

SCIENTIFIC REPORTS



OPEN

MicroRNA-101 targets von Hippel-Lindau tumor suppressor (*VHL*) to induce HIF1 α mediated apoptosis and cell cycle arrest in normoxia condition

Ning Liu, Wu-Yan Xia, Shan-Shan Liu, Hai-Yan Chen, Lei Sun, Meng-Yao Liu, Lin-Feng Li, Hong-Min Lu, Yu-Jie Fu, Pei Wang, Hailong Wu & Jian-Xin Gao

The activation/inactivation of HIF1 α is precisely regulated in an oxygen-dependent manner. HIF1 α is essential for hypoxia induced apoptosis and cell cycle arrest. Several recent studies indicated that the expression of miRNAs can be modulated by hypoxia. However, the involvement of miRNAs in the regulation of HIF1 α induction remains elusive. In present study, we demonstrated that *miR-101* was rapidly and transiently induced after hypoxia in breast cancer cells. Over-expression of *miR-101* significantly inhibited cell proliferation in breast cancer cells through increased apoptosis and cell cycle arrest in normoxia condition. This inhibitory phenomenon seems due to *miR-101*-mediated induction of HIF1 α , because we identified that *VHL*, a negative regulator of HIF1 α , is a novel target of *miR-101* and over-expression of *miR-101* decreased VHL levels and subsequently stabilized HIF1 α and induced its downstream target *VEGFA*. Furthermore, we demonstrated that siRNA-mediated knockdown of VHL or HIF1 α overexpression could also induce apoptosis and cell cycle arrest whereas enforced expression of VHL, administration of anti-*miR-101* oligos or treatment of 2-MeOE2, an inhibitor of HIF1 α , could rescue cells from such inhibition. These results reveal a novel regulatory mechanism of HIF1 α induction in normoxia and suggest that *miR-101* mediated proliferation inhibition may through HIF1 α mediated apoptosis and cell cycle arrest.

Hypoxia is one of the classical features in solid tumors. Hypoxia inducible factor-1 (HIF1), a heterodimer with HIF1 α and HIF1 β subunits, is the major activated transcriptional factor in response to hypoxia. Although HIF1 β is constitutively expressed, the protein level of HIF1 α is tightly regulated by the oxygen levels. When oxygen is adequate, HIF1 α is hydroxylated by prolyl hydroxylase proteins (PHDs) and in turn recognized by the von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase (VHL) for ubiquitin-mediated degradation¹. Under hypoxia, the hydroxylase activity of PHDs is dramatically reduced; thus HIF1 α is stabilized, dimerized with HIF1 β to form HIF1 and translocated to the nucleus². Because the increased protein level of HIF1 α is frequently observed and correlated with poor prognosis in many cancer types, a long-lasting concept believes that HIF1 α plays an oncogenic role in tumor growth^{3,4}. However, some recent well-designed studies challenged this concept. Carmeliet *et al.* showed that HIF1 α is required for hypoxia-mediated apoptosis and inhibition of cell proliferation⁵. HIF1 α deficiency apparently inhibited tumor growth and enhanced tumor invasion in microenvironment with sufficient oxygen supply⁶. HIF1 α stabilization due to loss of VHL decreased tumor growth⁷. Furthermore, HIF1 α interacted with Myc or Cdc6 to induce cell cycle arrest in the absence of hypoxic signal⁸. Therefore, the role of HIF1 α in tumor progression has been controversial.

MicroRNAs are evolutionally conserved short noncoding RNAs that negatively regulate the expression of both protein-coding and noncoding genes^{9,10}. Due to the partial complement to their targets, a microRNA can regulate multiple genes' expression simultaneously¹¹. Recent studies have indicated that hypoxia modulates the expression

State Key Laboratory of Oncogenes and Related Genes, Renji-Med X Clinical Stem Cell Research Center, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China. Correspondence and requests for materials should be addressed to H.W. (email: wuhailong2@hotmail.com) or J.-X.G. (email: jianxingao@sjtu.edu.cn)

of a specific set of microRNAs, termed “hypoxamirs”^{12–15}. Given adaptation to hypoxia is essential for solid tumor progression, it’s intriguing to explore the feedback regulatory loops between “hypoxamirs” and hypoxic pathway. In this regard, several hypoxamirs have been shown to regulate hypoxic pathway in either negative or positive feedback loops. For example, by directly targeting HIF1 α , two hypoxamirs, miR-20b and miR-199a, suppress hypoxia progression^{16,17}. miR-424, induced by hypoxia, targets cullin 2 (CUL2) to stabilize HIF1 α and enhance angiogenesis¹⁸.

miR-101 is evolutionally conserved in vertebrates. Down-regulation of *miR-101* has been observed in various types of cancers, including prostate, colon, bladder, gastric, ovarian and breast cancers^{19–25}. Ectopic expression of *miR-101* has been shown to induce cell apoptosis and inhibit cell proliferation^{26,27}. However, our previous study demonstrated that enforced expression of *miR-101* confers cells estrogen-independent growth ability in breast cancer²⁸. A recent report also showed that increased *miR-101* levels are negatively associated with the overall survival (OS) and disease-free interval (DFI) in patients with ovarian carcinoma and *miR-101* could target C-terminal binding protein-2 (CtBP2) to enhance the stemness of cancer cells²⁹. These controversial results indicate that *miR-101* has divergent and even antagonistic roles in tumor progression. Interestingly, Kim JH *et al.* showed that hypoxia could induce *miR-101* expression in human umbilical vein endothelial cells (HUVECs), human brain microvascular endothelial cells (HBMECs), astrocytes, HeLa, and U937 cells³⁰, suggesting that *miR-101* is a novel hypoxamir under the regulation of hypoxia. However, little is known about the roles of *miR-101* in regulating the components of hypoxia pathway.

In this study, we demonstrated that *miR-101* is an acute miRNA in response to hypoxia in breast cancer cells. *miR-101* could directly target *VHL*, a negative regulator of HIF1 α , resulting in HIF1 α induction in the absence of hypoxic signal. *miR-101* directed proliferation inhibition seems due to HIF1 α mediated apoptosis and cell cycle arrest.

Results

Hypoxia induced *miR-101* expression in breast cancer cells. Since Kim JH *et al.* showed induction of *miR-101* in HeLa and U937 cells 12 h after hypoxia, we then examined whether hypoxia could induce *miR-101* expression in breast cancer cells. MCF-7 and MDA-MB-231 cells were exposed to hypoxia condition (1% Oxygen) and *miR-101* levels were measured by TaqMan qPCR at indicated time points. Figure 1A showed that *miR-101* expression rapidly reached a peak at 1 h after hypoxia exposure, and then declined to the basal level in 24 h in MCF-7 cells (Fig. 1A). We also observed similar kinetic changes but with much less induction of *miR-101* in MDA-MB-231 cells after hypoxia (data not shown). Consistent with previous reports, the expression of VEGFA, a well known downstream target of hypoxia, was dramatically induced after hypoxia (Fig. 1B). Western blot showed that the induction of HIF1 α started in 1 h, reached its peak at 6 h and declined in 24 h after hypoxia (Fig. 1C). The induction of VEGFA was also confirmed by western blot (Fig. 1C). To further investigate the correlation between hypoxia and *miR-101*, we treated cells with the hypoxia-mimetic desferrioxamine (DFO). As shown in Fig. 1D,E, DFO treatment significantly increased HIF1 α and VEGFA levels in both MCF-7 and MDA-MB-231 cells. Interestingly, unlike the rapid induction of *miR-101* in hypoxia treatment, *miR-101* levels increased at 2 h and 6 h after DFO exposure in MCF-7 and MDA-MB-231 cells respectively (Fig. 1D,E).

