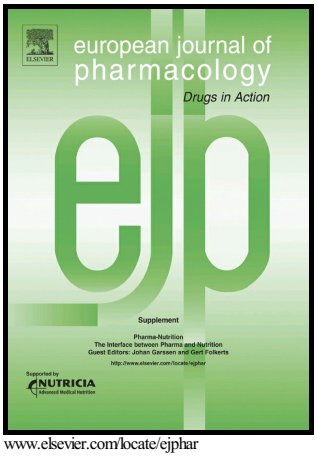

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MiR-132 plays an oncogenic role in laryngeal squamous cell carcinoma by targeting FOXO1 and activating the PI3K/AKT pathway

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ABSTRACT

Increasing evidence indicates that the dysregulation of microRNAs is involved in tumor progression and development. The purpose of the present study was to explore the expression of microRNA-132 (miR-132) and its function in laryngeal squamous cell carcinoma (LSCC). The results showed that miR-132 expression was markedly upregulated in LSCC tissues and cell lines. Functional analyses indicated that overexpression of miR-132 enhanced cell proliferation and tumor growth, which resulted in the downregulation of p27 and p21 and the upregulation of cyclin D1. In addition, luciferase activity indicated that miR-132 directly

targets FOXO1, and inhibits FOXO1 protein expression in LSCC cells. Further studies revealed that the ectopic expression of FOXO1 effectively reversed the cell growth induced by miR-132. Moreover, miR-132 also activated the PI3K/AKT pathway, which further decreased FOXO1 expression. In conclusion, these findings demonstrated that miR-132 plays an important oncogenic role in LSCC by modulating the PI3K/AKT/FOXO1 pathway at multiple levels, resulting in strong prognostic implication. Therefore, miR-132 might be a potential therapeutic strategy in LSCC.

Keywords: miR-132, FOXO1, PI3K/AKT pathway, laryngeal squamous cell carcinoma

1. Introduction

Laryngeal carcinoma is one of the most common malignancy neoplasms of the head and neck squamous cell carcinoma (Siegel et al., 2013). More than 90% of laryngeal tumors are diagnosed as laryngeal squamous cell carcinoma (LSCC). Although therapy approaches such as surgery and radiotherapy have been improved in recent years (Hunter et al., 2005), the overall 5-year survival rates for laryngeal carcinoma were less than 50% and still have not been significantly improved over the past 30 years, mainly due to metastasis and recurrence (Cosetti et al., 2008). Therefore, there is an urgent need to understand the molecular mechanisms responsible for LSCC metastasis.

MicroRNAs (miRNAs) are a class of approximately 22-nucleotide non-coding RNA molecules, which have been identified as key negative regulators of gene expression through

the endogenous RNA interference machinery (Bartel, 2009; Chen and Rajewsky, 2007). Accumulating evidence suggests that miRNAs play a role in diverse biological processes, including proliferation, apoptosis and tumorigenesis (Krol et al., 2010). Recent studies have also shown that dysregulation of miRNAs is implicated in invasion and metastasis in several human cancer types, and laryngeal carcinoma is not an exception (Kalfert et al., 2015). These studies suggest a critical role of miRNAs in tumorigenesis and development. Until now, miRNAs have been identified as both tumor suppressors and oncogenes, which is dependent on the role of their target genes.

An increasing number of studies has shown that lipid and protein phosphatase deregulation plays an important role in cancer development and progression through the constitutive activation of phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) signaling. There have been changes reported in the expression of many oncogenes and tumor suppressor genes in LSCC, such as p-AKT (JIA et al., 2009). These changes could affect cancer development by modulating downstream signal transduction pathways such as the well-known AKT signaling pathway (Knowles et al., 2011; Pedrero et al., 2005). Therefore, a deeper understanding of these molecular mechanisms will help us to identify new diagnostic and therapeutic approaches to this disease and improve the prognosis of LSCC patients. Human Forkhead box O (FOXO) proteins (FOXO1, FOXO3a, FOXO4, and FOXO6) are key effectors of PI3K/Akt signaling and regulate many biological processes, such as cell cycle regulation, cell differentiation, tumorigenesis, and oxidative stress responses (Burgering and Medema, 2003; Carter and Brunet, 2007; Fu and Tindall, 2008).

