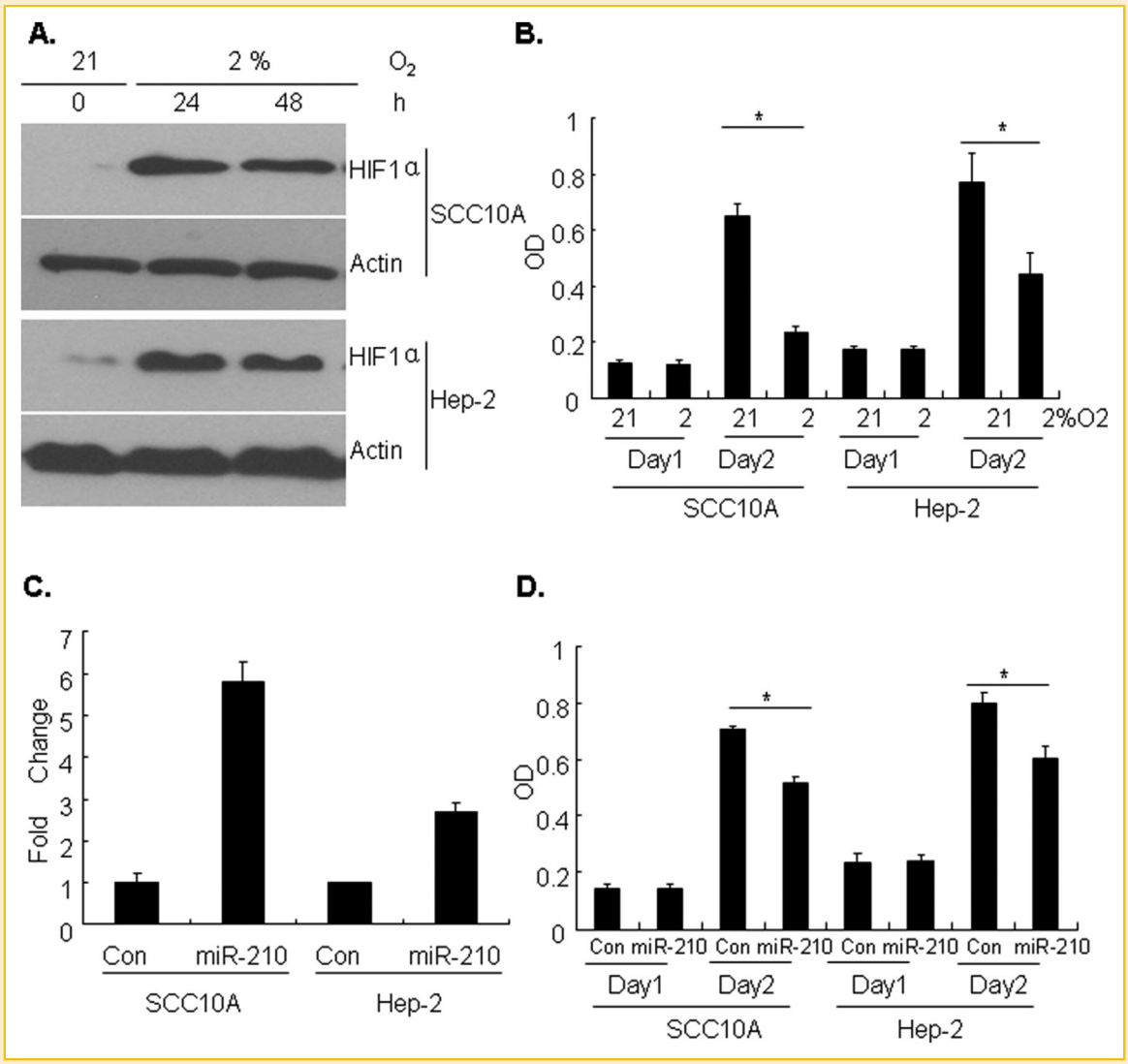

Fig. 1. High expression miR-210 inhibits proliferation of laryngocarcinoma cell. A. Western blot of HIF1α was shown to indicate hypoxia condition at different time point. Actin was used as a loading control. B. Hypoxia under 2% O_2 inhibits proliferation of Laryngocarcinoma cells. C. miR-210 expression is detected in SCC10A and Hep2 by real-time PCR. D. High expression of miR-210 inhibits proliferation of Laryngocarcinoma cell in SCC10A and Hep2. Error bar indicates standard deviation. Student's *t*-test was performed for statistical analysis, $^*P < 0.05$.