***miR-101* inhibits the cell proliferation in breast cancer cells.** To examine the functions of this novel hypoxamir in breast cancer cells, we stably expressed *miR-101* in MCF-7 and MDA-MB-231 cells after lentiviral infection. The ectopic expression of *miR-101* was confirmed by TaqMan qPCR in both cell lines (data not shown). Cell proliferation assay was performed. Figure 2A,B showed that over-expression of *miR-101* significantly inhibited the cell proliferation in both cell lines. To examine whether *miR-101* inhibits clonogenic survival, 2-dimension colony formation assay was performed. Compared to the control group, over-expression of *miR-101* significantly suppressed the colony formation to about 35% and 26% in MCF-7 and MDA-MB-231 cells respectively (Fig. 2C). To further assess whether *miR-101* suppresses cell proliferation, Edu incorporation was examined. Compared to the control group, *miR-101* expression dramatically impaired the incorporation of Edu in both MCF-7 and MDA-MB-231 cells (Fig. 2D).

***miR-101* induces apoptosis and cell cycle arrest in breast cancer cells in normoxia.** Since apoptosis and cell cycle arrest are tightly associated with reduced cell proliferation, we then determine whether *miR-101* could affect the apoptosis and cell cycle in breast cancer cells. Annexin V and Propidium iodide (PI) staining indicated that *miR-101* significantly induced cell apoptosis in both MCF-7 and MDA-MB-231 cells (Fig. 3A). DAPI staining further confirmed the *miR-101*-induced apoptosis in both cell lines (Fig. 3B). PI/FACS analysis demonstrated that *miR-101* over-expression caused significant G1-phase arrest in both cell lines (Fig. 3C). In addition, we examined the effects of *miR-101* on apoptosis and cell cycle of MCF-7 cells in hypoxia condition, but no significant changes were observed (Fig. S1). Given the essential role of CyclinD1 in regulating cell cycle, we started to check if *miR-101* mediated cell cycle arrest is due to the changes of CyclinD1. qPCR and western blot showed that neither mRNA nor protein levels of CyclinD1 was affected by *miR-101* in either normoxia or hypoxia condition (Fig. S2).

VHL is a direct target of *miR-101*. Previous studies have demonstrated that *miR-101* targets multiple genes, such as EZH2, COX-2, STMN1, ROCK2 and so on, to inhibit tumor growth^{19,20,31,32}. In this study, we focused on exploring the putative regulation loops between *miR-101* and hypoxia pathway. After miRNA database search, we identified that VHL, a negative regulator of HIF1 α , is a putative target of *miR-101* (miRDB v4.0). As shown in Fig. 4A, the VHL-3’UTR carries two putative binding sites of *miR-101*. We constructed a luciferase reporter vector carrying a VHL 3’UTR region containing those two binding sites. Compared to vector control, over-expression of *miR-101* significantly inhibited luciferase activity of VHL-3’UTR down to 40% as well as had

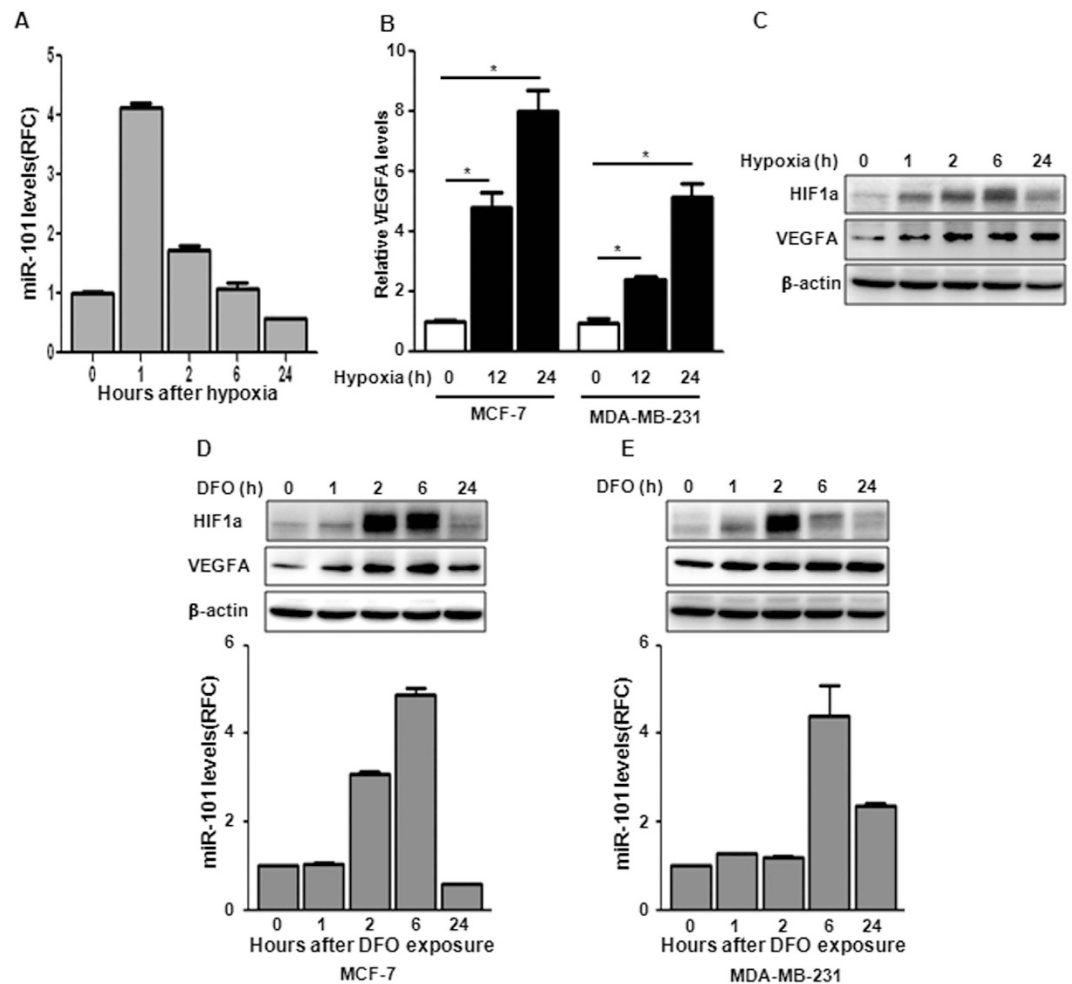


Figure 1. miR-101 is induced upon hypoxia exposure. (A) *miR-101* levels in MCF-7 after hypoxia exposure by qPCR. (B) VEGFA induction in MCF-7 and MDA-MB-231 cells by qPCR. (C) HIF1 α and VEGFA induction after hypoxia by western blot. (D,E) HIF1 α and VEGFA induction by western blot and *miR-101* expression by qPCR in MCF-7 (D) or MDA-MB-231 cells (E) after DFO treatment. Data were analyzed using Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

no effect on the luciferase activity of pGL3-control vector (Fig. 4B). Figure 4C showed that *miR-101* mediated reduction of luciferase activity was in a dose-dependent manner. To assess the specificity of *miR-101* mediated suppression, we employed anti-*miR-101* oligos to block the suppression of *miR-101*. Compared to the negative control oligo, introduction of the anti-*miR-101* reversed the *miR-101*-mediated suppression of luciferase activity (Fig. 4D). To further confirm whether *miR-101* mediated suppression of luciferase activity is attributed to the direct binding of *miR-101* to the VHL-3'UTR, we mutated those two putative binding sites individually or both. *miR-101* induced suppression was significantly attenuated in VHL-3'UTR vectors with either site 1 or site 2 mutation and was completely abolished in VHL-3'UTR with site1 and site2 double mutations (Fig. 4E). These results indicated that VHL is a direct target of *miR-101*. To examine if *miR-101* also targets on other negative regulators of hypoxia pathway, such as PHD1, PHD2, PHD3 and PHD4, we did an extensive search for putative *miR-101* targets by employing a prediction program with less stringency (FINDTAR3: <http://bio.sz.tsinghua.edu.cn/>). FINDTAR3 indicated putative bindings between *miR-101* and all those four negative regulators (Fig. S3). However, luciferase reporter assay failed to validate those interactions (Fig. S3). To determine whether *miR-101* could directly downregulate endogenous VHL expression, we performed western blot in MCF-7 and MDA-MB-231 cells with stable ectopic expression of *miR-101*. As shown in Fig. 4F, *miR-101* significantly suppressed VHL protein levels in both cell lines and this suppression was through post-transcriptional regulation because *miR-101* over-expression did not reduce VHL mRNA levels (Fig. 4G). In response to VHL downregulation, the expression of HIF1 α and VEGFA was increased (Fig. 4F).

