Long non-coding RNA regulates hair follicle stem cell proliferation and differentiation through PI3K/AKT signal pathway

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Abstract. Long non-coding RNAs (lncRNAs) are defined as non-coding transcripts (>200 nucleotides) that serve important roles in the proliferation and differentiation of stem cells. Hair follicle stem cells (HFTs) have multidirectional differentiation potential and are able to differentiate into skin, hair follicles and sebaceous glands, serving a role in skin wound healing. The aim of the present study was to analyze the regulatory role of lncRNA AK015322 (IncRNA5322) in HFTs and the potential mechanism of IncRNA5322-mediated differentiation of HFTs. The results demonstrated that lncRNA5322 transfection promoted proliferation and differentiation in HFTs. It was identified that lncRNA5322 transfection upregulated the expression and phosphorylation of phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) in HFTs. It was also observed that lncRNA5322 transfection upregulated microRNA (miR)-21 and miR-21 agonist (agomir-21) eliminated lncRNA5322-induced expression and phosphorylation of PI3K and AKT. The present study also demonstrated that agomir-21 blocked IncRNA5322-induced expression and phosphorylation of PI3K and AKT in HFTs. The results indicated that agomir-21 transfection also suppressed the IncRNA5322-induced proliferation and differentiation of HFTs. In conclusion, the results of the present study suggest that lncRNA5322 is able to promote the proliferation and differentiation of HFTs by targeting the miR-21-mediated PI3K-AKT signaling pathway in HFTs.

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Introduction

Long non-coding RNAs (lncRNA) are a class of small non-coding transcripts >200 nucleotides and have been identified to serve a role in the proliferation and differentiation of cells (1-3). In recent years, lncRNAs have become the focus of research into a number of human diseases, including metabolic and hereditary diseases, cancer and human stem cell differentiation (4-6). Evidence has indicated that lncRNA is associated with cellular signal pathway transduction, which suggests that lncRNA may integrate into the pluripotency network and be a target for patient-specific cell-based therapies (7-9). Molecular signaling mechanisms have confirmed that lncRNA is associated with a variety of cellular metabolism processes via regulating different signal pathways in human cells (HFTs) (10,11).

HFTs are adult stem cells and have a marked proliferation ability in the skin wound healing process (12,13). An immunohistochemical study suggested that HFTs are able to induce hair follicle growth by targeting Wnt10b (14). Shen *et al* (15) demonstrated that β-catenin induces HFT differentiation into transit-amplifying cells via upregulating c-myc activation. Another study indicated that the *in vivo* transcriptional governance of HFTs may be regulated by Wnt regulators (16). miR-128 is reported to regulate the differentiation of hair follicle mesenchymal stem cells into smooth muscle cells by targeting SMAD family member 2 (17). These reports suggest that HFTs may regulate cellular metabolism by regulating different molecule-mediated signal pathways.

The aim of the present study was to investigate the regulatory role of lncRNA AK015322 (IncRNA5322), which is regarded to be an important lncRNA for stem cells proliferation and differentiation (18), in the differentiation of HFTs. The results revealed the importance of the lncRNA5322/microRNA (miR)-21/phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway in the proliferation and differentiation of HFTs. It was also demonstrated that the proliferation and differentiation of hair follicle stem cells is based on the interaction between lncRNA5322 and miR-21, thereby regulating the PI3K/AKT signaling pathway in HFTs.

Materials and methods

Cells and reagents. HFTs were purchased from Beijing Jing-Meng High-Tech Stem Cell Technology Co., Ltd. (Beijing, China). They were cultured in Minimum Essential Medium (MEM) (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

Transfection of lncRNA5322 or miR-21 assay. LncRNA5322 (19) or miR-21 (5'-UCAACAUCAGUCAGAUAAGCUA-3') and negative control lncRNA-vector (18) or miR-vector (control, 5'-UAGCUUAUCAGACAGAUGUUGA-3') were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, USA). plncRNA5322 or pmiR-21 was cloned into the pBabe vector (Cell Biolabs, Inc., San Diego, CA, USA) to generate the plncRNA5322 and pmiR-21 vectors. Transfection of plncRNA5322, pmiR-21 and negative control vectors was performed using X-treme GENE RNA transfection reagent (Roche Applied Science, Rotkreuz, Switzerland). Transfection concentrations were 100 nM for plncRNA5322 and pmiR-21 or negative vector. After 48-h following transfection, cells were used to further analysis.