population of cells derived by transfection with an GV217/miR-210 plasmid (Fig. 1C). In order to elucidate the biological function of miR-210, we applied the miR-210 cell lines to carry out the cell proliferation assay. The data showed that overexpression of miR-210 down-regulated the cell proliferation (Fig. 1D) in normoxic condition.

Next, we investigate the function of miR-210 by choosing the SCC10A to further research. The data showed that overexpression of miR-210 leaded to a significant increase in the proportion of cells in G1/G0 phase, however, the proportion of cells in S phase significantly decreased. The proportion of cells in G2/M phase remained unchanged. These results showed that miR-210 resulted in cell cycle arrest (Fig. 2A). Interestingly, caspase-3/7 activity was significantly promoted in the overexpression of miR-210 cell compared with the control (Fig. 2B and C), which gave us a hint that overexpression of miR-210 may induce cell apoptosis. To summarize, the results show that miR-210 may inhibit proliferation of SCC10A cells mainly by inducing cell cycle arrest and apoptosis.

FGFRL1 IS ONE TARGET GENE OF MIR-210

In order to detect the meachnism of miR-210 inhibiting the cell proliferation, we carried out the analysis using miRNA target prediction programs miRanda. By miRNA target prediction, FGFRL1 was one of the target genes harboring the binding site of miR-210 in the 3′-UTR. To obtain further direct evidence that FGFRL1 is a target of miR-210, we characterized the binding site of miR-210 in the 3′-UTR of FGFRL1 mRNA. The results showed that miR-210 overexpression specifically decreased the luciferase activity in HEK293 cells (Fig. 3A)