Down-regulation of VHL induces increased apoptosis and cell cycle arrest. To answer whether *miR-101*-mediated apoptosis and cell cycle arrest are due to VHL suppression, we employed VHL siRNA to specifically knockdown VHL expression. Realtime PCR and western blot validated successful knockdown of VHL in both mRNA and protein levels respectively in MCF-7 and MDA-MB-231 cells 48 hours after siVHL transfection (Fig. 5A,B). The consequent increase of HIF1 α and VEGFA was also detected in cells with VHL

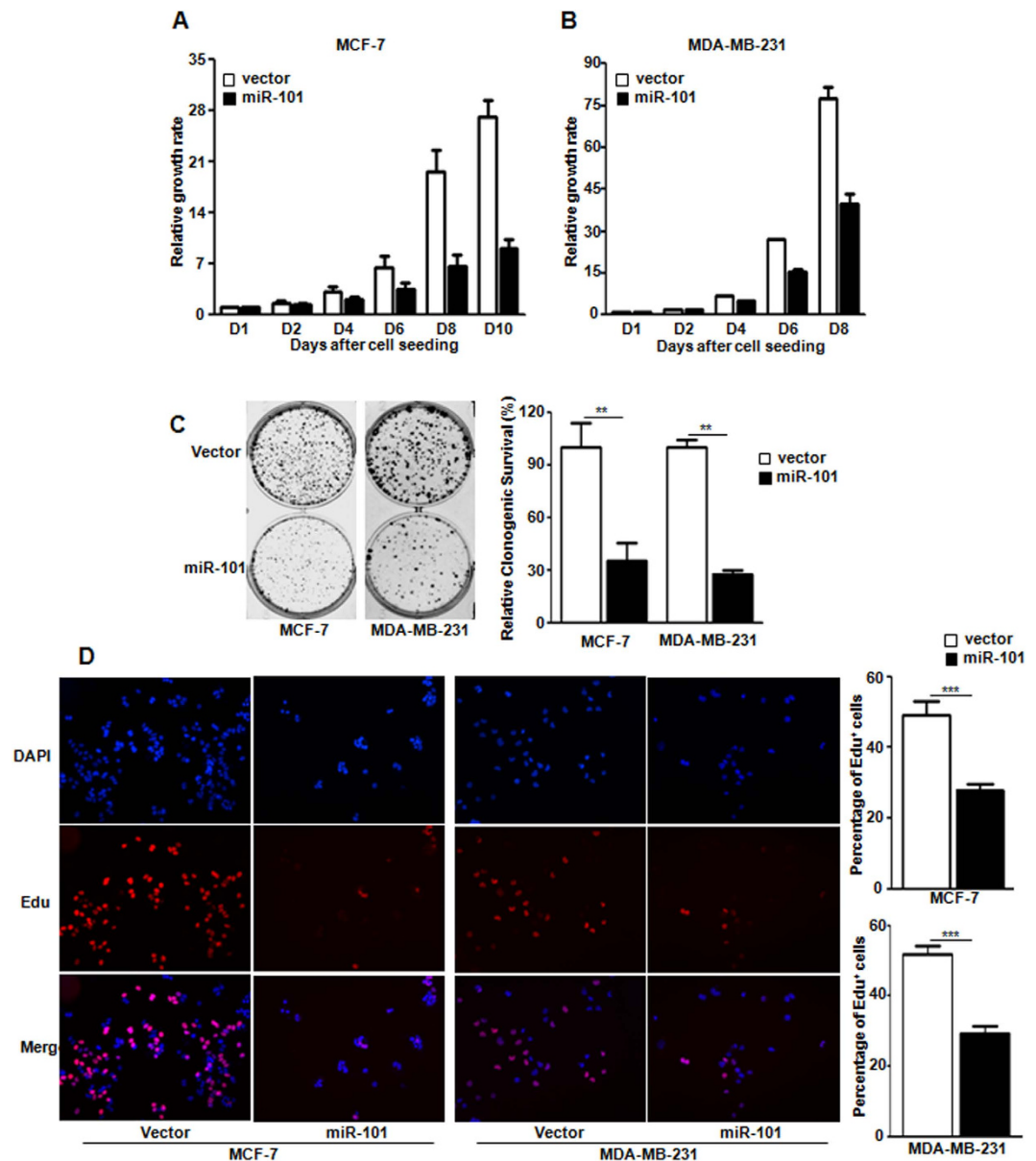


Figure 2. miR-101 inhibits cell proliferation. (A,B) Cell proliferation in MCF-7 (A) and MDA-MB-231 cells (B) with or without *miR-101* over-expression. ($p < 0.0001$ by two-way ANOVA). (C) Clonogenic survival in both MCF-7 and MDA-MB-231 cells with or without *miR-101* over-expression. (D) Edu incorporation in MCF-7 and MDA-MB-231 cells with or without *miR-101* over-expression. Data were analyzed using Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

suppression (Fig. 5B). Apoptosis and cell cycle assays were performed 48 hours post siVHL transfection. As shown in Fig. 5C–E, downregulation of VHL induced significant apoptosis and G1-phase arrest in MCF-7 and MDA-MB-231 cells compared to the negative control oligo.

Overexpression of *VHL* rescues cells from *miR-101*-mediated apoptosis and cell cycle arrest.

To further test whether *miR-101* functions upstream of *VHL* to induce cell apoptosis and G1-phase arrest, we over-expressed *VHL* in both cell lines with stable ectopic *miR-101* expression (Fig. 6A). Annexin V and PI staining showed that *VHL* over-expression significantly blocked *miR-101* induced apoptosis in both cell lines (Fig. 6B). FACS assay indicated that *miR-101* induced G1-phase arrest was significantly released upon *VHL* over-expression (Fig. 6C,D).

Anti-*miR-101* oligos block *miR-101* mediated apoptosis and cell cycle arrest. To answer whether inhibition of *miR-101* could rescue cells from *miR-101* induced apoptosis and cell cycle arrest, we transiently administrated anti-*miR-101* oligos into cells with stable *miR-101* over-expression. Compared with the negative control, released *VHL* repression was observed in cells transfected with anti-*miR-101* (Fig. 7A). Cell apoptosis

