# RESEARCH ARTICLE

# Ionizing Radiation-inducible *microRNA-21* Induces Angiogenesis by Directly Targeting *PTEN*

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## Abstract

    **Background:** Previous experimental studies have established that *MicroRNAs (miRNAs)* can function as oncogenes or tumor suppressors in the regulation of tumor biology or pathology. However, the effects of ionizing radiation (IR) on the expression levels of cellular miRNAs and their further effects on the biological behavior of tumor cells require further investigation. **Methods:** We determined the proliferation, migration and tube formation of HUVEC cells after ionizing radiation (control, 0h and 24h), and the changes of *miR-21*, VEFG and HIF-1α levels after ionizing radiation were measured by Western blot (WB). The effects of *miR-21* mimics and inhibitors on the protein and mRNA expression of *PTEN* were determined by RT-PCT and WB. Two independent luciferase reporter plasmids were constructed to detect changes in *PTEN* protein expression. **Results:** We found that both IR and *miR-21* increase proliferation, migration and tube formation of HUVEC cells. Ionizing radiation directly targets the inhibition of *PTEN* expression by causing an increase in *miR-21* expression, and induces the accumulation of VEGF and HIF-1α expression in cells by the PI3K / AKT signaling pathway. Simultaneous induction of ectopic expression of *PTEN* can rescue radiation-induced proliferation, migration and tube formation in tumor cells. **Conclusion:** *miR-21* promotes tumor cell proliferation and migration by targeting inhibition of *PTEN* expression, which may become a potential target for tumor therapy in the future.

    **Keywords:** MicroRNA 21- angiogenesis- *PTEN*- ionization- HIF-1α

## Introduction

    Ionizing radiation, as a high-energy physics damaging factors, can produce free radicals by ionizing stimulation, resulting in the alteration of a number of ionizing radiation inducible genes (IRIG). The IRIG, such as GADD45, ATM, p53 (Artuso et al., 1995; Long et al., 2007; Dean et al., 2009), are widely involved in cell cycle regulation, DNA damage repair, cell growth regulation and apoptosis. Recent studies have found a new class of non-coding RNA, micro RNA (miRNA) are also involved in the process of radiation-induced biological response (Kwon et al., 2013; Zhao et al., 2014; Kwak et al., 2015). Recent studies revealed that radiation can induce a series of miRNA expression and there is a close relationship between miRNA and radiation sensitivity, and the radiation-induced tumorigenesis and bystander effects. Therefore, it is of great significance to investigate how miRNA is involved in the biological effects induced by ionizing radiation and these will reveal its molecular biological mechanisms and provide potential clinical value.

    *miR-21* upregulation participated in various tumor progression, and is correlated with tumor metastasis (Bartel ,2004; Quintero et al., 2004; Griffiths-Jones et al., 2006; Suarez and Sessa, 2009), but the causes of *miR-21* upregulation and its direct role targeting tumor angiogenesis remains to be classified. Angiogenesis is essential for tumor growth and metastasis. The relavant marker, such as VEGF, plays a key role in regulating angiogenesis both in embryo and tumor (Melillo, 2004; Axelson et al., 2005), which is regulated by HIF-1 through transcriptional activation (Ghosh et al., 2009). In our study, we demonstrated of *miR-21* overexpression induced by ionization, which could increased HIF-1a and VEGF expression by PI3K/AKT/*PTEN* signaling pathway and induced tumor angiogenesis.

## Materials and Methods

### Cell lines and culture conditions

    Huvec cell lines were obtained from the American Type Culture Collection (ATCC). The cell line was cultured in RPMI 1640 (Gibco) supplemented with 10 % fetal bovine serum (FBS), and maintained at 37°C in 5 % $CO_2$. The total RNA isolation was performed using miniBEST universal RNA extraction kit (Takara Bio, Inc.). The cellular protein isolation was performed and its concentration was detected by the BCA protein kit (Pierce,

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Rockford, IL.) for the WB assays.

*RNAi system and plasmid constructon*

specific siRNAs targeting microRNA hsa-*mir-21* [miRBase: MI0000077] were bought from Genepharm. primers. The *miR-21* mimics sequences were: 5'-GGAUGGUACUUUCAUUGAATT-3'; *miR-21* inhibitor sequences were 5'-CGGCUUCCCAAUAACAGUATT-3', and the scramble sequence: 5'-UUCUCCGAACGUGUCACGUTT-3'. We also construct *PTEN* ectopic expression plasmid using pcDNA 3.0 vector.