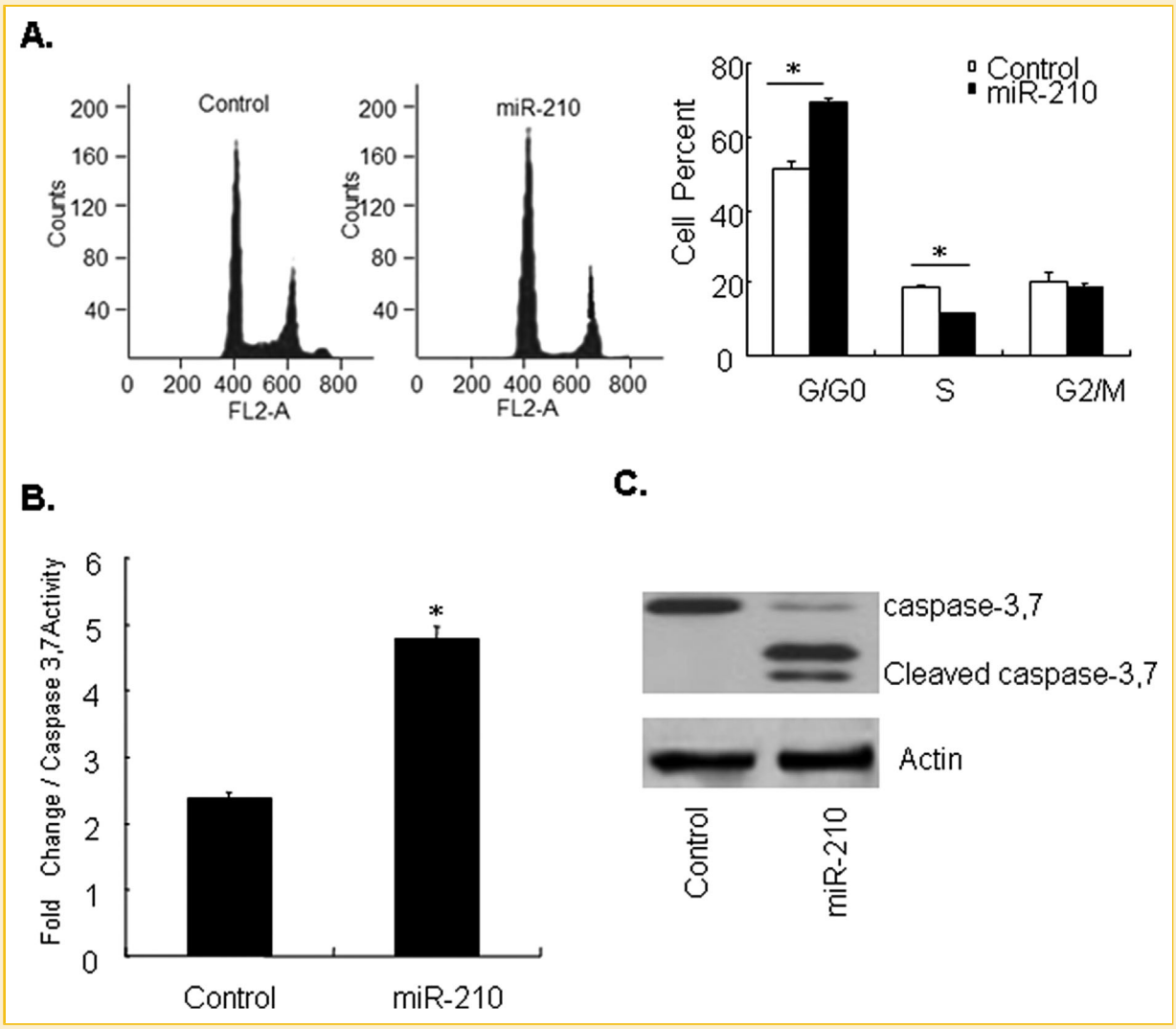

Fig. 2. High expression miR-210 induced cell apoptosis. A. The representative FACS profile was presented (left panel). The data about cell cycle was shown (Right panel, $^*P < 0.05$). B. The caspase-3,7 activity was investigated in SCC10A, $^*P < 0.05$. C. Western blot was shown to indicate caspase activation in SCC10A. Actin was used as a loading control.

and SCC10A (Fig. 3B). However, the repression was completely abolished when a mutant form of miR-210 expressing plasmid was cotransfected (Fig. 3A, B, and C). Furthermore, FGFRL1 protein level was decreased when miR-210 overexpressed in SCC10A (Fig. 3D).

In order to further verify the above result, we used immunohistochemistry to detect the expression of GLUT1 and FGFRL1 in the tissue of normal and LC. GLUT1 as a classical marker of hypoxia was not detected in the normal tissue, while FGFRL1 was high expression. On the contrary, GLUT1 was highly induced in the LC tissue. But the expression of FGFRL1 was inhibited (Fig. 2E). In the current study, we analyzed 211 samples of LC. The data showed that there was a statistically significant negative correlation between GLUT1 and FGFRL1 expression level by the analysis of Goodman–Kruskal Gamma (Table I). Together, the data indicate that FGFRL1 is a target gene of miR-210.

KNOCKDOWN OF FGFRL1 DECREASES CELL PROLIFERATION

In order to elucidate the function of FGFRL1, we applied the specific FGFRL1 siRNA to test the effect of FGFRL1 knockdown, which resulted in significant decrease of the level of FGFRL1 expression (Fig. 4A) and proliferation of SCC10A cells (Fig. 4B). Next, we examined the effects of FGFRL1 knockdown on the cell cycle. In addition, knockdown of FGFRL1 led to a significant promotion in the proportion of cells in G1/G0 phase and a decrease in S and G2/M phases (Fig. 4C). These data showed that knockdown of FGFRL1 induced cell cycle arrest in G1/G0. However, silence of FGFRL1 did not significantly change the caspase-3/7 activity (Fig. 4D), which indicated that FGFRL1 does not play role in apoptosis. Together, the results show that the down-regulation of FGFRL1 may inhibit proliferation of SCC10A cells mainly via cell cycle arrest.