Cells proliferation and differentiation. HFTs were cultured and treated with agomir-21 or PI3K inhibitor (0.5 mg/ml, Guangzhou RiboBio Co., Ltd., Guangzhou, China) at 37˚C in a humidified atmosphere containing 5% CO_2. Cell proliferation and differentiation were analyzed as previously described (20,21). For cell differentiation, HFTs were cultured in MEM for 12 h at 37˚C. HFT colonies growing on Matrigel (Corning China, Ltd., Shanghai, China) were loosely detached by dispase treatment for 5 min and washed 3 times with PBS. Cells were resuspended in Dulbecco's modified Eagle's medium (Sigma Aldrich; Merck KGaA) containing 20% FBS. Cells were maintained on 1% agar-coated slides and allowed to differentiate for another 18 days at 37˚C. Cells were subsequently fixed with 10% formalin for 1 h at 37˚C and stained with 60% Oil Red O in isopropanol as working solution for 10 min at 37˚C. The proportion of Oil Red O-positive cells was determined by counting stained cells under a light microscope at 40x magnification. For cell proliferation assay, HFTs were seeded in 96-well plates (10^3 cells/well) and cultured for 24 h at 37˚C. Cells proliferation was determined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from HFTs using the RNAeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA) and 1 μg total RNA was transcribed into cDNA using an RT kit (Qiagen Sciences, Inc.) for 1.5 h at 42˚C. The cDNA (10 ng) was subjected to a qPCR using a SYBR-Green Master Mix system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and sequences are as follows: Cyclin-dependent kinase (CDK)1 forward, 5'-CAGACTAGAAAGTGAAGAGGAAGG-3' and reverse, 5'-ACTGACCAGGAGGGATAGAATC-3'; CDK2 forward, 5'-TTGGCAGCACACTCTATG-3' and reverse, 5'-CCTCATTCGGCAAATAAACG-3'; LncRNA5322 forward, 5'-GACGAACTGACCGGTTGTCT-3' and reverse, 5'-GTGACAGAGGGATAGCGAGC-3'; and β-actin forward, 5'-CGGAGTCAACGGATTTGGTC-3' and reverse, 5'-AGCCTTCTCCATGGTCGTGA-3'. The following thermocycling conditions were applied: 45 amplification cycles consisting of denaturation at 95˚C for 30 sec, primer annealing at 63˚C for 45 sec with touchdown to 57˚C for 50 sec, and applicant extension at 72˚C for 60 sec. Relative mRNA expression changes were calculated using the $2^{-\Delta\Delta Cq}$ method (22). The results are expressed as the n-fold way compared to control.

Western blotting. HFTs were collected and lysed in radioimmunoprecipitation assay buffer (mammalian protein extraction reagent for the cells and tissue protein extraction reagent for the tissues; Thermo Fisher Scientific, Inc.) followed by homogenization at 4˚C for 10 min. Protein concentration was measured by a Biconchoninic Acid protein assay kit (Thermo Fisher Scientific, Inc). A total of 20 μg protein/lane was separated by 12.5% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated in blocking buffer (5% milk) for 2 h at 37˚C prior to incubation with primary antibodies at 4˚C overnight. The primary rabbit anti-mouse antibodies used in the present study were: CDK1 (ab133327; 1:1,000; Abcam, Cambridge, UK), CDK2 (ab76146; 1:1,000; Abcam), PI3K (ab182651; 1:200; Abcam), AKT (ab8805; 1:1,000; Abcam), phosphorylated (p)PI3K (ab182651; 1:1,000; Abcam), pAKT (ab38449; 1:500; Abcam) and β-actin (ab8226; 1:500; Abcam). A horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; Bio-Rad Laboratories, Inc.) was used as the secondary antibody. Bands were visualized using Western Blotting Luminol Reagent (Pierce™ Fast Western Blot kits, SuperSignal™ West Femto; Thermo Fisher Scientific, Inc.). Bands intensities normalized to β-actin. The density of the bands was analyzed by Quantity One software version 4.62 (Bio-Rad Laboratories, Inc.).

Luciferase reporter assay. The 3'-untranslated region (3'-UTR) sequence of PI3K and AKT, predicted to interact with lncRNA AK015322 or lncRNA vector (control) sequence within the predicted target sites (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml), was inserted into the pGL3 control vector (Promega Corporation, Madison, WI, USA). These constructs were designated as PI3K-3'-UTR and AKT-3'-UTR, respectively. For the reporter assay, HFTs cells were seeded in 24-well plates and transfected with the above constructs using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.), lncRNA AK015322 expression vector and negative control. After 48 h, the cells were collected and Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's protocols. Results were obtained from 3 independent experiments performed in duplicate.