Notably, FOXO1 is also a downstream molecule of the PI3K/AKT pathway. Activated AKT phosphorylates FOXO1, which is subsequently exported from the nucleus into the cytoplasm and degraded by proteasomes (Zhao et al., 2004). Moreover, it has been demonstrated that FOXO1 induces G1 phase cell-cycle arrest in glioma cells and renal cell carcinoma due to the inhibition of tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN), via the upregulation of p27 (Kuo et al., 2013).

In the current study, we demonstrated that the expression of miR-132 was significantly upregulated in LSCC cells, and acts as an important regulator in LSCC cell proliferation and tumor growth. Furthermore, we investigated the role of miR-132 in modulation of the PI3K/AKT pathway in LSCC cells, and discovered that FOXO1 is a novel direct target of miR-132. Our findings demonstrated that miR-132 might be a novel target for further studies into the therapy of laryngeal carcinoma.

2. Materials and Methods

2.1. Cell lines and human tissues

Two LSCC cell lines (Hep-2 and AMC-HN-8) and a normal human keratinocyte cell line (HaCaT) were obtained from Shanghai Institute Chinese Academy of Science (Shanghai, China), maintained in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (FBS), and 100 μM each of penicillin and streptomycin in a humidified atmosphere of 5 % CO_2 at 37°C. Human Laryngeal squamous cell carcinoma specimens (n = 10) and corresponding adjacent non-neoplastic tissues (n = 10) were obtained from patients of the Tianjin First Center

Hospital with documented informed consent in each case.

2.2. Transfection of miRNA mimics

The cells were placed in six-well plates (5×10^5 cells per well) in opti-MEM media (Qiagen, Duesseldorf, Germany) and were transfected with miR-132 mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfected cells were cultured for 6 h, and the culture medium was then replaced with fresh complete medium. The cells were harvested 24 h after transfection.

2.3. RNA extraction, reverse transcription, and real-time PCR

Total RNA from cells was extracted using TRIzol (Life Technologies) according to the manufacturer's instructions. Messenger RNA (mRNA) and miRNA were polyadenylated using a poly-A polymerase-based First-Strand Synthesis kit (TaKaRa Bio, DaLian, China) and reverse transcription (RT) of total mRNA was performed using a PrimeScript RT Reagent kit (TaKaRa) according to the manufacturer's protocol. Complementary DNA (cDNA) was amplified and quantified on ABI 7500HT system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I (Roche, Grenzach-Wyhlen, Germany). U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous controls. Relative fold expressions were calculated with the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method. The primer sequences showed in Table 1.

2.4. Western blotting

Forty-eight h after transfection, total protein was extracted from the Hep-2 cells using RIPA cell lysis buffer containing proteinase and phosphatase inhibitors. The protein concentration of cell lysates was quantified by BCA Kit. Equal quantities of protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA, USA). The membranes were blocked in 5% non-fat dry milk diluted with Tris Buffered Saline Tween-20 (TBST) (in mmol/L: Tris-HCl 20, NaCl 150, PH 7.5, 0.1% Tween 20) at room temperature for 1 h, and then probed with antibodies against p21, p27, cyclin D1, FOXO1, pAKT, AKT, pPI3K, and PI3K (Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. After extensive washing, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (diluted 1:2,000; Amersham Biosciences, UK).The immunoblots were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.5. MTT assay

Cell growth was measured by MTT assay, Hep-2 cells were seeded in 96-well plates at a concentration of 10^3 cells/well. The cells were then maintained at 37°C for 24, 48, and 72 h after transfection. The cells were treated with 100 μl 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) for 4 h at 37°C, followed by removal of the culture medium and the addition of 150 μl DMSO. The absorbance at 570 nm was measured in a Thermo Scientific Multiskan (Thermo Fisher Scientific, USA).