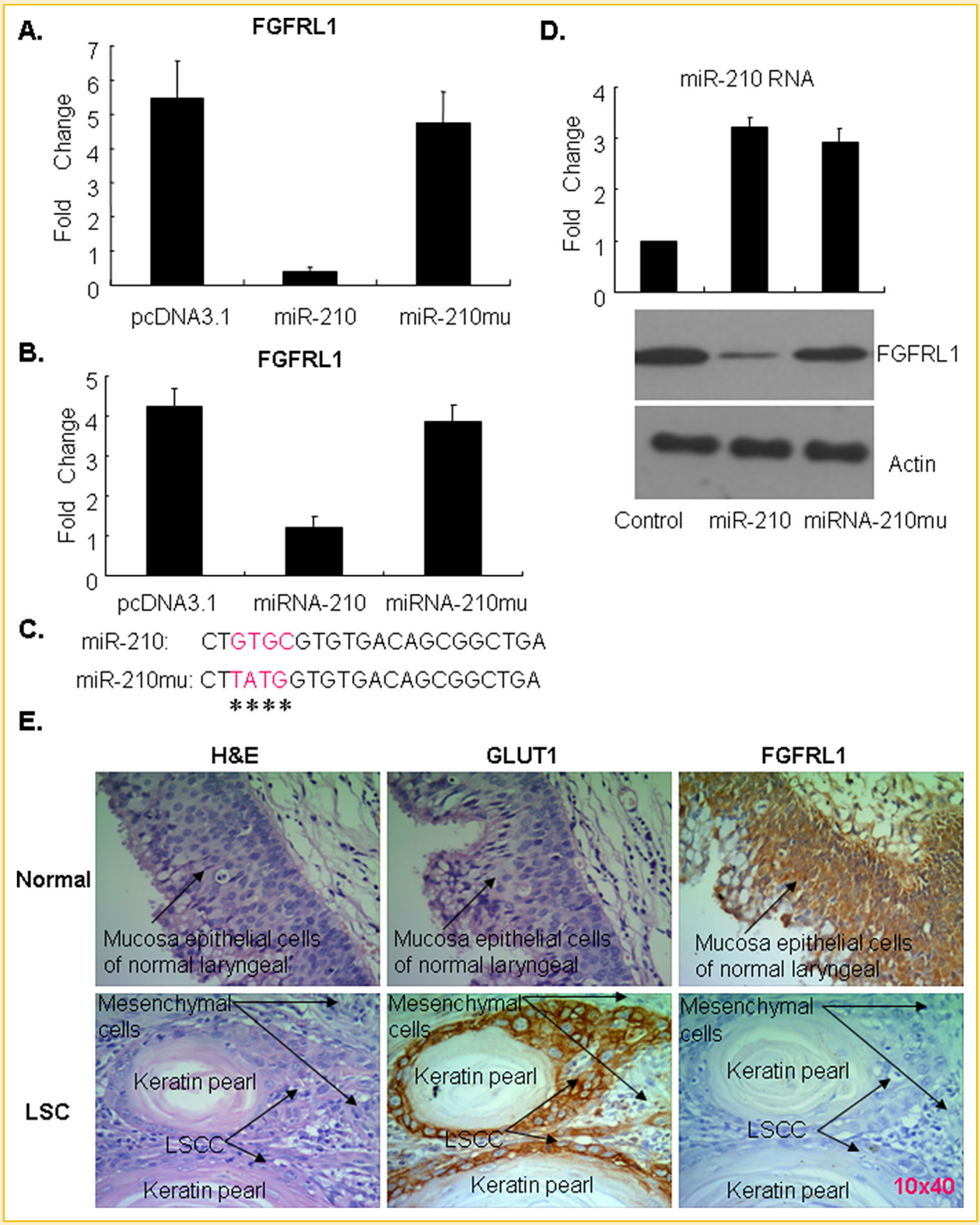

Fig. 3. FGFRL1 was a target gene of miR-210. A. Reporter luciferase activity is repressed or relieved by cotransfecting miR-210 wild-type or mutant expression vector with FGFRL1 3′-UTR constructs in HEK293 cells and SCC10A. B. Each reporter assay was repeated at least three times. Error bar indicates standard deviation. C. The mutations in miR-210 expression construct. The nucleotide mutation was introduced in the seed region of miR-210. D. High expression of miR-210 inhibits the expression of FGFRL1, while the mutations in miR-210 expression does not play role in the expression of FGFRL1. Real-time PCR done for miR-210 RNA extracted from control,miR-210-expressing and mutated miR-210-expressing cells (top panel). FGFRL1 expression was verified by immunoblotting with antibody to FGFRL1. Actin was used as a loading control (bottom panel). E. Representative results of immunohistochemistry of GLUT1 and FGFRL1 in the normal and LC tissues. Original magnification, 10 × 40.