*Transfection assays with RNAi or plasmids*

The pcDNA 3.0 plasmid or *miR-21* mimics and inhibitor were transiently transfected with Huvec cells. Briefly, the cells were plated at a density of $1*10^5$ cells/well in a six-well plate. After 24h, the plasmids or RNAi were diluted in Opti-MEM and incubated for 20 min at room temperature. Mixed lipofectant and the above solutions, and added into the plate. Then the cells were incubated for 12 h, rinsed and replenished with RPMI 1640 containing 10 % FBS. After 48 h, the cells were harvested and isolated the RNA and protein for RT-PCR and WB.

*Cell proliferation assay*

We used SRB method to perform the proliferation assay. Huvec were seeded in 96-well plates and cultured for 24 h. The cells were transfected with plasmid or RNAi or using conditioned media cultured for 24, 48, 72h, respectively. Then, the cells were fixed with 10% w/v of trichloroacetic acid (100 μL) at 4°C for 1 h, and washed with deionized water. Till air-dried, the samples were stained with 100 μL of SRB solution (in 0.4% w/v in acetic acid) for 20 min at room temperature. The plates were then washed with acetic acid (1%) and air dried. Tris-base (10 mM, 100 μL, pH 10) was added to each well for solubilization. Optical density (O.D) values were measured at 540 nm with a reference wavelength of 630 nm using microtiter plate reader (VERSMax).

*RNA isolation and RT-qPCR analysis*

Total RNA was isolated by using miniBEST universal RNA extraction kit (Takara Bio, Inc.). Reverse transcription use RT-qPCR kits (Takara Bio, Inc.) or miRcute miRNA cDNA kit (TianGen Biotech,Beijing) were used to evaluate expression of *PTEN* and *miR-21*. GAPDH and U6 expression was used to normalize for variance. The PCR Primers pairs used for each genes were: *miR-21* forward: CGCGGTAGCTTATCAGACTGATG, reverse: CAGTGCAGGGTCCGAGGTG; *PTEN* forward: CGACGGGAAGACAAGTTCAT, reverse: AGGTTTCCTCTGGTCCTGGT.

*WB assays*

Cells were lysed in RIPA lysis buffer at ice . An equal amount of protein (20μg) was subjected and fractionated using SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF). Blocking the PVDF membranes in 3% bovine serum albumin in TBST buffer

containing 0.1% Tween-20 for 1h at roomm temperature and incubating with the indicated primary antibodies at 4˚C overnight. Appropriate secondary antibodies were incubated at room temperature for 1 h and detected using the enhanced chemiluminescence detection system. The data were adjusted against loading control using GAPDH. The primary antibodies were listed as followed: anti-HIF-1α(BD Biosciences, Bedford, MA, USA); anti-*PTEN*, anti-PI3K, anti-p-PI3K, anti-AKT, anti-p-AKT, anti VEGF, anti-GAPDH (Cell Signaling Technology).

*Reporter plasmids and luciferase activity assays*

Cells were transiently transfected with pCMV-Luc-WT/MT reporter plasmids, which contain *PTEN*-3′-UTR regulatory region, according to the manufacturer's instructions as mentioned above. The *PTEN*--3′-UTR activity was normalized via co-transfection of empty vector. The luciferase activity in the cells was quantified using a dual luciferase assay system (Promega) 24 hours after transfection.

## Results

*Ionization induced Huvec proliferation, migration and tube formation*

To investigate the role of ionization on HUVEC, we used conditioned media to perform the proliferation, migration and tube formation assays. Three media were used, control, 0h and 24h conditional media. We could see that after 24 h ionization, the cell proliferation increased compared to the control groups (Figure 1A). Similarly, Huvec migration ability enhanced. The migrated cell numbers were much more than the control groups (Figure 1B). Besides, the tube formation capability was much stronger. The number of formed tubes increased after ionizationfor 24 h (Figure 1C). Our group previously found that high-dose radioation could up-regulate HIF-1α expression (Zhang et al., 2014). In this study, we also confirmed that 24 h radiation could up-regulate HIF-1α and VEGF expression (Figure 1D). Additionally, the concentration of VEGF also increased (Figure 1E).