2.6. BrdU labelling

Cells grown on coverslips (Fisher, Pittsburgh, PA,USA) at 70% confluence were incubated with bromodeoxyuridine (BrdU) for 1 h. Cells were then stained with anti-BrdU antibody (Upstate, Temecula, CA, USA) according to the manufacturer's instructions. Gray level images were acquired under a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany).

2.7. Tumor xenografts

Stable cell lines with high expressions of miR-132 were established by transfecting Hep-2 cells with a miR-132 mimics, followed by selection for 28 days to obtain stable cell lines. Up to 1×10^7 cells were mixed with matrigel (BD) and injected into athymic nude mice (six weeks old). The mice were monitored and the tumor size was measured daily, and tumor volumes were calculated as width (mm) × width (mm) × length (mm) × 0.5. The mice used in the experiments were handled in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. After 28 days, the mice were killed.

2.8. Luciferase reporter assay

Cells were plated in 100-mm cell culture dishes, proliferating to 60-80% confluence after 24 h of culture. The reporter constructs were transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. After 12 h incubation, the transfection medium was replaced; cells were harvested and washed with PBS, and lysed with passive lysis buffer (Promega). The cell lysates were analyzed immediately using a 96-well

plate luminometer (Berthold Detection System, Pforzheim, Germany). Luciferase and Renilla luciferase were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase activity of each lysate was normalized to Renilla luciferase activity. The relative transcriptional activity was converted into fold induction above the vehicle control value.

2.9. Statistical analysis

All statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). All data displayed as the mean ± S.D.. Student's t test or one-way ANOVA were performed to analyze the significance of differences between sample means obtained from three independent experiments. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of miR-132 is upregulated in LSCC

To analyze the expression of miR-132 in LSCC, qRT-PCR was employed to quantify the level of miR-132 in 10 pairs of LSCC tissues. As shown in Fig. 1A, miR-132 expression was significantly higher in cancer tissues compared with their paired paracarcinoma tissues. These results indicate that miR-132 expression is upregulated in human LSCC tissues. To determine the levels of miR-132 in LSCC cells, two LSCC cell lines (Hep-2 and AMC-HN-8) and a normal human keratinocyte cell line (HaCaT) were used to detect the level of miR-132 by real-time PCR. Our results demonstrated that the level of miR-132 was significantly increased in Hep-2 and AMC-HN-8 compared to that in HaCaT, as shown in Fig. 1B. These results

159 indicate that upregulation of miR-132 may play an important role in LSCC development.
160 Hep-2 cells were used to study further. In addition, the results from real-time PCR analysis
161 showed that miR-132 displayed evident upregulation in miR-132 group compared to miR-NC
162 group. These results confirmed that we effectively overexpressed miR-132 expression in
163 Hep-2 cells (Fig. 1C).

164 *3.2. MiR-132 upregulation promotes LSCC cell proliferation and modulated cell*
165 *cycle-related proteins*

166 To evaluate the effect of miR-132 on cell proliferation of LSCC, the cells were transfected
167 with miR-132 mimics or miR-NC. The results showed that miR-132 upregulation strongly
168 raised the viabilities of Hep-2 cells (Fig. 2A). Additionally, we also observed that
169 overexpression of miR-132 enhanced cell proliferation, as assessed by the Brdu-ELISA assay
170 (Fig. 2B), showing a positive correlation with the MTT assays. Taken together, these findings
171 demonstrated that overexpression of miR-132 had a proliferation promotion effect on LSCC
172 cells.

173 As miR-132 evidently promoted cell proliferation of LSCC, we assumed that miR-132
174 could accelerate the cell cycle. To confirmed this hypothesis, we examined its functions
175 regarding the expression of genes which regulate the G1/S transition, including the
176 cyclin-dependent kinase (CDK) inhibitors p21 and p27, and the CDK regulator Cyclin D1.
177 Our results suggested that p21 and p27 were strikingly downregulated at both mRNA (Fig. 2C)
178 and protein (Fig. 2D) levels while cyclin D1 levels were markedly upregulated in

miR-132-overexpressing Hep-2 cells, compared to NC transfected cells. These results provides further evidence that miR-132 plays an important role in LSCC cell proliferation. Altogether, our results indicated that miR-132 functionally modulates cell cycle regulators, p21, p27 and cyclin D1, thus making it relevant to cell proliferation and the cell cycle.