Statistical analysis. Data are presented as the mean ± standard deviation of triplicate dependent experiments and analyzed using Student t-tests or one-way analysis of variance followed by Tukey's honest significant difference test.

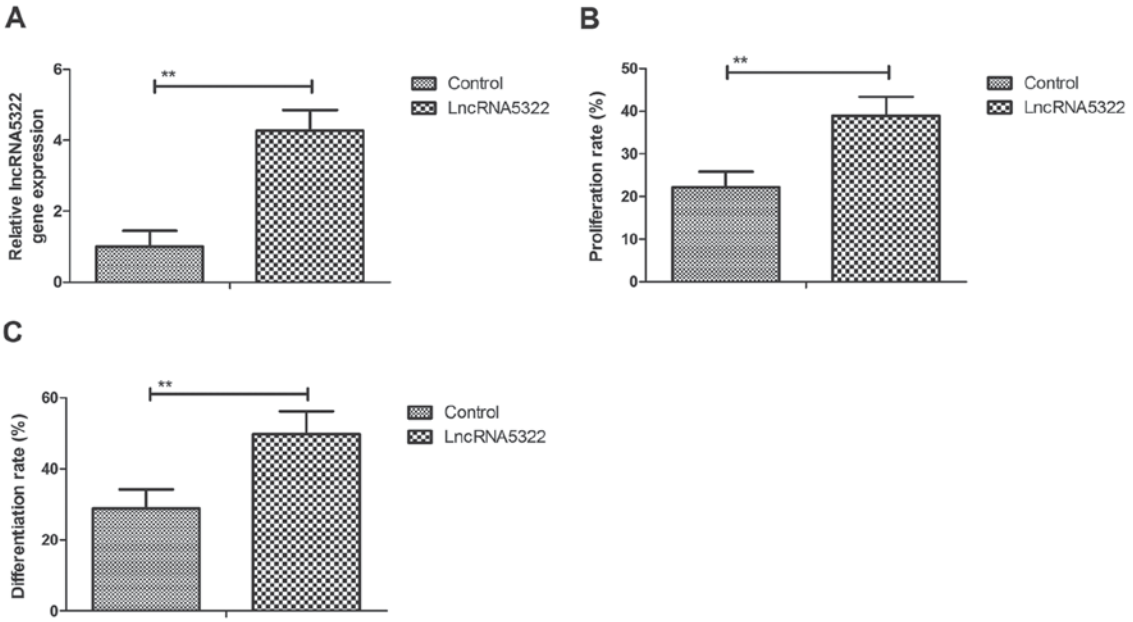

Figure 1. LncRNA5322 promotes the proliferation and differentiation of HFTs. Transfection with lncRNA5322 (A) increases lncRNA5322 expression (B) stimulates proliferation and (C) promotes differentiation in HFTs. **P<0.01. Lnc, long non-coding; HFT, hair follicle stem cell.