*miR-21 promoted Huvec proliferation, migration and tube formation*

Pervasive studies uncovered a series of differentially expressed microRNAs after cell irradiation, among which *miR-21* was detailed verified (Xu et al.,2014). Here, we saw that 24h ionization up-reguatlted *miR-21* expression level (Figure 2A), which implied that *miR-21* might work after radioation. We then transfected the cells with *miR-21* mimics and inhibitor to verify cell biological changes. We firstly confirmed the transfecttion efficiency. Figure 2B showed that *miR-21* mimics did increase its RNA expression, while inhibitor decreased its expression. Consistently, Huvecs with *miR-21* mimics possess a higher ability of proliferation (Figure 2C), migration (Figure 2D), and tube formation (Figure 2E), compared to the control groups. These phenotype changes might be due to PI3K/AKT/*PTEN* signalling pathway (Figure 2F).
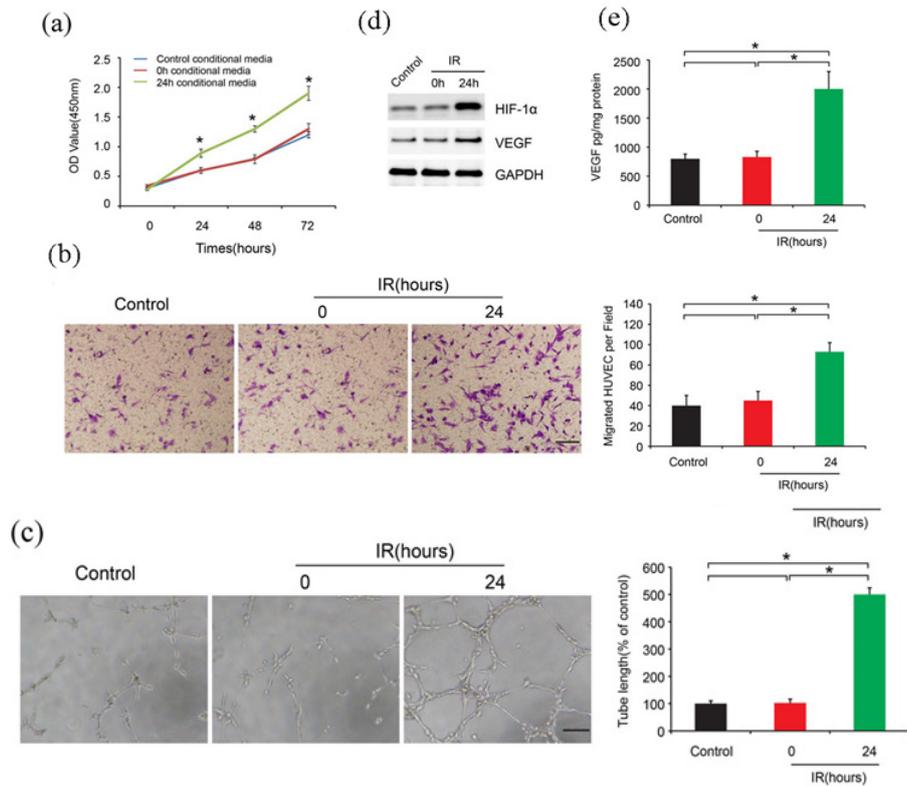
Figure 1. Ionization Induced Huvec Proliferation, Migration and Tube Formation. A.Huvec cells proliferation after 24h ionization. B. Huvec migration ability after 24h ionization. C. The tube formation capability after ionization. D. HIF-1α and VEGF expression after 24h ionization. E. The concentration of VEGF after 24h ionization.
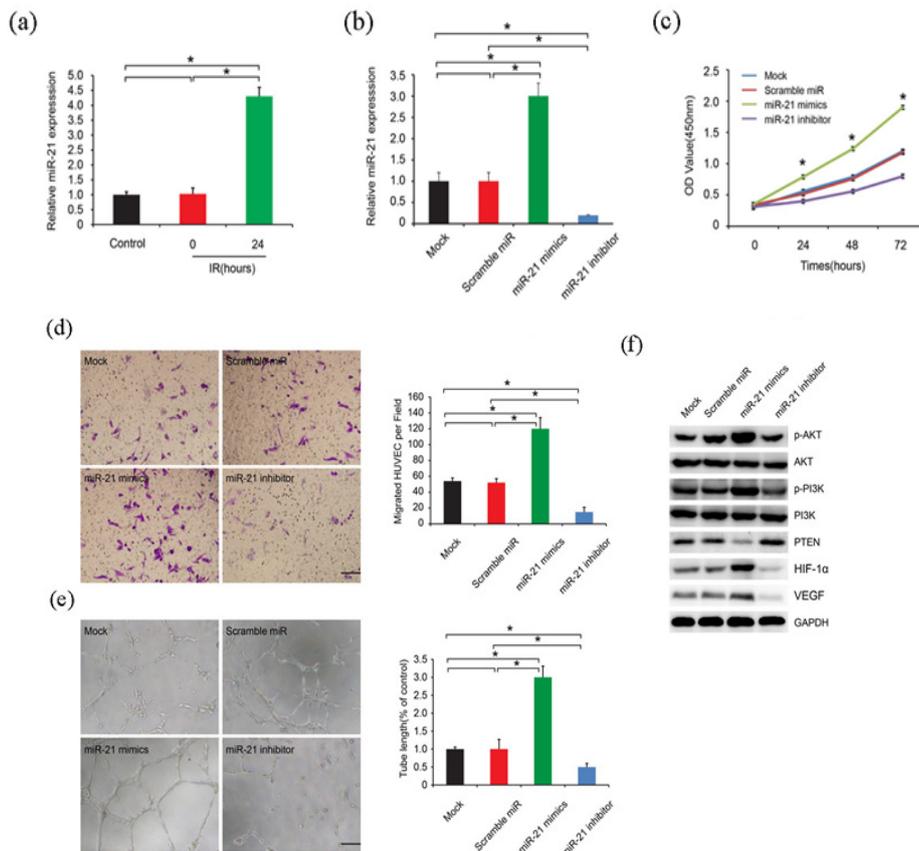