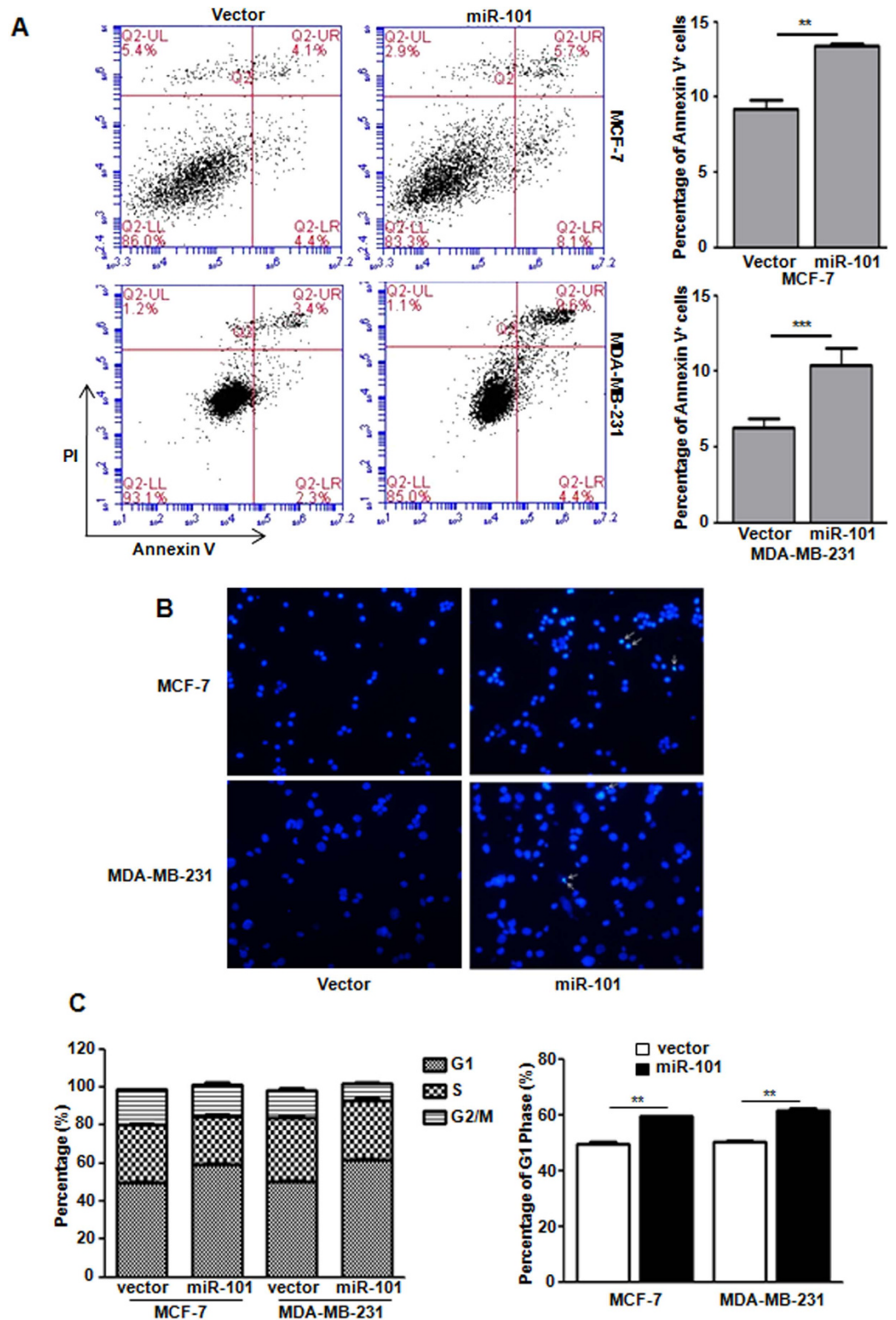


Figure 3. miR-101 induces apoptosis and cell cycle arrest. (A) Apoptosis assay in both MCF-7 and MDA-MB-231 cells with or without *miR-101* over-expression by Annexin V and PI double staining followed with flow cytometry analysis. (B) DAPI staining to detect apoptotic cells. (C) Cell cycle analysis in both MCF-7 and MDA-MB-231 cells with or without *miR-101* over-expression by PI staining followed by flow cytometry analysis. Data were analyzed using Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

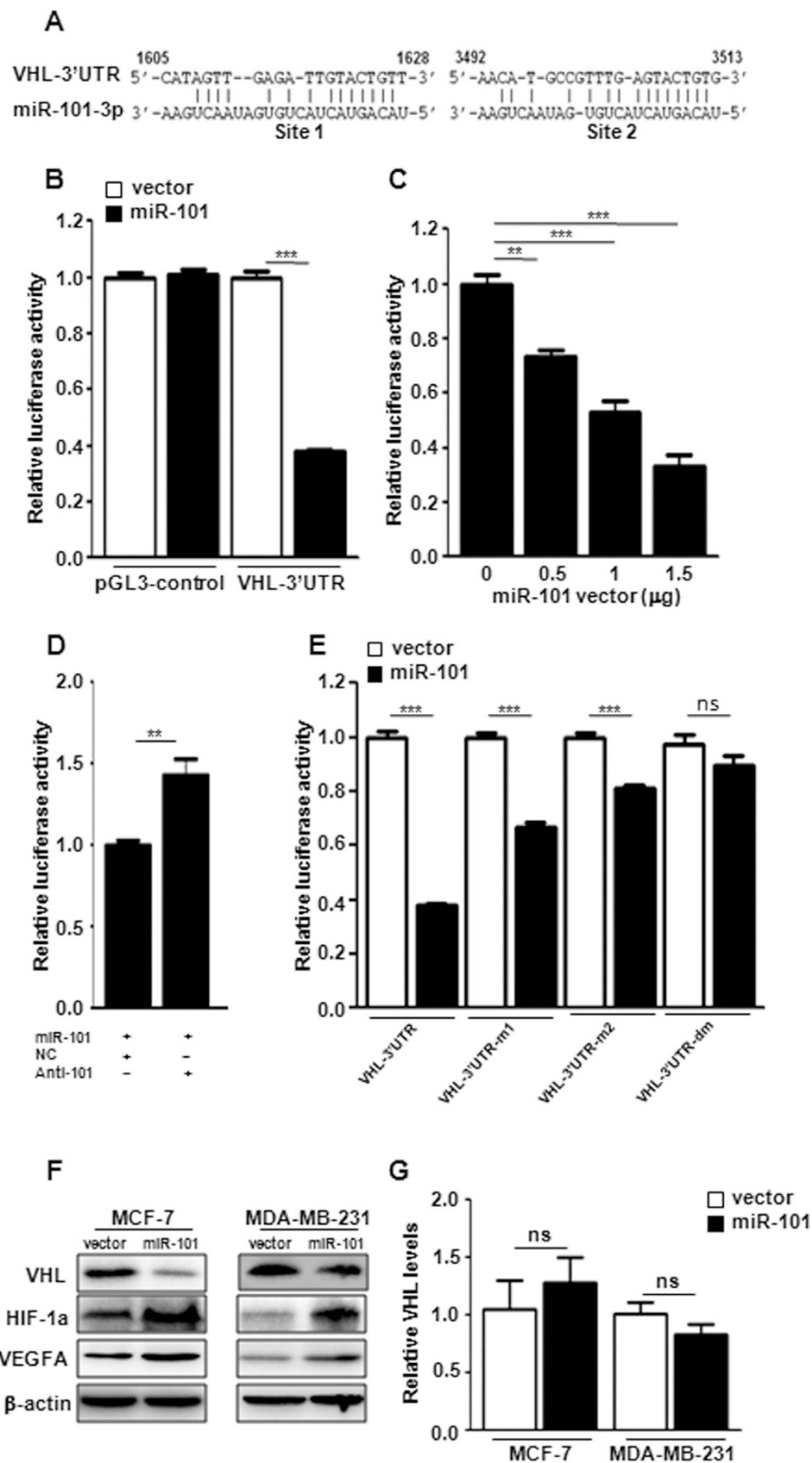


Figure 4. *VHL* is a direct target of *miR-101*. (A) Two putative binding sites of *miR-101* on the 3'UTR of *VHL*. (B) Suppression of the luciferase activity by *miR-101*. (C) Suppression of the luciferase activity by *miR-101* in a dose dependent manner. (D) anti-*miR-101* increased the luciferase activity in cells with *miR-101* over-expression. (E) Effect of *miR-101* on the luciferase activity of VHL-3'UTR, VHL-3'UTR-m1, VHL-3'UTR-m2 and VHL-3'UTR-dm. (F) HIF1 α , VEGFA, VHL level changes in MCF-7 and MDA-MB-231 cells with or without *miR-101* over-expression by western blot. (G) mRNA level changes of VHL in MCF-7 and MDA-MB-231 cells with or without *miR-101* over-expression by qPCR. Data were analyzed using Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