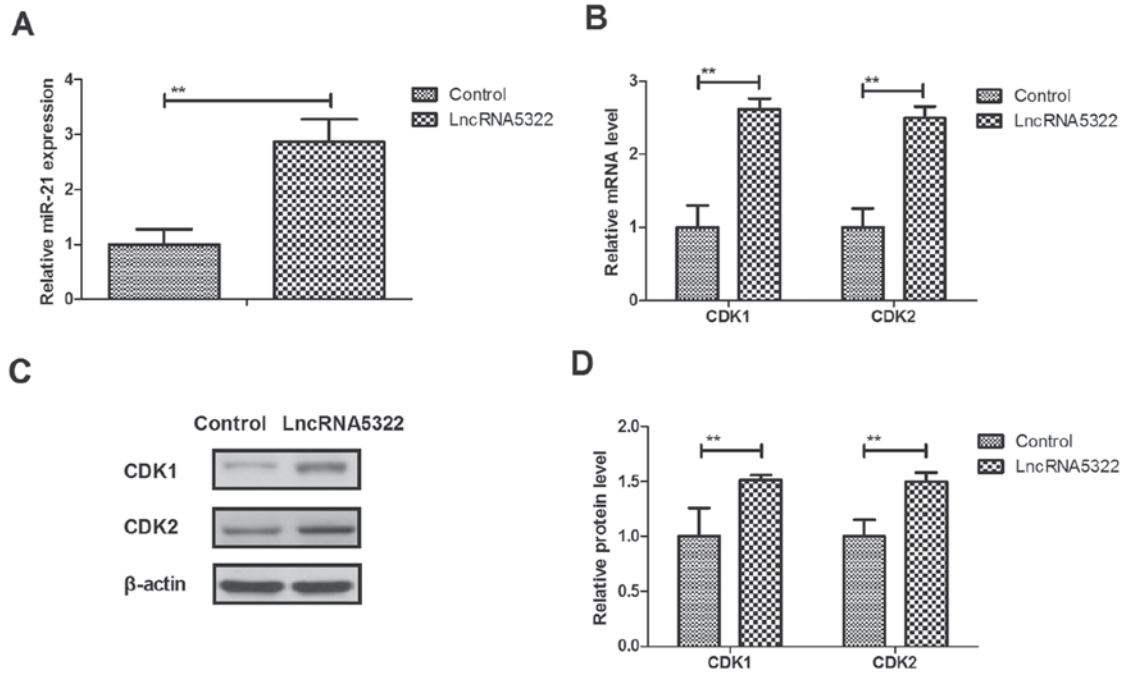

Figure 2. LncRNA5322 upregulates miR-21 expression and cell cycle during HFT differentiation. LncRNA5322 transfection upregulates (A) miR-21 expression as well as CDK1 and CDK2 (B) mRNA and (C) protein expression in HFTs. Band intensities were normalized to β-actin. (D) Effects of LncRNA5322 transfection on CDK1 and CDK2 expression in HFTs. **P<0.01. Lnc, long non-coding; miR, microRNA; HFT, hair follicle stem cell; CDK, cyclin-dependent kinase.

Significance was established using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

LncRNA5322 stimulates proliferation and differentiation of HFTs. The effect of lncRNA5322 on the proliferation and differentiation of HFTs was investigated. Transfection with lncRNA5322 increased lncRNA5322 expression in HFTs compared with the control (Fig. 1A). The results demonstrated that lncRNA5322 transfection stimulated proliferation of HFTs compared with control cells (Fig. 1B). The results also revealed that PlncRNA-1 transfection markedly promoted HFT differentiation (Fig. 1C). These results indicate that lncRNA5322 transfection induces the proliferation and differentiation of HFTs.

