Biochemistry and Cell Biology

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Journal:	<i>Biochemistry and Cell Biology</i>
Manuscript ID	bcb-2018-0301.R2
Manuscript Type:	Article
Date Submitted by the Author:	14-Nov-2018
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Keyword:	miR-3666, Lung cancer, BPTF
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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**miR-3666 inhibits lung cancer cell proliferation, migration and invasion by
targeting BPTF**

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Running Title: miR-3666 inhibits lung cancer cell growth

Key Words: miR-3666 • Lung cancer • BPTF

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Abstract

Our previous study suggested that BPTF overexpression was observed in lung adenocarcinoma, and closely associated with advanced clinical stage, more metastatic lymph nodes, present distant metastasis, low histological grade and poor prognosis. Down-regulation of BPTF inhibited lung adenocarcinoma cells proliferation and promoted lung adenocarcinoma cells apoptosis. The purpose of this study is to identify valuable microRNAs (miRNAs), which target BPTF to modulate lung adenocarcinoma cells proliferation. In our results, we found that miR-3666 was notably reduced in lung adenocarcinoma tissues and cell lines. By using miR-3666 mimics, cells proliferation, migration, and invasion were suppressed by miR-3666 overexpression,

while were enhanced by reduction of miR-3666. Moreover, bioinformatics analysis using Targetscan database and miRanda software suggested a putative targeting site in BPTF 3'-UTR. Furthermore, we verified that miR-3666 directly targeted to 3'-UTR of BPTF by luciferase reporter assay. Overexpression of miR-3666 negatively regulated protein expression of BPTF by western blot. Finally, PI3K/AKT and EMT was demonstrated to be inhibited by miR-3666 overexpression in lung cancer cells. In conclusion, our data indicate that miR-3666 might play an essential role in cell proliferation, migration and invasion by targeting BPTF and partly inhibited PI3K/AKT and EMT signaling pathways in human lung cancers.

1.Introduction

Lung cancer is a leading cause of human death worldwide(Siegel et al. 2013), and non-small cell lung cancer (NSCLC) comprises approximately 85% of all lung cancers(Chen et al. 2013; Grose et al. 2011). Although the diagnostic and therapeutic techniques have been improved in recent decades, poor prognosis of NSCLC still leads to a less than 20% five-year survival rate of the patients(Jemal et al. 2008). Therefore, it is crucial to improve the prognosis to meet the clinical requirements for NSCLC. BPTF, called bromodomain PHD-finger transcription factor, is an ISWI-containing ATP-dependent chromatin remodeling which plays a crucial role in regulation of genes essential for embryo development and tumor progression(Lee et al. 2016). Many cancers, including bladder cancers and melanomas, were reported harboring somatic mutations of BPTF(Balbas-Martinez et al. 2013; Gonzalez-Perez et al. 2013). In addition, some authors reported that BPTF indicated a negative prognosis in patients with hepatocellular carcinoma(Xiao et al. 2015a). Our previous study suggested that bromodomain PHD-finger transcription factor (BPTF) overexpression was observed in lung adenocarcinoma, and closely associated with advanced clinical stage, more metastatic lymph nodes, low histological grade(Dai et al. 2015). Although function and regulation of BPTF have been extensively studied, how BPTF is regulated remains incompletely understood in lung cancer development.

MicroRNAs (miRNAs) are a class of evolutionarily conserved small noncoding RNAs composed of 21~23 nucleotides that usually suppress the expression of protein-coding genes by targeting their 3'-untranslated region (3'-UTR)(Qin et al. 2017; Yang et al. 2017; Zhu et al. 2018). As post-transcriptional regulators of gene expression, miRNAs are involved in regulating many central biological processes, such as cell proliferation(Sun et al. 2018), differentiation(Zhou et al.

2018), apoptosis(Jiang et al. 2018), cell cycle progression(Zhang et al. 2014), migration and invasion(Yang et al. 2014). In recent years, aberrant miRNAs have been recognized to promote progression of lung cancer through enhancing oncogene expression or inhibiting tumor suppressor genes(Cui et al. 2015; Zhong et al. 2014). Thus, identification of novel miRNAs, which can be used for outcome prediction, is important for lung cancer prognosis.