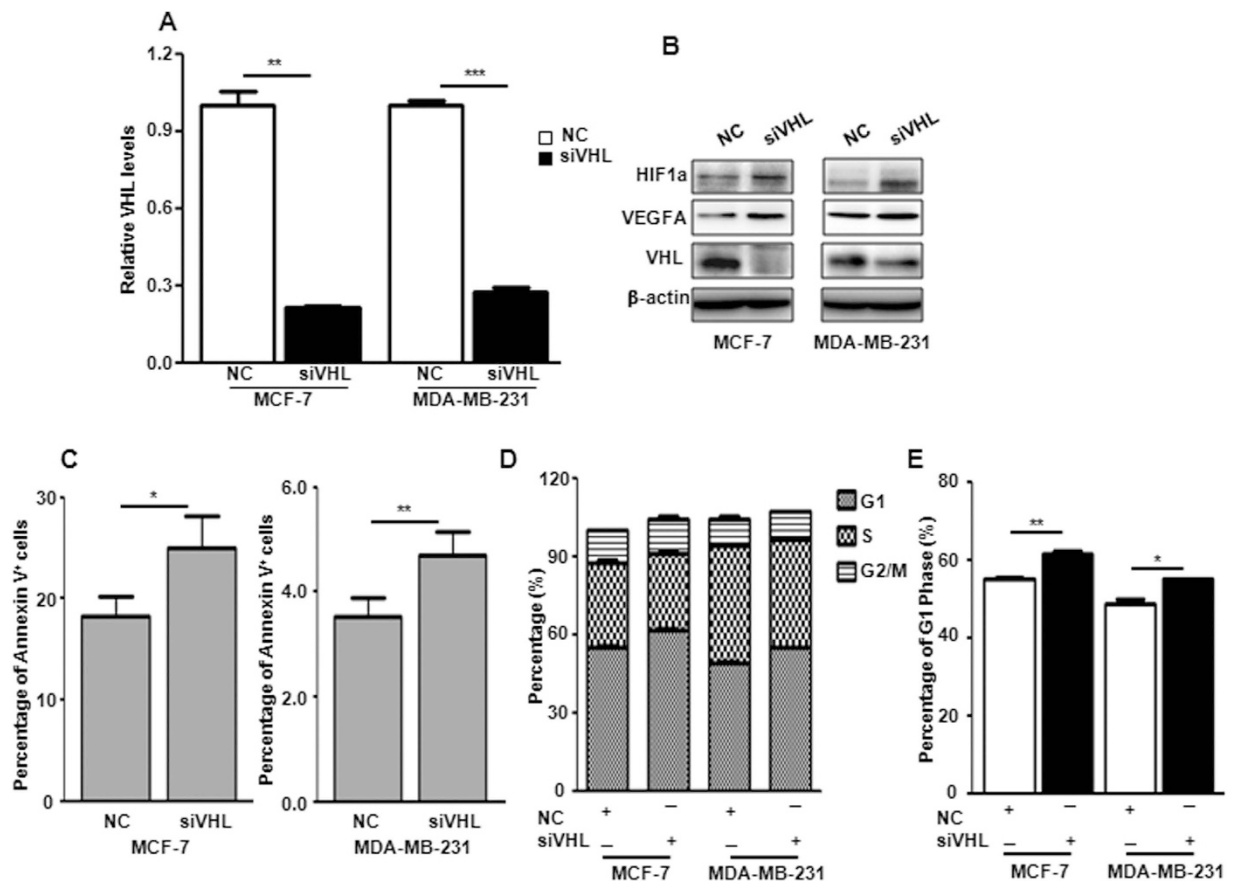


Figure 5. Knockdown of *VHL* induces apoptosis and cell cycle arrest. (A) *VHL* levels in MCF-7 and MDA-MB-231 cells transfected with siVHL by qPCR. (B) Protein level changes of *VHL*, *HIF1 α* and *VEGFA* in MCF-7 and MDA-MB-231 cells transfected with siVHL by western blot. (C–E) Percentage of Annexin V positive cells (C), G1, S and G2/M phases (D) and G1 phase (E) in MCF-7 and MDA-MB-231 cells transfected with siVHL. Data were analyzed using Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

and FACS assays showed that blocking of *miR-101* significantly rescued cells from *miR-101* induced apoptosis and G1 phase arrest in both breast cancer cell lines (Fig. 7B–D).

HIF1 α is required for *miR-101* mediated apoptosis and cell cycle arrest. To examine whether HIF1 α over-expression could induce apoptosis, MCF-7 cells with stable HIF1 α over-expression were generated after lentiviral infection. Ectopic HIF1 α expression was confirmed by western blot (Fig. 8A). Annexin V and PI staining indicated that HIF1 α over-expression increased the percentage of apoptosis cells to near 2-fold (Fig. 8C). To determine if HIF1 α is required for *miR-101* induced apoptosis and cell cycle arrest, we employed 2-MeOE, an inhibitor of HIF1 α , to treat MCF-7 cells with stable *miR-101* over-expression. 2-MeOE2 successfully inhibited HIF1 α activity as the VEGFA level was significantly decreased upon 500nM 2-MeOE2 treatment for 48h (Fig. 8B). As shown in Fig. 8D–F, inhibition of HIF1 α significantly rescued cells from *miR-101* mediated apoptosis and G1 phase arrest. Interestingly, consistent with a previous report that loss of HIF1 α promotes G1 to S phase transition, MCF-7-*miR-101* also showed significantly increased G1 to S progression upon 2-MeOE2 treatment (Fig. 8G).

Discussion

In present study, we demonstrated that *miR-101* is a novel hypoxamir in breast cancer cells, whose expression was rapidly and transiently induced in 1 hour after hypoxia. Through directing cell apoptosis and cell cycle arrest, ectopic expression of *miR-101* suppressed proliferation in breast cancer cells. To our knowledge, we for the first time identified that *VHL* is a direct target of *miR-101* and demonstrated that *miR-101* could increase HIF1 α protein levels by repressing *VHL* in normoxia condition. Furthermore, we showed that an increase of HIF1 α levels by either direct HIF1 α over-expression or by indirect knockdown of *VHL*, a negative regulator of HIF1 α , can induce apoptosis and cell cycle arrest. In contrast, HIF1 α downregulation by *VHL* over-expression, anti-*miR-101* oligo administration or 2-MeOE2 treatment rescued cells from *miR-101*-mediated apoptosis and cell cycle arrest in breast cancer cells.

Hypoxic microenvironment commonly exists in many solid tumors, and is tightly associated with poor prognosis and resistance to therapy. HIF1 α expression is induced in cells under hypoxia exposure and maintained at a basal level under normoxia⁴. Either the normoxic silencing or the hypoxic induction of HIF1 α is orchestrated