3.3. MiR-132 upregulation promotes LSCC tumorigenicity in vivo

To test whether miR-132 could enhance LSCC tumorigenesis, miR-132-overexpressing and negative control cells were inoculated into nude mice. Tumors were measured daily in two dimensions with calipers. The growth curve of tumor xenografts showed that the over-expression of miR-132 significantly enhanced tumor growth compared with the negative control (Fig. 2E). After 28 days, the mice were killed, and the tumors were removed. The average weight of the tumors was measured, demonstrating that the high miR-132 markedly increased the tumor weight compared to the negative control (Fig. 2F). These findings indicated that the overexpression of miR-132 was able to promote LSCC growth.

3.4. FOXO1 is identified as a functional downstream target of miR-132

To investigate how miR-132 affects the development of LSCC, we searched for potential regulatory targets of miR-132 using three prediction tools (miRanda, PicTar, and TargetScan), and selected FOXO1 as a potential downstream target gene (Fig. 3A). To further examine the effect of miR-143-3p on NFATc1 expression, we reexamined the effect of miR-132 on the mRNA and protein expression of FOXO1. As shown in Fig. 3B and 3C, the upregulation of miR-132 could significantly suppress both mRNA and protein level of FOXO1 expression

compared with the control group. Furthermore, we performed luciferase reporter assay to further validate whether FOXO1 is a direct target of miR-132. The results showed that miR-132 decreased luciferase activity with FOXO1 3'-UTR, but had no effect on luciferase reporter with mutated miR-132- binding elements (Fig. 3D).

3.5. FOXO1 is involved in miR-132-induced cell proliferation of LSCC

As shown above, over-expression of miR-132 promoted proliferation of LSCC, to further elucidate the function of FOXO1 in miR-132-mediated proliferation. We co-transfected miR-132 mimics with FOXO1 over-expression vectors harboring no specific miR-132 binding sequences in the 3'-UTR. We also observed that the luciferase activity of the FOXO1 reporter was decreased in miR-132-overexpressing cells. The inhibitory effects of miR-132 could be abolished upon transfection with FOXO1 (Fig. 4A). In addition, the MTT assay showed that the ectopic expression of FOXO1 effectively reversed the cell growth induced by miR-132 overexpression (Fig. 4B). Thus, our results demonstrate that miR-132 was able to enhance the proliferation of LSCC, at least in part, by the suppression of FOXO1.

3.6. MiR-132 activates the PI3K/AKT pathway in LSCC

FOXO1 transcription factor, a major PI3K-AKT downstream effector, regulates the expression of genes that are critical for progress. Given that the expression of FOXO1 was downregulated by miR-132 in LSCC. We hypothesized that miR-132 might modulate the PI3K/AKT pathway by targeting FOXO1 downstream. As shown in Fig. 4C, miR-132 upregulation could activate AKT and PI3K activity, indicated by a decrease in phospho-AKT

and phospho-PI3K in Hep-2 cells. At the same time, the level of FOXO1 was decreased. These results demonstrated that miR-132 activates the PI3K/AKT pathway in LSCC. To further confirm whether overexpression of miR-132 promotes LSCC proliferation by activating PI3K/AKT signaling, the cells were treated with an AKT inhibitor (MK-2206) and a PI3K inhibitor (LY294002). The results demonstrated that LSCC cell proliferation was dramatically suppressed (Fig. 4D). Together, these findings demonstrated that overexpression of miR-132 augmented proliferation of Hep-2 cells through activating PI3K/Akt pathway.