To develop inhibitor for BPTF, we predicted novel and potential miRNAs that could target and regulate BPTF by bioinformatics algorithms. A novel miRNA, miR-3666, contained the predicted binding sites for 3'-UTR of BPTF. miR-3666 has been found to function as tumor suppressor frequently and aberrantly expressing in many types of human cancer, such as cervical cancer(Li et al. 2015), colorectal cancer(Liu et al. 2018), Thyroid Carcinoma(Wang et al. 2016). However, the role of miRNA-3666 in lung cancer and the related mechanism which it is involved has not yet completely been investigated.

2. Materials and Methods

2.1. Sample collection

For the use of these clinical materials for research purposes, prior written informed consent from all the patients and approval from the Ethics Committees of the first affiliated Hospital of Dalian Medical University). Forty-eight fresh lung adenocarcinoma tissue specimens and adjacent non-tumor lung tissue specimens were obtained from the first affiliated Hospital of Dalian Medical University, Liaoning, China. Clinical samples were collected at the time of diagnosis before any therapy. All specimens had confirmed pathological diagnosis by two pathologists.

2.2. Cell culture and transfection

Human lung adenocarcinoma cells (H1299, H1437, A549 and H1975), large cell lung cancer cell H460 and normal human bronchial epithelial cell line (HBE) were obtained from ATCC (Manassas, VA). Cells were maintained in either DMEM medium or RPMI 1640 medium supplemented with 10% fetal bovine serum. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. miR-3666 mimics are chemically synthesized small double-strands miRNAs that mimic mature miR-3666, miR-3666 inhibitors are chemically synthesized and specially modified miRNA that can decrease the function of miRNA by specifically binding to mature miRNA molecules. The mimics-miR-3666, mimics-NC, inhibitor-miR-3666 and inhibitor-NC were purchased from RiboBio (Guangzhou, China) and used

at 20mM Opti-MEM transfection media and lipofectamine 2000 (both from Invitrogen, USA) were used to transfect the cells once they reached 80% confluency. The qRT-PCR was used to test the transfection efficiencies of mimics-miR-3666 and inhibitor-miR-3666 in A549 and H460 cells following 48h transfection.

2.3. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from lung cancer tissues and cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was reverse transcribed from 2μg total RNA using a Reverse Transcription Kit (Takara, Biochemical, Tokyo, Japan). cDNA was then amplified with a SYBR® Premix Ex Taq™ II (Perfect Real-Time) kit (Takara). The relative expression ratio of miR-3666 in each tissue and cell line was calculated using the $2^{-\Delta\Delta CT}$ method.

2.4. Western blotting

Western blot analysis was performed, total protein was isolated from cells after transfection, and protein concentration was measured using the BCA protein assay kit (Beyotime, Shanghai, China) followed by the manufacturer's instruction. Samples were electrophoresed using 10% SDS-PAGE, then proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with specific primary antibodies (1:1000) at 4°C overnight after blocking in skim milk. After being washed with TBST three times for 15 min each, the membranes were incubated with HRP-conjugated anti-rabbit IgG antibodies (1:2000) at room temperature for 1h. The protein antibodies included BPTF (Abcam, USA), phospho-PI3K p85 (Tyr458)/p55 (Tyr199), PI3K, phospho-Akt (Ser473), Akt, p-p38MAPK, p38MAPK, pTyr202/Y204-ERK1/2, ERK1/2, GAPDH (Cell Signaling Technology, Beverly, MA), E-cadherin, Vimentin (proteintech Group, Inc., Chicago, USA), Quantity One Software (Bio-Rad, USA) was used to analyze the intensity of blots.