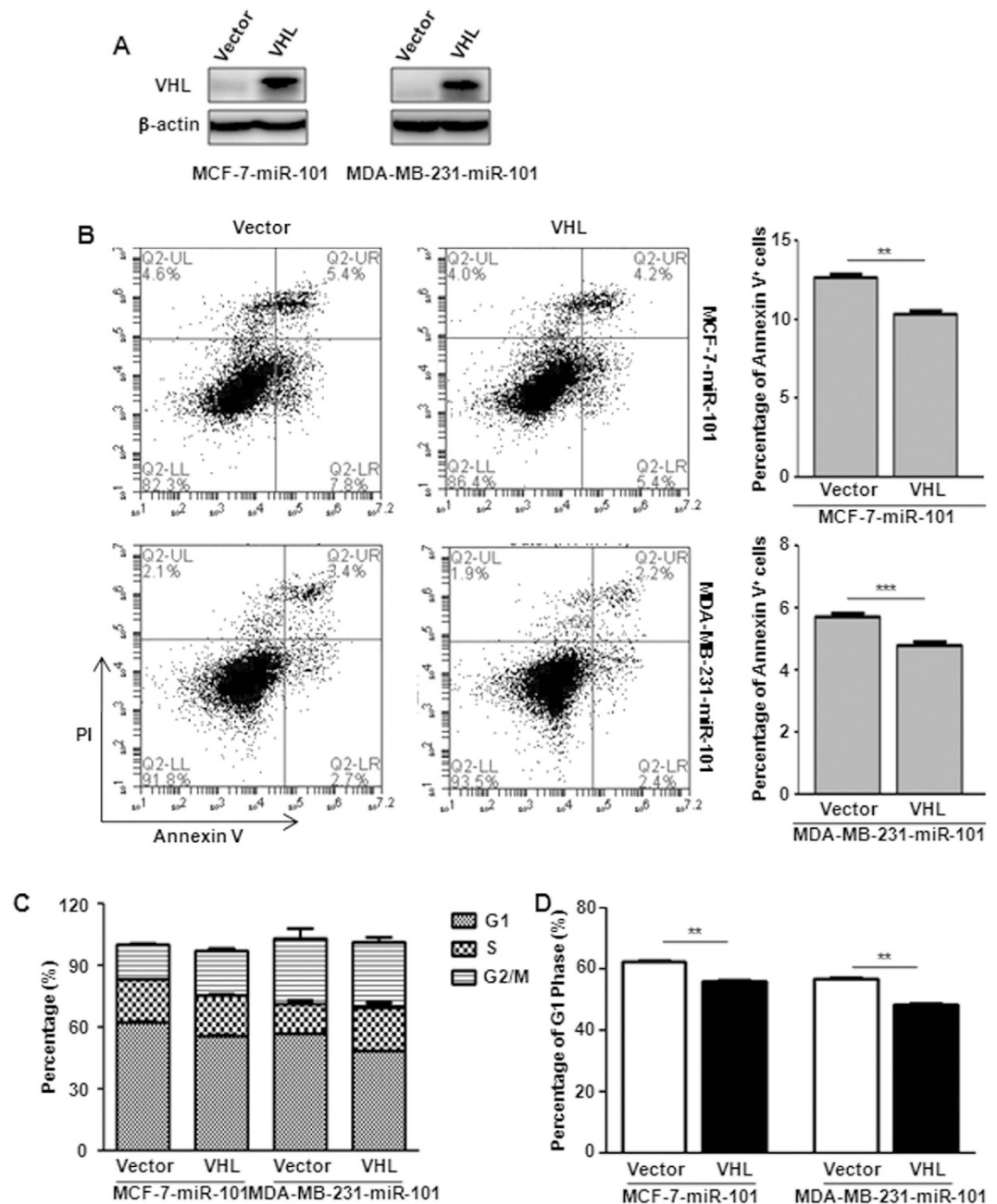


Figure 6. *VHL* restoration rescues cells from *miR-101*-mediated apoptosis and cell cycle arrest. (A) Ectopic expression of *VHL* in MCF-7-miR-101 and MDA-MB-231-miR-101 cells by western blot. (B) Apoptosis assay in both MCF-7-miR-101 and MDA-MB-231-miR-101 cells with or without *VHL* over-expression by Annexin V and PI double staining followed with flow cytometry analysis. (C,D) Percentage of G1, S and G2/M phases (C) and G1 phase (D) in MCF-7-miR-101 and MDA-MB-231-miR-101 cells with or without *VHL* over-expression. Data were analyzed using Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

modulated by multiple negative or positive regulators³³. Hypoxia induced HIF1 α dimerizes with HIF1 β to transcribe the expression of hundreds of downstream genes. miRNAs, a class of novel negative regulators of gene expression, have emerged in the downstream targets of HIF1 α under hypoxia. These hypoxia mediated miRNAs are termed hypoxamiRs, including miR-210, miR-10b, miR-155, miR-372/373 and miR-424 whose expression was up-regulated, and miR-199a, miR-20b and miR-200b whose expression was down-regulated under hypoxia^{16-18,34-41}. Interestingly, some of those hypoxamiRs, such as miR-20b, miR-199a and miR-424, can form negative or positive feedback loops to modulate hypoxia pathway¹⁶⁻¹⁸.

miR-101, a conserved miRNA, was first reported as a tumor suppressor in prostate cancer. Thereafter, accumulating evidence had demonstrated the tumor suppression feature of *miR-101* in many other tumor types including colon cancer, hepatocellular carcinoma, bladder cancer, gastric cancer, non-small cell lung cancer, breast cancer, pancreatic cancer and esophageal cancer^{20-22,25,26,42-44}. However, by adopting an unbiased

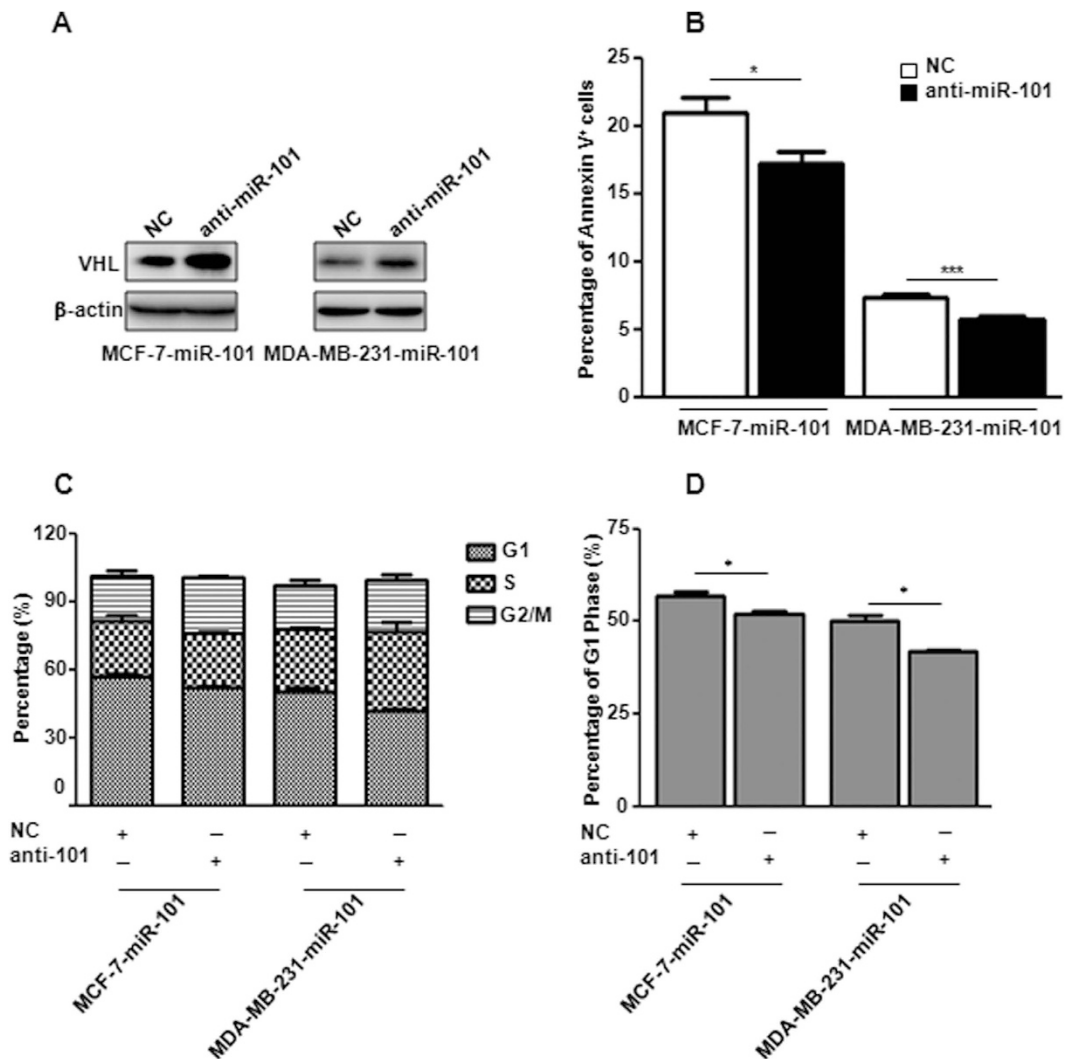


Figure 7. Blocking of *miR-101* releases cells from apoptosis and cell cycle arrest. (A) VHL level changes in MCF-7-miR-101 and MDA-MB-231-miR-101 cells transfected with anti-miR-101 oligos by western blot. (B) Percentage of Annexin V positive cells in MCF-7-miR-101 and MDA-MB-231-miR-101 cells transfected with anti-miR-101 oligos. (C,D) Percentage of G1, S and G2/M phases (C) and G1 phase (D) in MCF-7-miR-101 and MDA-MB-231-miR-101 cells. Data were analyzed using Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