TABLE I. The Expression Analysis of Glut1 and FGFRL1 in LC Tissue

	Glut1		
	Low	Middle	High
FGFRL1			
Low	6	14	79
Middle	5	9	23
High	55	12	8

Goodman–Kruskal Gamma Coefficient = −0.820, $P < 0.05$.

EXPRESSION OF MIR-210 REPRESSES TUMOR XENOGRAFT GROWTH

To investigate the biological effect of miR-210 in vivo, we implanted the control cell lines and the cells that stably express miR-210 subcutaneously into nude mice, in which the tumor growth of the cells highly expressing miR-210 was significantly inhibited (Fig.5A). Since miR-210 showed a clear inhibitory effect on tumor growth, we analyzed the sizes of harvested xenograft samples and their corresponding miR-210 expression level. As shown in Figure 5B, there is a statistically significant negative correlation between xenograft size and miR-210 expression level, further indicating miR-210 expression is growth inhibitory (Fig. 5B). Taken together, our data indicates that ectopic expression of miR-210 is primarily inhibiting tumor growth, since a significantly higher miR-210 expression still remains in these tumors whose growth was inhibited.

In order to further verify if the miR-210 target we identified is also regulated by miR-210 in vivo, we examined expression level of FGFRL1 protein, which is actually increased in cells transfected with a FGFRL1 expression vector without the 3′-UTR of FGFRL1 and miR-210 (Fig. 5C). Overexpression of FGFRL1 with no 3′-UTR significantly decreased the effection of miR-210 inhibiting cell proliferation compared to the control (Fig. 5D). These findings further suggest that FGFRL1 increases the proliferation of SCC10A cells by inhibiting cell cycle arrest. We also found that the inhibitory effect elicited by miR-210 on tumor growth was partly rescued by expressing FGFRL1 in vivo (Fig. 5E). All in all, high miR-210 expression inhibits tumor growth by down-regulating the expressing of FGFRL1 in vitro and in vivo.

DISCUSSION

Head and neck squamous cell carcinoma (HNSCC) is an aggressive malignancy and has a severe impact on the life quality of patients and survivors. More than two-thirds of patients present with advanced disease at time of diagnosis, which negatively affects the survival of these patients [Zuo et al., 2011; Boeckx et al., 2014]. Laryngocarcinoma is one of the most common malignant tumors in HNSCC and the mechanism involves genetics [Jiang et al., 2012]. Despite advances in our understanding and treatment of this disease, treatment resistance and relapse are common and have been related to tumor hypoxia. miR-210 is a significant marker of hypoxia and is robustly induced by HIF-1α directly through its interaction with its promoter sequence in response to hypoxia in HNSCC cell lines [Harris, 2010; Gee et al., 2010]. In the paper, we want to detect the function of miR-210 in laryngocarcinoma cells. The results show that high miR-210 expression induced by hypoxia, inhibits tumor growth by down-regulating the expressing of FGFRL1 (Fig. 5F).

MiR-210 is up-regulated in the most of solid tumors including clear cell renal cell carcinoma [Zhang et al., 2007], head and neck cancer [Gee et al., 2010], breast cancer [Hong et al., 2012], and prostate cancer [Porkka et al., 2007], which the expression levels correlate with clinical outcome negatively. In these reports, the overexpression of miR-210 may actually reflect the hypoxic status of the tumor samples instead of its biological function in tumorigenesis. Therefore, the overexpression of miR-210 is a well-known independent indicator of poor outcome of patient in these cases because it could predict poor prognosis [Huang et al., 2009; Camps et al., 2008].