2.5. Bioinformatics analysis and Luciferase reporter assay

Targetscan and miRanda database were used to analyze the putative target genes of miR-3666. The BPTF wild-type (WT) and mutant (MUT) 3'-UTR were created and cloned to the firefly luciferase-expressing vector the pmiR-RB REPORT luciferase reporter plasmid (RiboBio, China). For the luciferase assay, H460 cells were seeded in 24-well plates, and co-transfected with the pmiR-BPTF-WT/pmiR-BPTF-MUT and mimics-miR-3666/NC-mimics-miR-3666 by using lipofectamine 2000 (Invitrogen, USA). Luciferase activities were determined with the

Dual-Luciferase Reporter System (Promega, USA).

2.6. Cell proliferation and colony formation assay

Cell proliferation was assessed using the CCK8 assay (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Cells were seeded into 6-well plate overnight and treated with mimics-miR-3666, mimics-NC, inhibitor-miR-3666 and inhibitor-NC respectively for 24h. Cells were then harvested, resuspended, counted and single cells were planted into 96-well plate. Ten microliters of CCK-8 was added to each well at 0h, 24h, 48h, 72h, 96h and the absorbance was measured at 450 nm after incubated at 37°C for 2h. The different groups of cells (1000 cells/well) were cultured in triplicate into 6-well plates for 14 days and washed twice with phosphate buffer solution (PBS). Colonies were stained with crystal violet for 15 minutes at room temperature and colonies that contained more than 50 cells were counted to evaluate the cell's viability.

2.7. Cell migration and invasion assay

Matrigel-uncoated and -coated transwell inserts (8 µm pore size; Millipore) were used to evaluate cell migration and invasion. The insert upper standing in 24-well cell culture plate were coated with 100µl 1mg/mL Matrigel Matrix (BD Falcon, USA) in serum-free RPMI-1640 medium and air-dried. 2×10^4 transfected cells were suspended in 150µL serum free medium into the upper chamber, 700µL 1640 medium containing 20% FBS was placed in the lower chamber. After 48h incubation, the cells on the inner layer were softly removed with a cotton swab and outer cells were fixed in 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet dye for 20 min. Cells were counted at five randomly selected views, and the average cell number per view was calculated. The insert upper standing in 24-well cell culture matrigel-uncoated plate was used to assess cell migration ability.

2.8. Statistical analysis

GraphPad Prism 5.0 (GraphPad Software Inc., USA) software was used for statistical analysis. Qualitative data were analyzed by the chi-square test. Independent tests were used to compare differences between two groups. One-way ANOVA with Bonferroni post-hoc tests were performed to compare differences among more than two groups. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. miR-3666 was downregulated in lung cancer tissue and cell lines

To explore the possible role of miR-3666 in lung cancer, we detected its expression level in normal bronchial epithelial cell (HBE) and lung cancer cell lines (H1437, H460, A549, H1299, H1975) by qRT-PCR. The results showed that miR-3666 was expressed in all detected cell lines and downregulated in lung cancer cell lines compared with normal bronchial epithelial cell (HBE) (Fig 1A). In addition, we investigated and analyzed the expression level of miR-3666 in lung cancer tissues and adjacent tissues by qRT-PCR assay. In line with lung tumor cells, the expression of miR-3666 in lung cancer tissue was significantly decreased compared with adjacent tissues (Fig 1B). Considering the crucial function of BPTF in lung cancer cell reported in our previous studies, we also measured the expression of BPTF in lung cancer tissues and adjacent tissues. The results indicated that BPTF protein level in lung cancer tissues was upregulated compared with adjacent tissues (Fig 1C). Therefore, our preliminary results showed that low expression of miR-3666 and high expression of BPTF may associate with lung cancer formation.

3.2. BPTF is a direct target of miR-3666

Bioinformatics analysis by Targetscan and miRanda revealed that BPTF might be a potential target of miR-3666, based on putative target sequences at the BPTF 3'-UTR (Fig. 2A). To confirm that the binding of miR-3666 to BPTF relies on this site, a complementary sequence to the miR-3666 seeds in the BPTF 3'-UTR was mutated to abolish potential miR-3666 binding (Fig 2A). By performing a dual-luciferase reporter assay in lung cancer cells H460, we found that miR-3666 overexpression significantly reduced the Luciferase activity cotransfected with reporter containing wild-type BPTF 3'-UTR compared with control cells that cotransfected with reporter containing mutated-type BPTF 3'-UTR (Fig 2B). Furthermore, Western Blot results showed that ectopic miR-3666 expression decreased the BPTF protein level compared with the NC in both H460 and H1299 cells (Fig 2C). All above data consistently suggested that BPTF is a direct target of miR-3666.