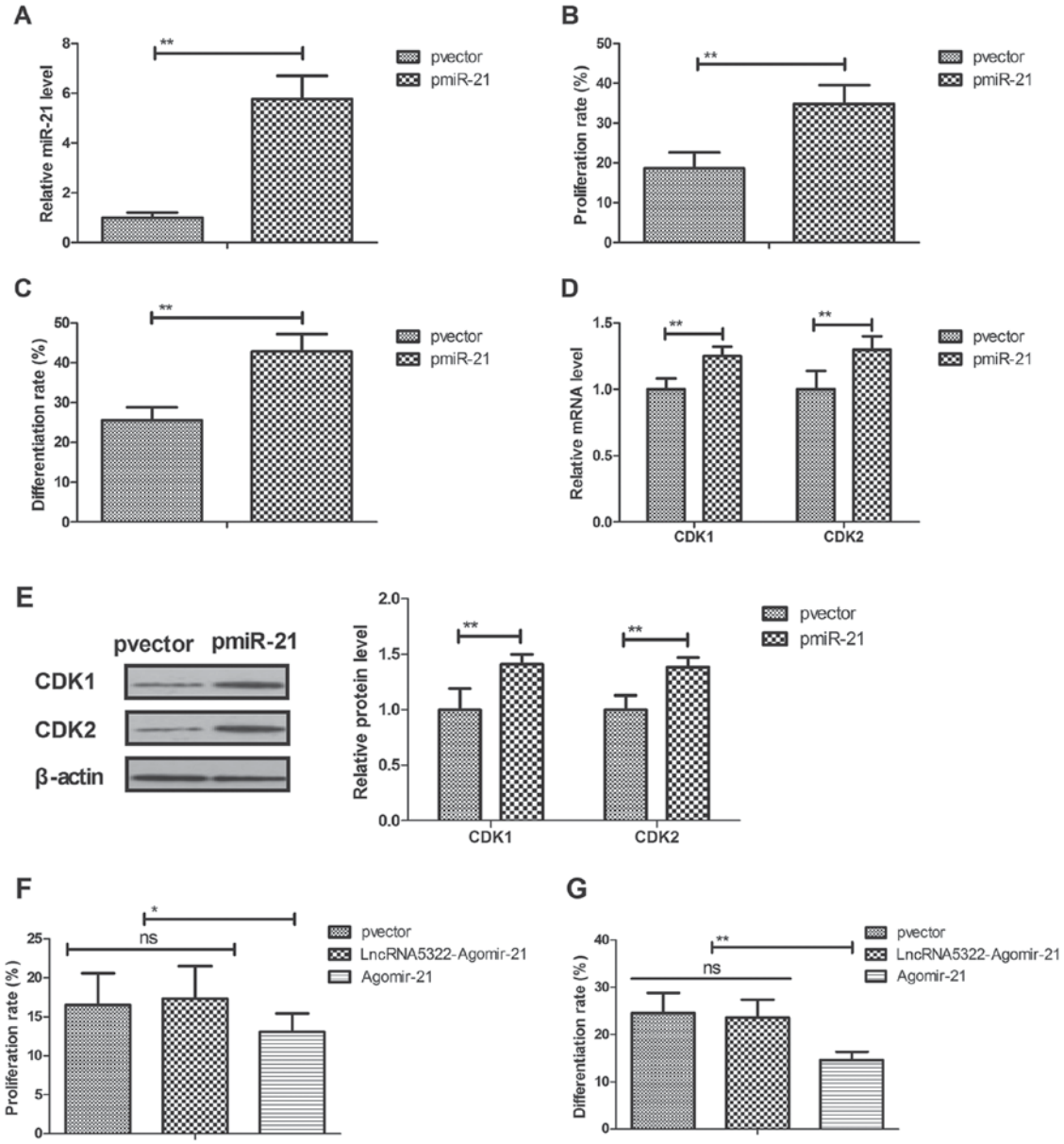

Figure 3. Transfection with miR-21 promotes HFT proliferation and differentiation. Transfection with miR-21 increases (A) miR-21 expression, (B) proliferation and (C) differentiation, as well as the expression of CDK1 and CDK2 (D) mRNA and (E) protein expression during HFT differentiation. Bands intensities were normalized to β-actin. Agomir-21 transfection blocks lncRNA5322-mediated (F) proliferation and (G) differentiation of HFTs. *P<0.05, **P<0.01. miR, microRNA; HFT, hair follicle stem cell; CDK, cyclin-dependent kinase; lnc, long non-coding; ns, not significant.

LncRNA AK015322 upregulates miR-21 expression and cell cycle during HFTs differentiation. miR-21 transfection has been reported to promote the differentiation of hair follicle-derived neural crest stem cells into Schwann cells (23). In the present study, it was demonstrated that miR-21 expression levels were upregulated by lncRNA5322 transfection-induced differentiation (Fig. 2A). The results revealed that lncRNA5322 transfection promotes CDK1 and CDK2 mRNA and protein expression in HFTs (Fig. 2B-D). These results suggest that LncRNA5322 upregulates miR-21 expression and increases CDK1 and CDK2 expression during HFT differentiation.

miR-21 transfection promotes HFT proliferation and differentiation. The role of miR-21 in the proliferation and differentiation of HFTs was investigated. Results demonstrated that miR-21 transfection (pmiR-21; Fig. 3A) promoted HFT proliferation and differentiation (Fig. 3B and C). Transfection with miR-21 also upregulated CDK1 and CDK2 mRNA and protein expression levels during HFTs differentiation (Fig. 3D and E). Agomir-21 transfection blocked lncRNA5322-mediated HFT proliferation and differentiation (Fig. 3F and G). These results indicate that transfection with miR-21 promotes HFT proliferation and differentiation and increases cyclin expression during HFT differentiation.