miRNA library screening system, we previously demonstrated that over-expression of *miR-101* conferred cells estrogen-independent growth ability in MCF-7 and T47D cells, suggesting the oncogenic potential of *miR-101* in breast cancer²⁸. Interestingly, the oncogenic role of *miR-101* has been recently reported by Dr. Weiping Zou's group in ovarian cancer²⁹. They demonstrated that Myeloid-derived suppressor cells (MDSCs) in ovarian carcinoma could induce the expression of *miR-101* and in turn enhance the stemness of cancer cells²⁹. Thus, these controversial data indicate that *miR-101* plays a dual role as either a tumor suppressor or an oncogene in tumor progression. In present study, we demonstrated that *miR-101* was induced at the very early stage of hypoxia and *miR-101* could target VHL, resulting in stabilization of HIF1 α in normoxia condition.

Emerging evidence has indicated that HIF1 α has differential roles in tumor progression greatly dependent on the microenvironment of the tumor⁶. Blouw found that HIF1 α deficient astrocytomas showed greater proliferation and invasion abilities when inoculated into the brain their natural habitat (with sufficient oxygen supplement) instead of the subcutaneous habitat (a poorly vascularized environment)⁶, suggesting the tumor suppressive role of HIF1 α in the tumor with adequate oxygen supply. In gliomas with mutations in isocitrate dehydrogenase 1/2 (IDH1/IDH2), HIF1 α also presents as a tumor suppressor⁴⁵. Koivunen found that the most common IDH mutant IDH1R132H can activate PDHs to suppress HIF1 α level, in turn promoting the proliferation and anchorage-independent growth abilities of immortalized astrocytes⁴⁵. Therefore, HIF1 α plays a suppressive role when the microenvironment of tumor is oxygen sufficient. Consistent with these previous findings, we demonstrated that *miR-101* can target VHL, an inhibitor of HIF1 α , to increase HIF1 α level and in turn suppress cell proliferation via HIF1 α mediated apoptosis and cell cycle arrest. Our finding suggested that the *miR-101*/VHL/HIF1 α axis may play a tumor suppressive role when adequate oxygen was supplied to the tumor.

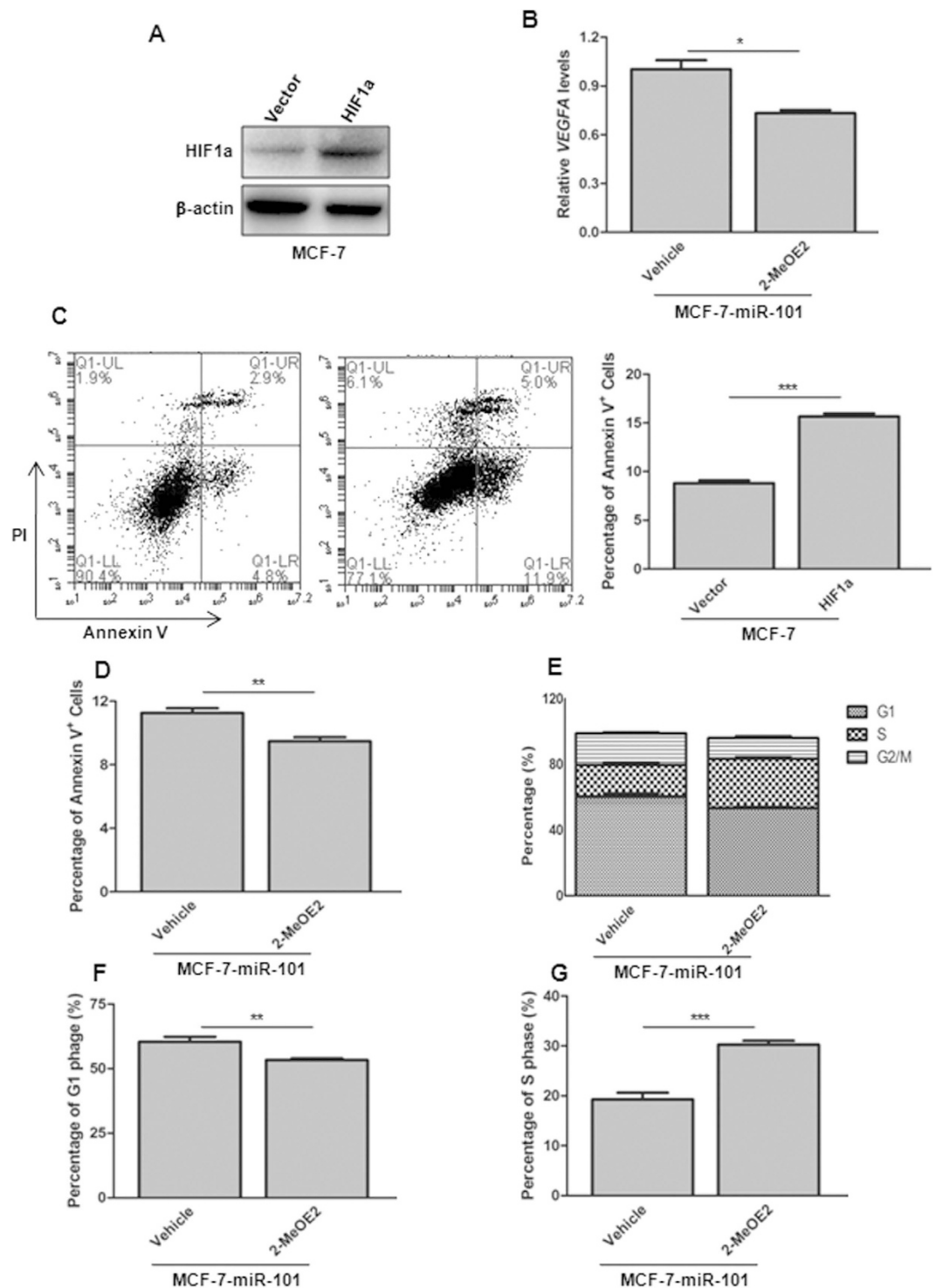


Figure 8. HIF1 α is attributed to *miR-101* mediated apoptosis and cell cycle arrest. (A) HIF1 α level change in MCF-7 cells infected with HIF1 α expression vector by western blot. (B) VEGFA level change in MCF-7-miR-101 cells after 48 h 2-MeOE2 treatment by qPCR. (C) Apoptosis assay in MCF-7 cells with HIF1 α over-expression by Annexin V and PI double staining followed with flow cytometry analysis. (D) Apoptosis assay in MCF-7-miR-101 cells after treated with 2-MeOE2. (E–G) Percentage of G1, S and G2/M phases (E), G1 phase (F) and S phase (G) in MCF-7-miR-101 cells after 2-MeOE2 treatment. Data were analyzed using Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A recent paper reported that HIF1 α plays a positive role in the proliferation in MDA-MB-231 cells⁴⁶, which is opposite to our findings. However, it's not convincing that Shi just employed a simple approach, siRNA mediated

HIF1 α knockdown, in their study to address the positive role of HIF1 α . It's also inappropriate for Shi to use only one siRNA duplex against HIF1 α , because the phenotypes in Shi's study may be probably caused by off-targeting.