3.3. miR-3666 negatively regulated NSCLC cell growth, migration and invasion

The downregulation of miR-3666 in lung cancer cell lines and tissues suggested that miR-3666 may be a tumor suppressor during the development and progression of lung carcinoma. To validate the physiological function of miR-3666, we performed the “miRNA-gain-of-function” and “miRNA-loss-of-function” assays. The CCK8 assay showed that cell viability was significantly decreased when miR-3666 was overexpressed, whereas cell viability was promoted

by silencing miR-3666 expression in both H460 and H1299 cells (Fig 3B and Fig 4B). We also observed that transfection with miR-3666 mimics obviously reduced the number of colonies formed, while miR-3666 inhibitor increased the colonies formed in H460 and H1299 cells (Fig 3D and Fig 4D), suggesting that miR-3666 can regulate lung cancer cell growth. Subsequently, we investigated the effects of miR-3666 on NSCLC cell migration and invasion by transwell assays. The migration assay showed that cell migration was suppressed when miR-3666 was upregulated, while miR-3666 downregulation significantly accelerated the migration in H460 and H1299 cells (Fig 3C and Fig 4C). Additionally, compared with control cells, overexpression of miR-3666 in H460 and H1299 markedly decreased the ability of cell invasion, whereas cells cultured in miR-3666 inhibitor medium were distinctively more invasive (Fig 3C and Fig 4C). Together, these results suggested that miR-3666 regulated lung cancer cell growth, migration and invasion.

3.4. Overexpression of miR-3666 suppressed PI3K-AKT signaling pathway and EMT-related protein

To elucidate the underlying mechanism of miR-3666 overexpression in regulating lung cancer cell survival, we detected the phosphorylation level of key protein of PI3K/AKT signaling pathway. The results showed that miR-3666 overexpression slightly decreased the phosphorylation of PI3K and AKT (Fig 5A and 5B). We also examined the phosphorylation level of MAPK related protein, P-p38 MAPK and P-ERK1/2. The results indicated that there was no significantly change on the phosphorylation of p38 MAPK and ERK1/2 after miR-3666 was upregulated in H460 and H1299 lung cancer cells (Fig 5A and 5B). In addition, we also detected the protein levels of epithelial–mesenchymal transition (EMT)-related factors which was commonly associated with cell migration and invasion. The results showed that E-cadherin levels were increased, while Vimentin levels were reduced after miR-3666 upregulation in H460 and H1299 lung cancer cells (Fig 5A and Fig 5B). These results indicated that miR-3666 might regulated cell growth, migration and invasion via PI3K/AKT and EMT signaling pathway.

3.5. Overexpression of BPTF reversed the cellular effect caused by miR-3666 overexpression.

To investigate whether miR-3666 affected the biological function of lung cancer cells by targeting BPTF, we overexpressed miR-3666 together with BPTF in lung cancer cell line H460. The migration and invasion assay results indicated that overexpression of BPTF was able to rescue

the decreases of migration and invasion ability caused by miR-3666 overexpression (Fig 6A). We also evaluated the effect of BPTF on cell proliferation caused by overexpression of miR-3666 in lung cancer cell H460 by colony formation assay. Consistently, BPTF transfection in miR-3666 overexpressed H460 cell also rescued the cell growth inhibition of miR-3666 (Fig 6B). To further verify the potential molecular mechanism by which BPTF rescue the decrease of lung cancer cell growth, migration and invasion caused by miR-3666. We firstly analyzed a series of pro-survival proteins which might be affected by BPTF. The results showed that the phosphorylation of PI3K and AKT proteins are enhanced compared with control (Fig 6C). Additionally, the expression of E-cadherin, a key molecule involved in cell invasion and migration, was significantly increased, while Vimentin protein levels were reduced (Fig 6C). To some extent, these data indicated that miR-3666 may inhibit lung cancer cell migration, invasion by targeting BPTF.