LncRNA AK015322 upregulates PI3K/AKT expression and phosphorylation during HFT differentiation. A previous study indicated that the PI3K/AKT signaling pathway is associated with stem cell differentiation (24). The association between lncRNA5322 and the PI3K/AKT signaling pathway during HFT differentiation was analyzed. The results revealed that lncRNA5322 transfection increased PI3K and

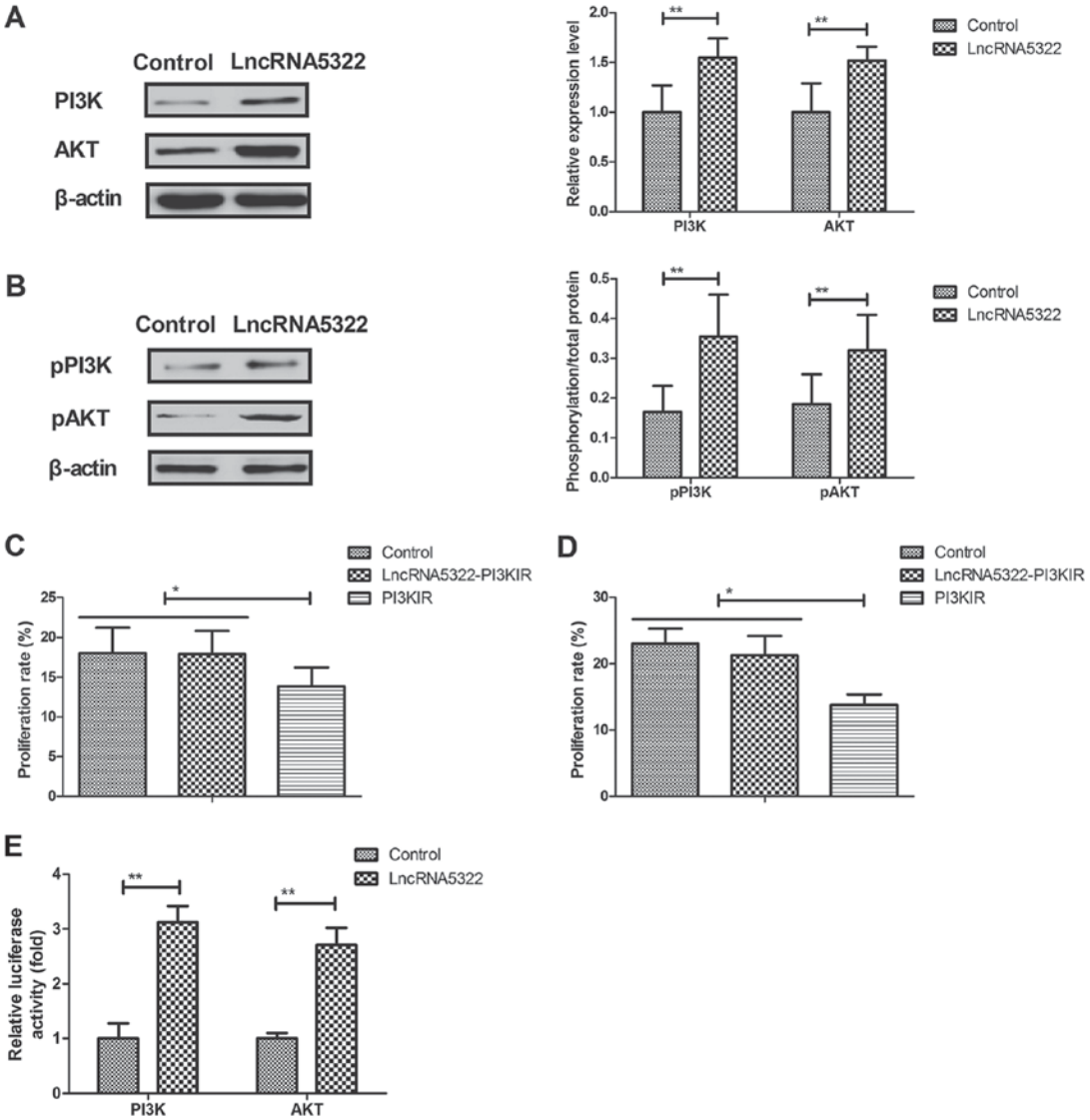

Figure 4. LncRNA5322 upregulates PI3K/AKT expression and phosphorylation during HFT differentiation. lncRNA5322 transfection increases PI3K and AKT (A) expression and (B) phosphorylation in HFTs. Bands intensities were normalized to β-actin. PI3K inhibitor inhibits lncRNA5322-induced (C) proliferation and (D) differentiation of HFTs. (E) LncRNA5322 transfection increases the luciferase activity of PI3K and AKT in HFTs *P<0.05, **P<0.01. Lnc, long non-coding; PI3K, phosphoinositide-3-kinase; AKT, protein kinase B; HFT, hair follicle stem cell.