4. Discussion

MiRNAs are naturally existing small non-coding RNAs which control the expression of a large number of genes by binding to specific sites in the target mRNA, resulting in mRNA cleavage/degradation or translational repression. Recent advances in the understanding of the molecular mechanism of laryngeal cancer have revealed that multiple miRNAs play an important role in laryngeal carcinogenesis. However, the underlying mechanism responsible for miR-132 in laryngeal carcinogenesis remains unknown. Thus, in the present study, our study revealed that the expressions of the miR-132 was markedly up-regulated in LSCC tissues and cell lines. Additionally, ectopic expression of miR-132 enhanced cell proliferation by blocking the G1/S-phase transition *in vitro*, and accelerated tumor growth. Collectively, these findings indicated that miR-132 may have an essential role in the tumorigenesis and progression of laryngeal cancer.

238 FOXO1 is a transcription factor for p27, p21, FasL, and Bim, which function as tumor
239 suppressors by blocking the G1/S transition and inducing apoptosis (Ho et al., 2008; Lam et
240 al., 2013). Despite mounting evidence of a tumor suppressive role for FOXO1, the
241 mechanism by which FOXO1 activity is regulated remains unclear. Recently, it has been
242 shown that FOXO1 activity is generally regulated by post-transcriptional modification rather
243 than genetic aberration (Haftmann et al., 2012; Mei et al., 2012). Other mechanisms reported
244 to be important for the regulation of FOXO1 are PI3K/AKT signaling and miRNA (Haftmann
245 et al., 2012; Xie et al., 2012). Among the miRNAs, miR-27a, miR-96, miR-182, and miR-183
246 suppressed FOXO1 expression in breast cancer, melanoma, and Hodgkin lymphoma
247 (Haftmann et al., 2012; Xie et al., 2012). However, the association between miR-132 and
248 FOXO1 expression has never been addressed. Thus, the identification of target genes may
249 elucidate miR-132 function and the molecular mechanisms by which it mediates LSCC
250 progression. Our study is the first to have identified FOXO1 as a genuine target of miR-132
251 by bioinformatics analysis, suggesting a crucial functional role of FOXO1 in larynx
252 carcinoma tumorigenesis.

253 A central transducer of growth and proliferative signaling, the PI3K/AKT signaling
254 pathway plays an essential role in maintaining tumor cell proliferation, and constitutive
255 activation of PI3K/AKT signaling is involved in the initiation and progression of various
256 human cancers, resulting in poor prognosis. It has been well documented that p21 (Tinkum et
257 al., 2013) and p27 (Cappellini et al., 2003) expression can be transcriptionally regulated by
258 FOXO1, and the transcriptional activity of FOXO1 is in turn modulated by AKT

phosphorylation (Hou et al., 2014). Several reports have demonstrated that PI3K/AKT pathway alterations play an important role in the development of a variety of human carcinomas. FOXO1 is key downstream factor for PI3K/Akt signaling and regulate many biological processes. Therefore, we hypothesized that miR-132 might modulate the PI3K/AKT pathway by targeting FOXO1 downstream. Our results revealed that miR-132 overexpression remarkably increased PI3K and Akt phosphorylation, and activates PI3K/AKT signaling, while PI3K/AKT inhibitors suppressed the miR-132-induced proliferation. In addition, we further demonstrated that miR-132 activated the PI3K/AKT pathway by targeting FOXO1at the downstream level. These studies implicate miR-132 as an oncogenic miR and indicate that miR-132 may have an important role in laryngeal cancer development.

In conclusion, for the first time, the present study demonstrates that miR-132 is upregulated in LSCC and over-expression of miR-132 promotes cell proliferation and tumor growth. These findings also demonstrate a functional link between miR-132 and FOXO1 in LSCC, indicating that FOXO1 may be a novel direct target of miR-132. This study demonstrated that miR-132 downregulated FOXO1 in both direct and indirect manners by binding the 3'-UTR of FOXO1 and by activating the PI3K/AKT pathway in LSCC. Based on all of the data, it was inspiring to speculate that miR-132 could be a significant diagnostic and prognostic biomarker for larynx carcinoma. Further exploration of the precise role of miR-132 in the pathogenesis of a variety of tumors and in PI3K/AKT signaling pathway activation will

increase our knowledge of the molecular regulation of cancer progression and may allow the development of new therapeutic strategies against larynx carcinoma.