4. Discussion

In recent studies, miRNAs have received more and more attention for their high diagnostic and prognostic significance in cancer progression (Iorio and Croce. 2012), including lung cancer (Lin et al. 2013). Elucidating the association of miRNAs with lung cancer progression is of great importance to identify novel therapeutic targets and to improve the clinical outcome of this disease. In this study, we proposed a novel role for miR-3666 in coordinating the expression of BPTF in lung cancer cells. miR-3666 was frequently altered in multiple tumor types and was associated with the pathogenesis and progression of cancer. For example, miR-3666 was significantly decreased in cervical cancer cell lines as well as clinical specimens, and that miR-3666 overexpression decreased cell invasion by targeting the ZEB1 expression (Li et al. 2015). Besides, miR-3666/E2F7 may play a key role in regulating colorectal cancer development (Liu et al. 2018). Although One study suggested that miR-3666 could target SIRT7 to inhibit NSCLC cell growth (Shi et al. 2016), the molecular mechanism was not fully understood. We found that miR-3666 was lowly expressed in lung cancer cell lines and tumor tissues by comparison with normal lung cell and noncancerous lung tissues. Our *in vitro* experiments further suggested that miR-3666 exerted its anti-oncogene function by regulating the proliferation, migration and invasion of lung cancer cells. Biological prediction and cellular experiments results further showed that BPTF was a direct target of miR-3666.

A number of studies have demonstrated that the PI3K/AKT, MAPK (Chen et al. 2013), and

EMT signaling pathway(Xiao et al. 2015b) was excessively activated in tumors and promotes tumor progression. To investigate the underline molecular mechanisms that regulated cell proliferation migration and invasion, we examined PI3K/AKT, MAPK and EMT signaling pathways alteration. miR-3666 overexpression suppressed the phosphorylation of PI3K, AKT in lung cancer cell lines, the phosphorylation of ERK and P38 showed no difference. Additionally, the protein level of E-cadherin were reduced, while Vimentin levels was increased, indicating that EMT was Involved in migration and invasion of tumor cells. In brief, miR-3666 might exert its growth-regulating effect, at least in part, by modulating the PI3K/AKT and EMT signaling pathways in lung cancer cells.

In conclusion, we went a step to certify that miR-3666 was a tumor suppressive microRNA to modulating lung cancer cell proliferation, migration and invasion. The potential mechanism might be the inactivation phosphorylation of PI3K/AKT and EMT as an effect of miRNA-3666. However, Our subject also has its shortcomings, firstly, further detailed analyses are necessary to determine whether miR-3666 inhibited lung cancer cell proliferation, migration and invasion by decreasing the phosphorylation of PI3K/AKT and regulating EMT was directly related with targeting BPTF. Secondly, due to the limited sample size in this study, more studies would be needed to further confirm the prognostic significance of miR-3666 in NSCLC patient.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the the National Natural Science Foundation of China (Grant No. 81702831), Key project of Science and Technology Development Foundation of Nanjing Medical University (2016NJMUZD063).

Disclosure Statement

The authors declare no Disclosure Statement.

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Figure legends

Figure 1 miR-3666 was downregulated in lung cancer tissues and cell lines. (A) The levels of miR-3666 expression in lung cancer lines and normal HBE cells by qPCR. (B) The levels of miR-3666 expression in 48-paired human lung cancer and adjacent normal tissues. (C) The expression of BPTF in lung cancer specimens and adjacent normal tissues was detected by Western Blot. The mean $\pm$ SD in the graph presents the relative levels from three replications. * P <0.05.