AKT expression and phosphorylation levels in HFTs (Fig. 4A and B). It was also demonstrated that PI3K inhibitor (PI3KIR) inhibited lncRNA5322-induced proliferation and differentiation of HFTs (Fig. 4C and D). Notably, the results of a luciferase gene report assay demonstrated that lncRNA5322 transfection increased the luciferase activity of PI3K and AKT (Fig. 4E). These results suggest that lncRNA5322 regulates proliferation and differentiation in HFTs via the PI3K/AKT signal pathway.

Agomir-21 blocks lncRNA AK015322-induced upregulation of the PI3K/AKT signaling pathway during HFT differentiation. The effects of agomir-21 on the PI3K/AKT signaling pathway in HFTs were investigated. It was demonstrated that agomir-21 transfection decreased PI3K and AKT expression and phosphorylation levels in HFTs (Fig. 5A and B). Agomir-21 transfection also blocked the lncRNA5322-induced upregulation of the PI3K/AKT signaling pathway during HFT differentiation (Fig. 5C and D).

Discussion

LncRNA has demonstrated a potential role in the progression of multilineage differentiation of HFTs (25,26). The molecular characteristics and multipotency of HFT differentiation has been reviewed in bulge cells and dermal papilla mesenchyme cells as well as in the mechanism of hair growth (27). LncRNAs have been reported to be associated with mesenchymal stem cell differentiation via triple helix formation (28). The present study analyzed the regulatory effects of lncRNA5322 on HFT proliferation and differentiation and explored the potential mechanisms of the lncRNA5322-mediated signaling pathway. The results suggest that lncRNA5322 regulates HFT proliferation and differentiation via regulation of the miR-mediated PI3K/AKT signaling pathway.

A previous study regarding gene therapy and novel wound treatments reported that it is necessary to consider epidermal cells and HFTs as distinct populations (29). Hu *et al* (19) stated that lncRNA5322 is able to promote proliferation of C18-4