Conflict of interest The authors have no conflict of interest.

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342

343 **Figure legends**

344 **Fig. 1** Identification the overexpression of miR-132 in LSCC tissues and cell lines. **(A)** The
345 expression level of miR-132 was measured in 10 pairs of LSCC tissues and adjacent normal
346 tissues by qRT-PCR. T: LSCC tissues; N: adjacent normal tissues. **(B)** The expression of
347 miR-132 was detected by using qRT-PCR in Hep-2, AMC-HN-8 and HaCaT cells. **(C)** The
348 mRNA levels of miR-132 in Hep-2 cells transfected with miR-132 mimic or its negative
349 control (miR-NC) by real-time PCR. All data are presented as mean ± SD. #P<0.05 vs.
350 HaCaT; *P<0.05 vs. miR-NC.

351 **Fig. 2** miR-132 upregulation promotes LSCC cell proliferation and modulated cell
352 cycle-related proteins. **(A)** MTT assays revealed that upregulation of miR-132 promotes the
353 growth of Hep-2 cells. **(B)** Cell proliferation was determined by using BrdU-ELISA assay. **(C)**
354 The mRNA levels and **(D)** the protein expressions of p21, p27 and cyclin D1 were determined
355 by real-time PCR and Western Blot, respectively. α-Tubulin was used to serve as the loading
356 control. All data are presented as mean ± SD. *P<0.05 vs. miR-NC. Overexpression of
357 miR-132 promoted LSCC tumorigenicity *in vivo*. **(E)** Growth curves for tumor formation
358 after the implantation of Hep-2 cells. Mean tumor volumes are plotted. **(F)** Histograms of the
359 mean tumor weights of each group. Bars represent the mean ± SD of three independent
360 experiments. *P<0.05 vs. miR-NC.

Fig. 3 MiR-132 regulates the expression of FOXO1. **(A)** Predicted miR-132 target sequences in the 3'-UTRs of FOXO1. **(B)** The mRNA expression of FOXO1 was detected using qRT-PCR. **(C)** Results of the Western blot analysis of FOXO1 expression after treatment with the miR-132 mimic. The expression of FOXO1 in Hep-2 cells was lower in the miR-132 upregulation group compared with the control group. **(D)** Luciferase reporter assay of the indicated cells transfected with the pGL3-FOXO1-3'-UTR reporter or pGL3-FOXO1-3'-UTR-Mut reporter and miR-132 mimic or negative control. **P*<0.05 vs. miR-NC.

Fig. 4 FOXO1 mediates miR-132-induced cell proliferation of LSCC. **(A)** Relative FOXO1 reporter activities in the indicated cell lines. **(B)** MTT assay of Hep-2 cells transfected with either scramble, miR-132 mimic, miR-132 and FOXO1. **P*<0.05 vs. negative control. MiR-132 regulates the PI3K/AKT/FOXO1 pathway and involved in proliferation of LSCC. **(C)** Western blot analysis was performed for pPI3K, PI3K, pAkt, Akt, and FOXO1 respectively. **(D)** miR-132-induced proliferation was abrogated by AKT inhibitor (MK-2206) and PI3K inhibitor (LY294002) in LSCC cells as detected by MTT assay. **P*<0.05 vs. miR-NC, #*P*<0.05 vs. miR-132 mimic.

Table 1 Primer sequence for target genes

Gene	Sequence (5'- 3')
FOXO1	ACATTTCGTCCTCGAACCAGCTCA

	ATTTCAGACAGACTGGGCAGCGTA
	CGATGCCAACCTCCTCAACGA
p21	
	TCGCAGACCTCCAGCATCCA
	TGCAACCGACGATTCTTCTACTCAA
p27	
	CAAGCAGTGATGTATCTGATAAACAAGGA
	AACTACCTGGACCGCTTCCT
cyclin D1	
	CCACTTGAGCTTGTTCACCA
	GACTCATGACCACAGTCCATGC
GAPDH	
	AGAGGCAGGGATGATGTTCTG