Figure 2 miR-3666 directly targeted BPTF in lung cancer cells. (A) Predicted miR-3666 targeting sequence in BPTF 3'-UTR (WT BPTF 3'-UTR). Target sequences of BPTF 3'-UTR were mutated (MUT BPTF 3'-UTR). (B) Dual-luciferase reporter assay of H460 cells transfected with WT BPTF 3'-UTR or MUT BPTF 3'-UTR reported together with 50 nM of miR-3666 mimics or negative control (NC) oligoribonucleotides compared with the relative luciferase activity in WT BPTF 3'-UTR plus miR-NC group. * P <0.05. (C) Western blot detected the expression level of BPTF when H460 cells were treated with miR-3666 mimics. The mean $\pm$ SD in the graph presents the relative levels from three replications. * P <0.05.

Figure 3 The effects of miR-3666 on the cell proliferation, migration, invasion and colony formation by the lung cancer cell line H460 cells. Cells were transfected with miR-3666 mimics or the negative control (NC), miR-3666 specific inhibitor or negative control (NC) respectively. The negative control (NC) cells included a non-targeting sequence. (A) miR-3666 overexpression and inhibition levels were analyzed by qRT-PCR compared with the miR-3666 expression level in the NC group. (B) A CCK-8 assay was used to show the effect of miRNA-3666 overexpression or silencing on the H460 cell proliferation rate. (C) A Transwell assay was used to show the effect of miRNA-3666 overexpression or silencing on H460 migration and invasion. (D) A colony formation assay was used to show the effect of miRNA-3666 overexpression and silencing on the H460 colony formation. The mean $\pm$ SD in the graph presents the relative levels from three replications. * P <0.05.

Figure 4 The effects of miR-3666 on the cell proliferation, migration, invasion and colony formation by H1299 cells. Cells were transfected with miR-3666 mimics or the negative control (NC), miR-3666 specific inhibitor or negative control (NC) respectively. The negative control (NC) cells included a non-targeting sequence. (A) Overexpression levels or inhibition levels of miR-3666 expression were analyzed by RT-qPCR compared with the miR-3666 expression level in the NC group. (B) A CCK-8 assay was used to show the effect of miR-3666 overexpression or inhibition on the H1299 cell proliferation rate. (C) A Transwell assay was used to show the effect of miR-3666 overexpression or inhibition on H1299 migration and invasion. (D) A colony formation assay was used to show the effect of miR-3666 overexpression or inhibition on the H1299 colony formation. The mean $\pm$ SD in the graph presents the relative levels from three replications. * $P < 0.05$.

Figure 5 miR-3666 inactivated the PI3K/AKT and EMT signaling pathway. Western blot analysis of PI3K/AKT, their activation and epithelial-mesenchymal transition (EMT)-related factors: E-cadherin and Vimentin in H460 cells and H1299 cells transfected with a negative control (NC) or miR-3666 mimics(A). the quantitative measurement was shown below the Western Blot in H460 cells (B). the quantitative measurement was shown below the Western Blot in H1299 cells (C). The mean $\pm$ SD in the graph presents the relative levels from three replications. * $P < 0.05$.

Figure 6 Overexpression of BPTF reversed the cellular effects caused by miR-3666 overexpression. (A) Cell migration and invasion was detected by migration and Matrigel invasion assay in H460 cells transfected with BPTF overexpression plasmid and miR-3666 mimics. (B) Cell growth ability was measured by colony formation assay. (C) Western Blot was used to detected the expression of BPTF, phosphorylated and total PI3K and AKT, E-cadherin, and Vimentin protein in H460 after transfected with miR-3666 mimics or co-transfected with miR-3666 mimics and BPTF plasmids. The mean $\pm$ SD in the graph presents the relative levels from three replications. * $P < 0.05$.

Figure 1 miR-3666 was downregulated in lung cancer tissues and cell lines. (A) The levels of miR-3666 expression in lung cancer lines and normal HBE cells by qPCR. (B) The levels of miR-3666 expression in 48-paired human lung cancer and adjacent normal tissues. (C) The expression of BPTF in lung cancer specimens and adjacent normal tissues was detected by Western Blot. The mean $\pm$ SD in the graph presents the relative levels from three replications. * $P < 0.05$.