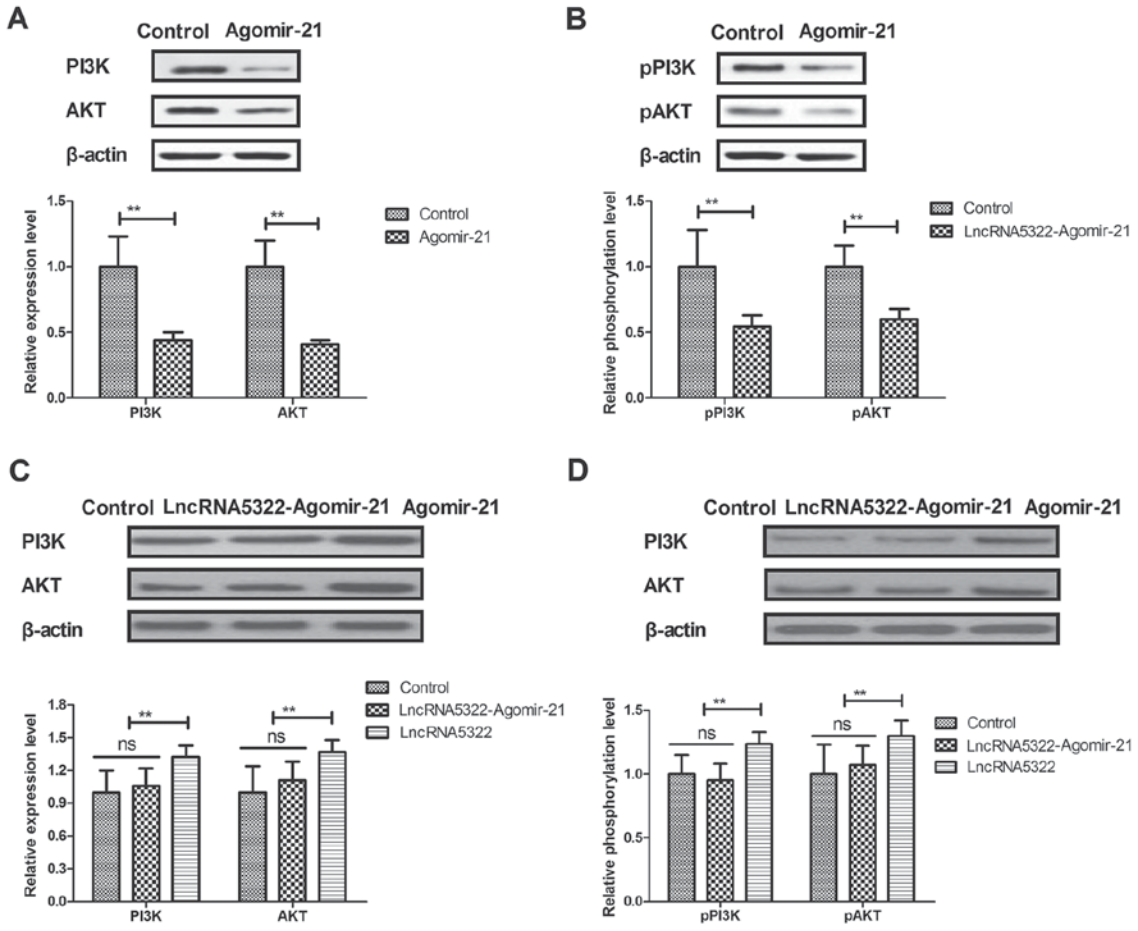

Figure 5. Agomir-21 blocks lncRNA5322-induced upregulation of the PI3K/AKT signaling pathway during HFT differentiation. Agomir-21 transfection decreases PI3K and AKT (A) expression and (B) phosphorylation in HFTs. Agomir-21 transfection blocks lncRNA5322-induced PI3K and AKT (C) expression and (D) phosphorylation during HFT differentiation. Bands intensities were normalized to β-actin. **P<0.01. Agomir-21, microRNA-21 agonist; PI3K, phosphoinositide-3-kinase; AKT, protein kinase B; Lnc, long non-coding; HFT, hair follicle stem cell.

spermatogonial stem cells by acting as a decoy for miR-19b-3p. In the present study, it was demonstrated that lncRNA5322 also stimulates the proliferation of and upregulates miR-21 expression in HFTs. Evidence has suggested that miR-21 promotes the differentiation of hair follicle-derived neural crest stem cells into Schwann cells (23). In the present study, it was observed that miR-21 transfection stimulates the proliferation and differentiation of HFTs, whereas agomir-21 blocks lncRNA5322-induced proliferation and differentiation. This suggests that lncRNA5322 may regulate proliferation and differentiation of HFTs via miR-21 expression.

To investigate the mechanism by which lncRNA5322 regulates the proliferation and differentiation of HFTs, the PI3K/AKT signaling pathway was assessed. The results demonstrated that the expression and phosphorylation of PI3K and AKT in lncRNA5322-transfected HFTs were significantly increased. A previous study reported that PI3K us able to regulate bone morphogenetic protein 2-induced β-catenin activation in human bone marrow stem cells (30). A further study has indicated that the PI3K/AKT signaling pathway serves a critical role in neuron differentiation from human neural stem cells (31). The results of the present study demonstrate that lncRNA5322 or miR-21 transfection lead to upregulation of the PI3K/AKT signaling pathway in HFTs. Deng *et al* (32) suggested that miR-21 reduced hydrogen peroxide-induced apoptosis in c-kit+ cardiac stem cells *in vitro* via PTEN/PI3K/AKT signaling. The findings of the present study indicate that agomir-21 blocks lncRNA5322-induced upregulation of the PI3K/AKT signaling pathway during HFT differentiation. These results shed light on a potential novel signaling pathway responsible for lncRNA5322-mediated HFT differentiation.

The present study revealed that lncRNA5322 stimulates proliferation following 24 h transfection. However, cell cycle analysis was not performed following lncRNA5322 transfection in HFTs. Another limitation is that HFT differentiation was only analyzed using Oil Red O staining, not differentiation markers or fluorescence-activated cell sorting analysis. Additionally, previous studies have reported other potential molecular pathways that may be associated with the proliferation (33-37) and differentiation of HFTs, including the Wnt signal transduction pathway, forkhead box P1-mediated oxidative stress and epidermal growth factor receptor/extracellular signal-regulated kinases/AKT, c-Jun N-terminal kinases/c-Jun and TGF-β pathways (38). The present study only investigated the PI3K/AKT signaling pathway in HFTs. Therefore, further experiments, including cell cycle analysis of HFTs and differentiation markers, are required to confirm the results of the present study. Other potential molecular pathways of HFT proliferation and differentiation should be considered.

In conclusion, this study revealed that lncRNA5322 transfection promotes miR-21 expression and induces the proliferation and differentiation of HFTs via upregulating the PI3K/AKT signaling pathway. miR-21 is a direct target of lncRNA5322 in HFT differentiation, which provides a potential insight into the repair mechanism of injured skin by tissue engineering.

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