In fact, several previous studies had also indicated the connection between *miR-101* and hypoxia pathway. For example, *miR-101* induction has been observed in HUVECs, HBMECs, astrocytes, HeLa, and U937 cells under hypoxia treatment³⁰; inhibition of *miR-101* could rescue cardiomyocytes from apoptosis induced by hypoxia/reoxygenation⁴⁷. Thus, previous data and the present study strongly implicated that *miR-101* mediated inhibition on cell proliferation in breast cancer cells seems due to HIF1 α -induced apoptosis and cell cycle arrest. Recently, *miR-101* based targeted therapy had been reported in treatment of hepatocellular carcinoma in a mouse model with a great success³². However, when we appreciate the great potential of *miR-101* in tumor therapy, necessary cautions must be emphasized given the dual role of *miR-101* in tumor progression.

Methods

Cell Culture. HEK293T and human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 0.1 mg/ml Penicillin-Streptomycin (Gibco) and incubated at 37°C in a humidified chamber supplemented with 5% CO₂.

Hypoxia exposure. Cells were maintained in a hypoxic chamber flushed with a gas mixture containing 94%N₂ and 5%CO₂. Under these conditions, O₂ levels in the medium were determined to be 1%. For DFO treatment, cells were treated with DFO (500 μ M). Protein and RNA were isolated at indicated time points.

Plasmid construction and transfection. HIF α over-expression vector was purchased from Addgene (Plasmid# 19365). To generate miR-101 expression vectors, a 420bp fragment carrying pre-miR-101 was amplified from the MCF-7 genomic DNA by the high fidelity polymerase Phusion enzyme (New England Biolabs, Ipswich, MA) using PCR primers:

5' tctagaTATTTTCAGCCTCACCCTTGCT
5' tctagaCCCATGTTACAAAACAAGGCA.

The amplified fragment was cloned into the pLVX-IRES-ZsGreen vector (Clontech) at the Xba1 site.

To generate the luciferase reporter vector carrying the VHL-3'UTR region carrying the two putative binding sites of miR-101, we amplified a 2243bp VHL-3'UTR region from the genome DNA of MCF-7 by the high fidelity polymerase Phusion enzyme (New England Biolabs, Ipswich, MA) using PCR primers:

5' tctagaGGAGTAGCCTGGACTGTTTCAT
5' tctagaTCCTTGGACAACACCAAAAACAC

The amplified fragment was cloned into the pGL3-control vector (Promega) at the Xba1 site.

To generate the VHL-3'UTR reporter vectors with mutated *miR-101* binding sites, we used *Phusion Site-Directed Mutagenesis* Kit (Life Technologies) to directly mutate those two binding sites by using primers:

VHL-UTR-mut1f: p-catagttgagattCACACACtcatagctttta
VHL-UTR-mut1r: p-taaaaaccaaccaaatctgccctaaa
VHL-UTR-mut2f: p-acatgccgtttgaCACACACgttttgggttg
VHL-UTR-mut2r: p-tttttttgtttttggttcttttg

VHL siRNA and anti-*miR-101* oligos were purchased from Shanghai GenePharma.

Quantitative real time-polymerase chain reactions (qRT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen). RNA was reverse-transcribed by RevertAidFirst Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green PCR real-time PCR Master MIX (Toyobo). miRNA was isolated using mirVanaTM miRNA Isolation Kit(Life Technologies). The TaqMan[®] microRNA reverse transcription kit (Applied Biosystems) was used to synthesize the cDNA for *miR-101*. The expression level of U6 snRNA was used as an internal control for normalization.

Cell proliferation assay. 20000/well cells were seeded into a six-well plate and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), changing half of the medium every other day. Then counting cell number at indicated time points.

Luciferase assay. The dual-luciferase assay was performed 48 h after transfection according to the manufacturer's instructions (Promega, Madison, WI, USA). The Renilla luciferase was used as a control reporter. The ratio of Renilla luciferase to firefly luciferase was normalized to the negative control. Three independent experiments were performed.

Apoptosis assay. Cells were harvested and washed twice with pre-cooled PBS and then were incubated in the binding buffer with a mixture containing annexin V (Biolegend) and propidium iodide (Sungene Biotech) for 15 min in darkness. Cells were measured by flow cytometry. Three independent experiments were performed.

Cell cycle assay. The cells were fixed in chilled 75% ethanol and stained with a propidium iodide (PI) solution contains 100 μ g/ml RNase (Tiangen Biotech) and 50 μ g/ml PI (Biolegend) in PBS for cell cycle analysis.

Colony formation assay. 1000/well cells were seeded into a six-well plate and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) for 14 days, changing half of the medium every other day. Colonies were fixed in 4% paraformaldehyde for 20 min at room temperature, and then

stained with crystal violet for 30 min at room temperature. The numbers of colonies were counted and pictures were captured.

Western blot. Cells were collected and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Thermo Fisher, Rockford, IL, USA) containing 0.02% complete Protease Inhibitor EASY packs EDTA-Free (Roche Applied Science). Then the protein was separated using 12% polyacrylamide gel, and transferred to polyvinyl difluoride membranes. After blocking with 5% bovine serum albumin (BSA) in TBS/Tween20 (TBST) and incubated with specific primary antibodies at 4 °C overnight, followed by a 5 min wash with TBS-T, which was repeated three times. After this, the membranes were incubated with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h, followed a 5 min wash with TBST, which was repeated three times. Then the membranes were analyzed with the ECL chemiluminescent detection system (Bio-Rad).

The following antibodies were used in this study: anti-actin monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HIF1 α polyclonal (Cell Signaling Technology), anti-VEGFA polyclonal, anti-cyclinD1 polyclonal (ProteinTech Group, Chicago, IL, USA) and anti-VHL polyclonal (Cell Signaling Technology).

EdU (5-ethynyl-2'-deoxyuridine) incorporation assay. Cells were seeded on cover slips for 48 h. The assay was performed with EdU cell proliferation assay kit (Thermo Fisher, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, EdU was added to the culture media for 2 h and the final concentration was 10 μ M. After labeling, cells were fixed in 4% formaldehyde. Then stained cells with Edu and counterstained with Hoechst, mounted in standard mounting media and imaged by fluorescence microscopy.

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Acknowledgements

This study was supported by grants from SJTU Interdisciplinary Research Grant (YG2013MS36 to HW), National Natural Science Foundation of China (81372233 to HW, and 81372188 and 81372188 to JXG, and 81402287 to LFL), the State Key Laboratory of Oncogenes and Related Genes in China (JXG, No. 90-14-06), the Special Fund for Innovation and Development of Science and Technology and Cultivation Fund for Major Projects and Innovative Team to JXG, Shanghai Jiao Tong University, China; Tumor Stem Cell Project (#90-14-06 to JXG), Shanghai Cancer Institute, China; The University Doctorate Research Fund for Freshly Recruited Teachers (No. 20130073120010 to LFL), Ministry of National Education, China; the Fund for Key Disciplines and Specialties, Shanghai Health and Family Planning Committee, China (JXG), the Fund for Key Disciplines and Specialties, Shanghai Health and Family Planning Committee, China (JXG), and Startup Funds to JXG from Renji Hospital and School of Medicine, Shanghai Jiao Tong University, China.

Author Contributions

N.L. and H.W. conceived and designed the experiments. N.L., W.Y.X. and H.W. performed the experiments. N.L. and H.W. analyzed the data and wrote the manuscript. W.Y.X., S.S.L., H.Y.C., L.S., M.Y.L., J.X.G., L.F.L., H.M.L., Y.J.F. and P.W. edited the manuscript. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Liu, N. *et al.* MicroRNA-101 targets von Hippel-Lindau tumor suppressor (VHL) to induce HIF1 α mediated apoptosis and cell cycle arrest in normoxia condition. *Sci. Rep.* **6**, 20489; doi: 10.1038/srep20489 (2016).



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