Figure 2. miR-21 Promoted Huvec Proliferation, Migration and Tube Formation. A. miR-21 expression level after 24h ionization. B. confirmation of miR-21 overexpression and knockdown. C. huvec cell proliferation after transfected with miR-21 mimics and inhibitor. D. huvec cell migration ability after transfected with miR-21 mimics and inhibitor. E. Tube formation ability after transfected with miR-21 mimics and inhibitor. F. PI3K/AKT/*PTEN* signalling pathway relative protein expression after transfected with miR-21 mimics and inhibitor.
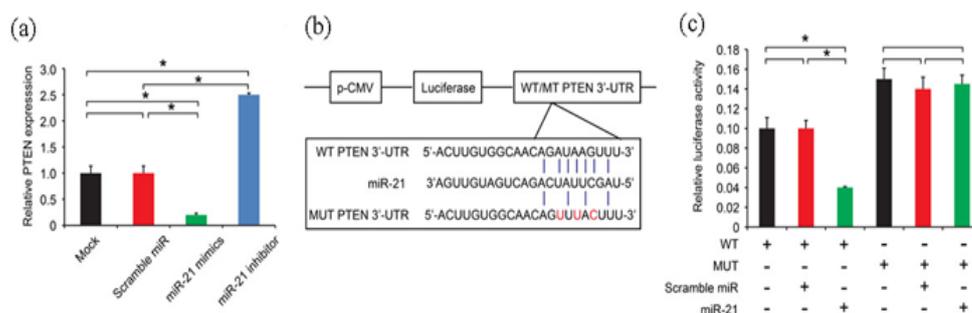
Figure 3. miR-21 Directly Targeted *PTEN*. A. *PTEN* mRNA level after transfected with miR-21 mimics and inhibitor B. two independent luciferase reporter plasmids were generated, containing cloned microRNA response elements (MREs) to miR-21 from the *PTEN* 3′-UTR regulatory region and its mutant type. C. luciferase activity in cells were transiently transfected with constructed plasmid with or without miR-21.