254x190mm (300 x 300 DPI)

Figure 2 miR-3666 directly targeted BPTF in lung cancer cells. (A) Predicted miR-3666 targeting sequence in BPTF 3'-UTR (WT BPTF 3'-UTR). Target sequences of BPTF 3'-UTR were mutated (MUT BPTF 3'-UTR). (B) Dual-luciferase reporter assay of H460 cells transfected with WT BPTF 3'-UTR or MUT BPTF 3'-UTR reported together with 50 nM of miR-3666 mimics or negative control (NC) oligoribonucleotides compared with the relative luciferase activity in WT BPTF 3'-UTR plus miR-NC group. *P<0.05. (C) Western blot detected the expression level of BPTF when H460 cells were treated with miR-3666 mimics. The mean $\pm$ SD in the graph presents the relative levels from three replications. *P<0.05.

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Figure 3 The effects of miR-3666 on the cell proliferation, migration, invasion and colony formation by the lung cancer cell line H460 cells. Cells were transfected with miR-3666 mimics or the negative control (NC), miR-3666 specific inhibitor or negative control (NC) respectively. The negative control (NC) cells included a non-targeting sequence. (A) miR-3666 overexpression and inhibition levels were analyzed by qRT-PCR compared with the miR-3666 expression level in the NC group. (B) A CCK-8 assay was used to show the effect of miRNA-3666 overexpression or silencing on the H460 cell proliferation rate. (C) A Transwell assay was used to show the effect of miRNA-3666 overexpression or silencing on H460 migration and invasion. (D) A colony formation assay was used to show the effect of miRNA-3666 overexpression and silencing on the H460 colony formation. The mean $\pm$ SD in the graph presents the relative levels from three replications. *P<0.05.

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Figure 4 The effects of miR-3666 on the cell proliferation, migration, invasion and colony formation by H1299 cells. Cells were transfected with miR-3666 mimics or the negative control (NC), miR-3666 specific inhibitor or negative control (NC) respectively. The negative control (NC) cells included a non-targeting sequence. (A) Overexpression levels or inhibition levels of miR-3666 expression were analyzed by RT-qPCR compared with the miR-3666 expression level in the NC group. (B) A CCK-8 assay was used to show the effect of miR-3666 overexpression or inhibition on the H1299 cell proliferation rate. (C) A Transwell assay was used to show the effect of miR-3666 overexpression or inhibition on H1299 migration and invasion. (D) A colony formation assay was used to show the effect of miR-3666 overexpression or inhibition on the H1299 colony formation. The mean $\pm$ SD in the graph presents the relative levels from three replications. *P<0.05.

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Figure 5 miR-3666 inactivated the PI3K/AKT and EMT signaling pathway. Western blot analysis of PI3K/AKT, their activation and epithelial-mesenchymal transition (EMT)-related factors: E-cadherin and Vimentin in H460 cells and H1299 cells transfected with a negative control (NC) or miR-3666 mimics(A). the quantitative measurement was shown below the Western Blot in H460 cells (B). the quantitative measurement was shown below the Western Blot in H1299 cells (C). The mean $\pm$ SD in the graph presents the relative levels from three replications. *P<0.05.

254x166mm (300 x 300 DPI)

Figure 6 Overexpression of BPTF reversed the cellular effects caused by miR-3666 overexpression. (A) Cell migration and invasion was detected by migration and Matrigel invasion assay in H460 cells transfected with BPTF overexpression plasmid and miR-3666 mimics. (B) Cell growth ability was measured by colony formation assay. (C) Western Blot was used to detected the expression of BPTF, phosphorylated and total PI3K and AKT, E-cadherin, and Vimentin protein in H460 after transfected with miR-3666 mimics or co-transfected with miR-3666 mimics and BPTF plasmids. The mean $\pm$ SD in the graph presents the relative levels from three replications. * $P < 0.05$.

254x190mm (300 x 300 DPI)