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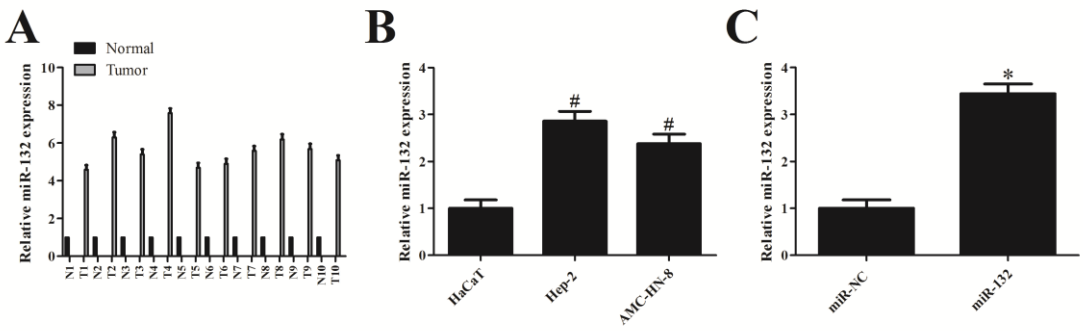

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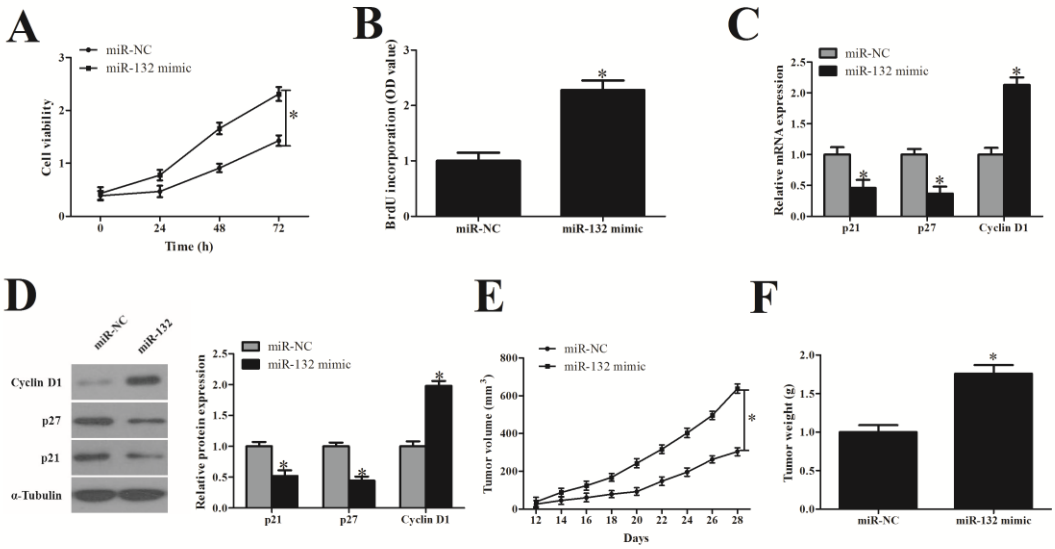