Some reports show that miR-210 has some validated targets including the DNA repair enzyme RAD52 [Crosby et al., 2009], the receptor tyrosine kinase ligand ephrin-A3 [Fasanaro et al., 2008], and the transcription factor E2F3 [Giannakakis et al., 2008]. In the present study, we found FGFRL1 is another target of miR-210. Our data show that miR-210 targets to the FGFRL1 3′-UTR and suppresses FGFRL1 expression in LSCC. Moreover, knockdown of FGFRL1 by siRNA inhibited SCC10A cell proliferation, whereas overexpression of FGFRL1 effectively rescued the miR-210-induced suppression of SCC10A cell proliferation. Together, these findings show that miR-210 might exert its inhibition effect in SCC10A mainly by targeting FGFRL1. Interestingly, ectopic expression of miR-210 inhibits cell proliferation in the current study. This was consistent with the result that HIF1a can inhibit tumor growth [Maranchie et al., 2002; Raval et al., 2005] because it induces some genes leading to cell-cycle arrest and cell death [Goda et al., 2003; Kim et al., 2009]. In agreement with our findings, the miR-210 is deleted/downregulated in ovarian cancer [Giannakakis et al., 2008] and esophageal squamous cell carcinoma [Tsuchiya et al., 2010], which suggests a potential tumor suppressor function of miR-210. This conclusion is also consistent with Huang's report that FGFRL1 could partially rescue the suppression of tumor growth caused by ectopic expression of miR-210 in tumor xenografts [Huang et al., 2009]. However, targeting of FGFRL1 by miR-210 appears to explain only part of the action of miR-210.

We also confirmed that miR-210 induced cell apoptosis associated with activation of caspases, which is consistent with the reports indicating that miR-210 increases apoptosis in pulmonary arterial endothelial cells (HPAECs) in vitro [Chan et al., 2009]. However, FGFRL1 siRNA did not. In fact, FGFRL1 silencing and miR-210 overexpressions led to very distinctive consequences, which suggestes that the effect of miR-210-induced cell apoptosis was rather mediated by targeting other genes. Therefore, miR-210-induced cell cycle arrest and apoptosis might be regulated by other targets of miR-210. Though miR-210 was reported to promote cell-cycle progression by activating c-Myc through inhibiting MNT [Zhang et al., 2009]. However, in our systems we did not observe that overexpressing miR-210 offered an growth advantage of cells in vitro, and miR-210 actually repressed tumor growth in vivo.