*miR-21 directly targeted PTEN*

microRNA usually act as tumor suppressor silencing in tumor progression (Farazi et al., 2013). Former WB experiment confirm that *miR-21* mimics could downregulate *PTEN* protein expression. We aim to detect whether *miR-21* could regulate *PTEN* mRNA level. *PTEN* mRNA level was increased after transfected with *miR-21* mimics, while decreased with *miR-21* inhibitor
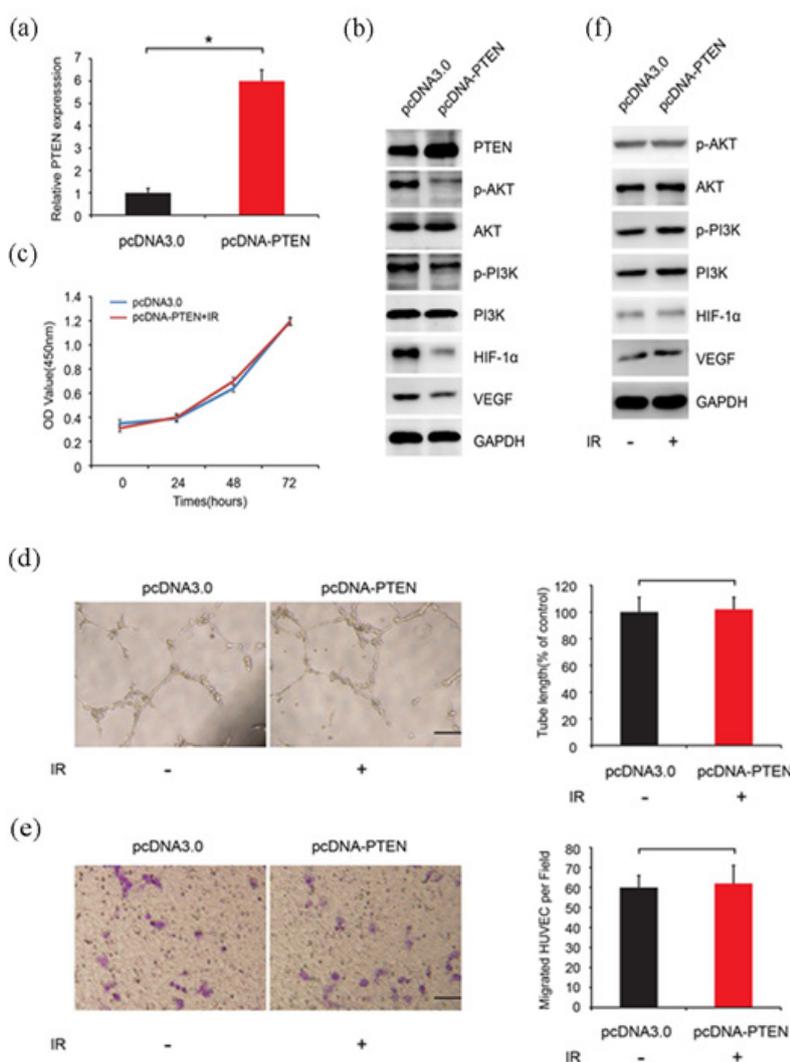


Figure 4. *PTEN* Rescued Irradiation-induced Cell Proliferation, Migration and Tube Formation. A. The construction pcDNA-*PTEN* verification. B. *PTEN*, PI3K, AKT, HIF-1α and VEGF protein level in two group, pcDNA3.0 (empty vector) and pcDNA-*PTEN* with irradiation. C. pcDNA-*PTEN* cell proliferation ability exposed to irradiation. D. pcDNA-*PTEN* cell migration ability exposed to irradiation. E. The pcDNA-*PTEN* cell tube formation bility exposed to irradiation. F. *PTEN*, PI3K, AKT, HIF-1α and VEGF protein level in two group, pcDNA3.0 (empty vector) and pcDNA-*PTEN* with irradiation.

(Figure 3A). Two independent luciferase reporter plasmids, containing cloned microRNA response elements (MREs) to *miR-21* from the *PTEN* 3′-UTR regulatory region and its mutant type, were constructed to demonstrate the direct target genet (Figure 3B). The cells were transfected with constructed plasmid, and found that transfection with pCMV-Luc-WT *PTEN* 3' UTR caused an obvious decrease in luciferase activity compared with empty vector group. However, the activity made no difference when transfected with mutant type plasmid (Figure 3C).

*PTEN rescued irradiation-induced cell proliferation, migration and tube formation*

Since irradiation could increase *miR-21* expression, which thereafter silenced *PTEN* expression, we constructed stably transfected *PTEN* cell lines in order to verify whether *PTEN* could rescue the cell phenotype changes. The construction verification was shown in Figure 4A and B. *PTEN* mRNA and protein level both increased. We set our experiment in two group, pcDNA3.0 (empty vector) and pcDNA-*PTEN* with irradiation. Intriguingly, even if exposure to irradiation, the phenotypes of pcDNA-*PTEN* cells did not change, such as proliferation (Figure 4C), migration (Figure 4D) and tube formation (Figure 4E). WB analysis showed that ectopic expression of *PTEN* down-regulated PI3K, AKT, HIF-1α and VEGF expression (Figure 4B), however, this phenomenon disappeared when the pcDNA-*PTEN* cells exposed to irradiation (Figure 4 F).