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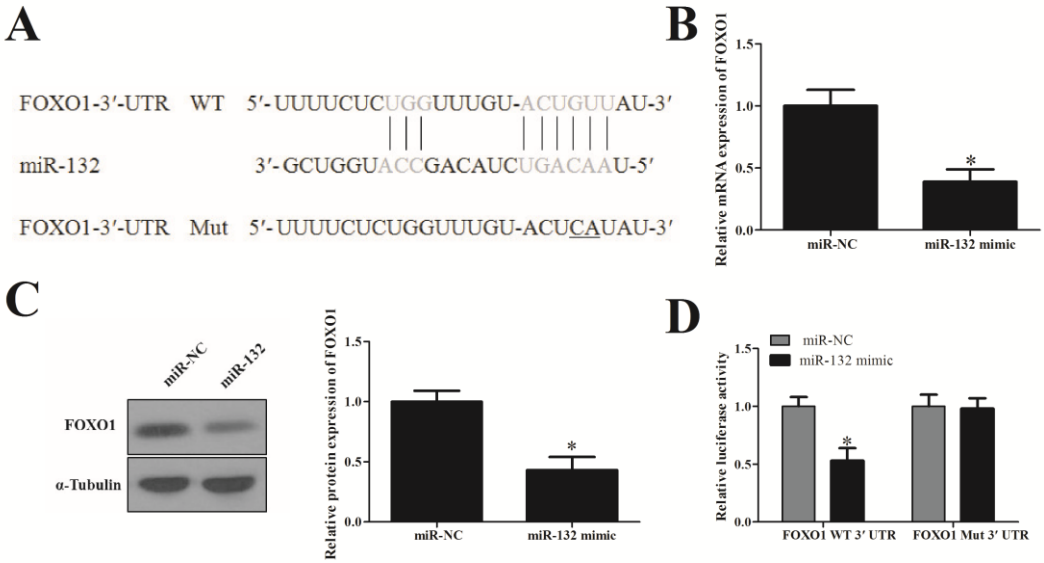

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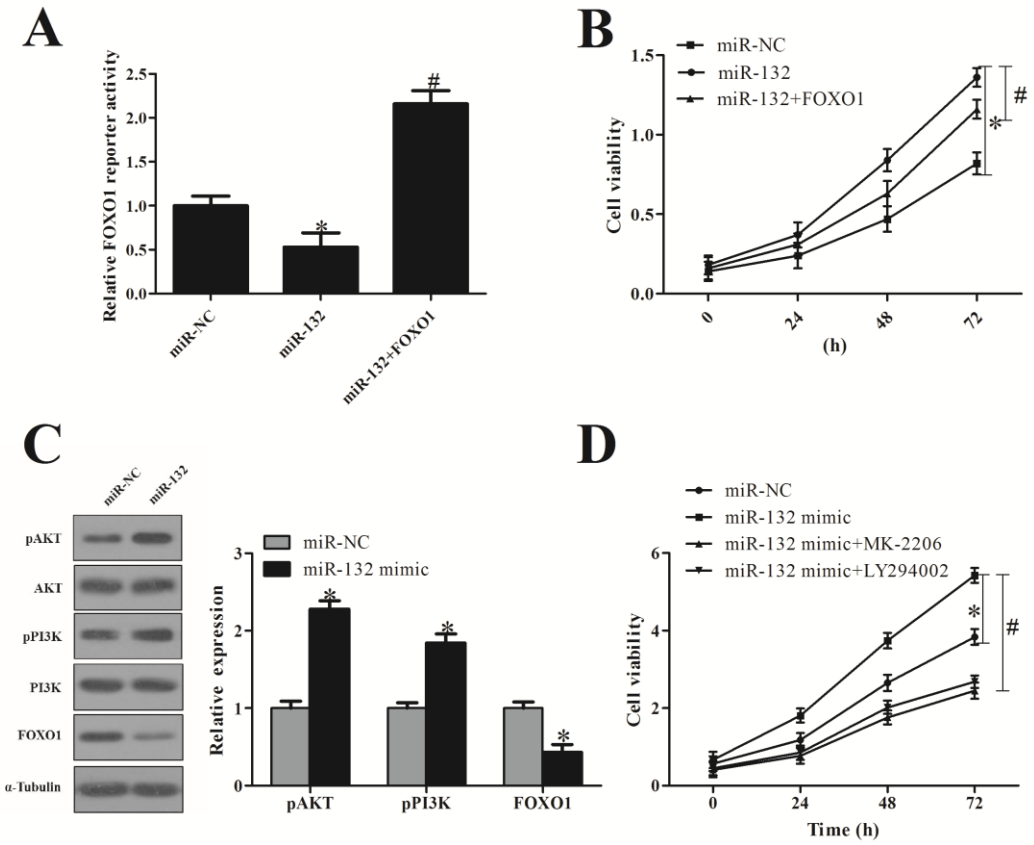

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