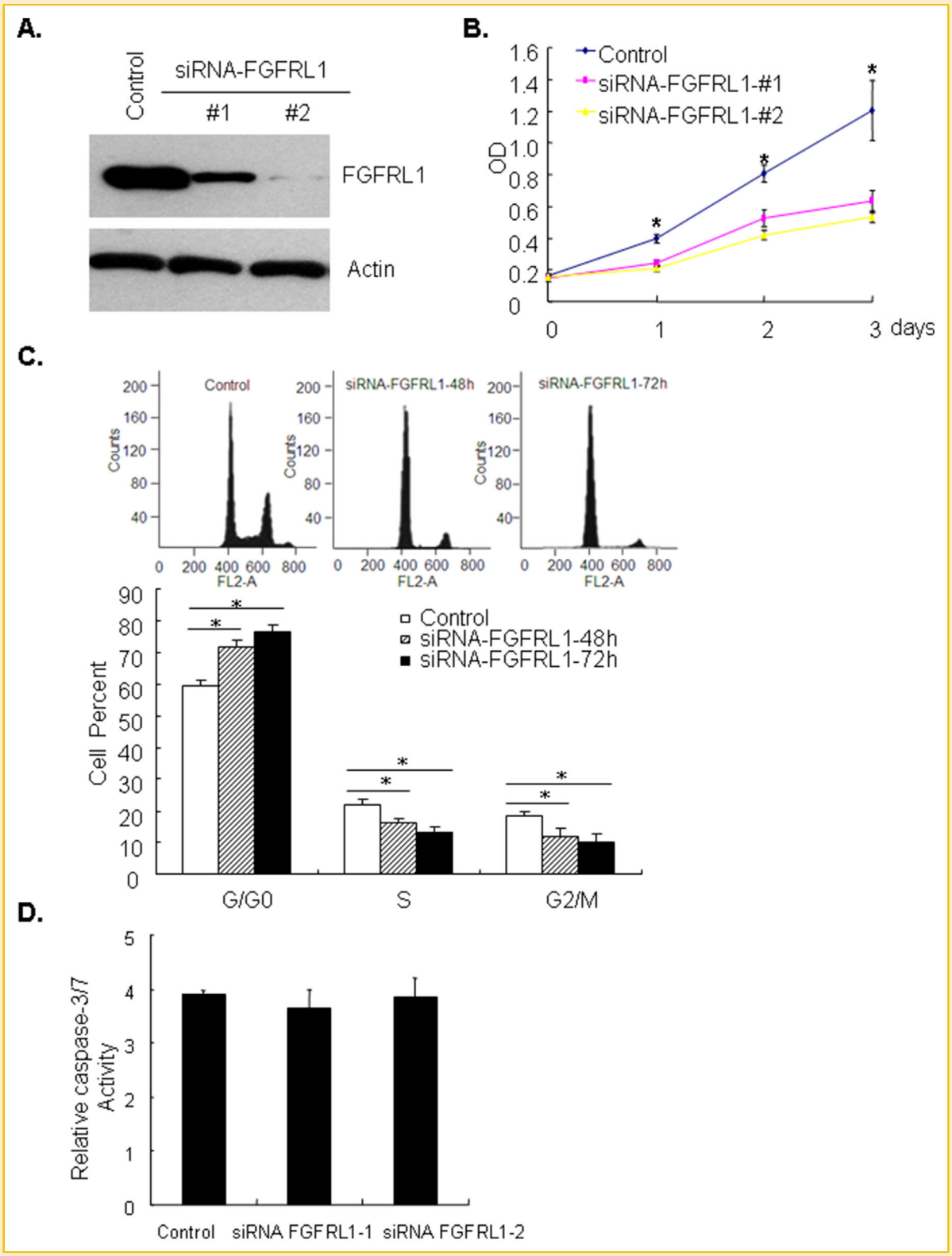

Fig. 4. miR-210 represses cell growth in vitro. A. SCC10A cells were left untreated or transfected with FGFRL1 siRNA. After 48 h, whole cell lysates were prepared and FGFRL1 expression was analyzed by immunoblotting. B. Silence of FGFRL1 inhibits proliferation of Laryngocarcinoma cell SCC10A. Each reporter assay was repeated at least three times. Error bar indicates standard deviation. Student's *t*-test was performed for statistical analysis, $^*P < 0.05$. C. The representative FACS profile was presented (top panel). The data about cell cycle was shown (bottom panel, $^*P < 0.05$). D. The caspase-3,7 activity was investigated.