## Discussion

Abnormally expressed miRNAs are detected in many cancer types and play critical roles in tumor biology, such as cell apoptosis and metastasis (Lu et al., 2005; Esquela-Kerscher and Slack, 2006; Hwang and Mendell, 2007; Landgraf et al., 2007). However, little is known about the functional role of miRNAs in participating the angiogenesis, which is a critical process in cancer development (Soucek et al., 2007), stimulating by hypoxia, cytokines, and growth factors (Semenza,2001, Goh et al., 2007; Liao and Johnson, 2007). Hypoxia inducible factor-1α (HIF-1α) is a pivotal transcriptional factor regulates angiogenesis by inducing the VEGF expression, interleukin-8 (IL-8), and fibroblast growth factor (Seagroves et al., 2001). Pervious research implied that the synthesis of HIF-1α is regulated through oxygen-independent mechanisms involving oncogene activation, such as H-Ras, myc, and src, tumor suppressor genes such as p53, *PTEN*, and VHL,and various growth factors (Kung et al., 2000; Vleugel et al., 2006; Zhou and Brune, 2006; Huang, 2008). *PTEN* is a pivotal transcriptional factor for angiogenesis (He et al., 2016). We hypothesized that miRNAs might take part in *PTEN* regulation at the transcriptional level and that the dysregulation of miRNAs might contribute to the onset of angiogenesis. In this study, we identified *miR-21* as an IR-inducible miRNA that promotes angiogenesis directaly targeting *PTEN* via PI3K/AKT signaling pathway by increasing VEGF and HIF-1α expression.

Ionized radiation could alter the microRNA profiles, such as let-7 family, *miR-34, miR-15,miR-21, miR-200b* (Simone et al., 2009, Marta et al., 2015). Thereinto, miR-21 was most highly regulated miRNA in both solid and haematological malignancies (Volinia et al., 2006), and it is associated with clinicopathological factors in various malignancies (Chan et al., 2008; Markou et al., 2008; Li et al., 2009; Gao et al., 2010; Hiroki et al., 2010; Motoyama et al., 2010). Pervious studies show that *miR-21* promotes proliferation, migration, invasion and cell cycle (Kuang and Nie, 2016; Li et al., 2016; Yan et al., 2016). In this study, we want to illustrate how *miR-21* influence angiogenesis. We used HUVEC cells to verify our hypothesis. We used conditioned media (control, 0h and 24h) to perform the proliferation, migration and tube formation assays. We could see that after 24h ionization, the cell proliferation and migration ability increased compared to the control groups. Besides, the tube formation capability was much stronger. In this study, we also confirmed that 24h radiation could up-regulate HIF-1α and VEGF expression, which is vital for angiogenesis in cancer development. But the underlying mechanism remained to be cover. Among IR-induced genes, *miR-21* is essential for tumor development. We have showed that *miR-21* level was upregulated after ionized radiation. *miR-21* promoted Huvec proliferation, migration and tube formation. The downregulation or upregulation of many proteins or signaling pathway in the cell angiogenesis, such as CD64/PI3K/Akt (Chen et al.,2016), MAPK/ERK (Chen et al., 2016), Wnt/β-catenin signaling pathway (Wang et al.,2016), were demonstrated. Here, we focus on an critical transcriptional factor *PTEN* participating in PI3K/Akt signaling pathway. We confirm that *miR-21* mimics could down-regulate *PTEN* protein and mRNA expression. Two independent luciferase reporter plasmids were constructed, and found that transfection with pCMV-Luc-WT *PTEN* 3' UTR caused an obvious decrease in luciferase activity compared with control group. However, transfected with mutant type plasmid did not make obvious difference.We could conclude that *miR-21* could directly target *PTEN* expression. We then carried out the rescue experiments. Interestingly, we showed that *PTEN* rescued irradiation-induced cell proliferation, migration and tube formation.

In our own study, we identified *miR-21* as a potent factor of IR-induced angiogenesis. IR induces *miR-21* expression, which in turn causes the downregulation of *PTEN* and finally induces angiogenesis through increased VEGF and HIF-1α through PI3K/AKT pathway.

In summary, we revealed a novel ionization dependent mechanism for regulating *PTEN* expression and inducing angiogenesis, which could be exploited to develop a novel anticancer strategy, especially for tumors that with low expression of *PTEN*. Dissecting the underlying mechanism of activity of *miR-21* in detail will help us understand how cell angiogenesis is regulated during ionized radiation, and is a focus of our future studies.

## Acknowledgements

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