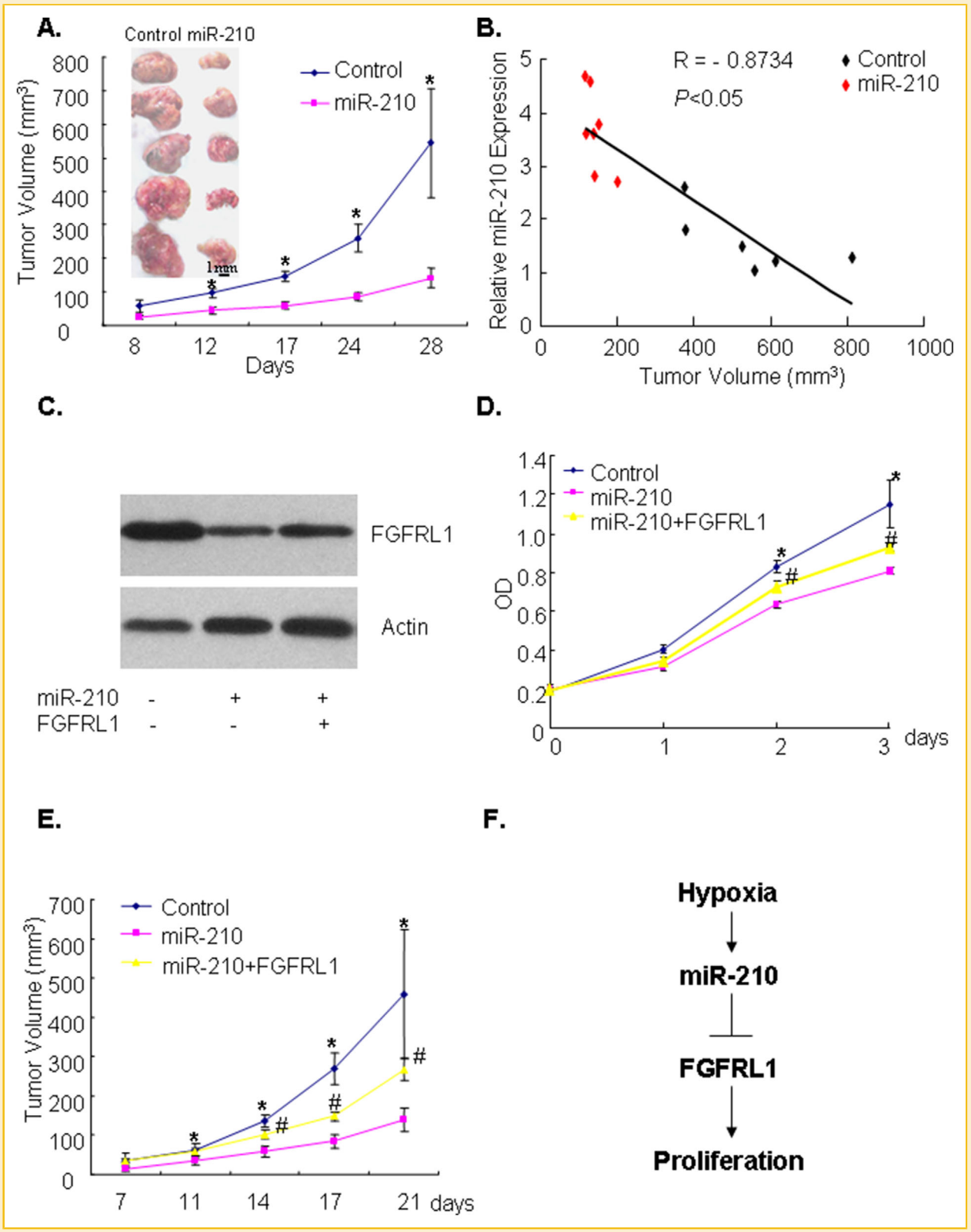

Fig. 5. miR-210 represses cell growth in a mouse xenograft model. A. SCC10A cells with ectopic expression of miR-210 and control cells were injected s.c. in nude mice at a density of 2×10^6 cells. The pictures of xenograft tumors were shown in the top panel. B. Shown is negative correlation between the size of xenograft tumors and corresponding miR-210 expression level. C. SCC10A cells were left untreated or transfected with ectopic expression of miR-210, or transfected with ectopic expression of miR-210 and FGFRL1 without a 3′-UTR region. After 48 h, whole cell lysates were prepared and FGFRL1 expression was analyzed by immunoblotting. D. FGFRL1 could partially rescue the suppression of cell growth caused by ectopic expression of miR-210 in Laryngocarcinoma Cell SCC10A. Error bar indicates standard deviation. Student's *t*-test was performed for statistical analysis, *,# $P < 0.05$. E. SCC10A cells with ectopic expression of miR-210, expressing miR-210 and FGFRL1 coding sequence without a 3′-UTR region and control cells were injected s.c. in nude mice at a density of 2×10^6 cells. F. Schematic representation of miR-210 signaling pathways inhibiting the cell proliferation in SCC10A cells.

Therefore, further studies are needed to detect deeply the molecular mechanism of decreased growth by miR-210.

In conclusion, we identified miR-210 as a robust hypoxia-inducible microRNA under hypoxia. In LSCC, our finding reveals a new mechanism that miR-210 inhibits the proliferation via inducing cell cycle arrest and apoptosis by the targeting of FGFRL1.

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