LncRNA PlncRNA-1 regulates proliferation and differentiation of hair follicle stem cells through TGF-β1-mediated Wnt/β-catenin signal pathway

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Abstract. The present study demonstrated that hair follicle stem cells (HFSc) have multidirectional differentiation potential and participate in skin wound healing processes. Long non-coding RNAs (lncRNAs) are defined as non-protein coding transcripts longer than 200 nucleotides, which are important in the proliferation and differentiation of cells. The purpose of the present study was to investigate the role of PlncRNA-1 in the proliferation and differentiation of HFSc. Results revealed that PlncRNA-1, transforming growth factor (TGF)-β1, Wnt and β-catenin expression levels were significantly downregulated in HFSc. PlncRNA-1 transfection promoted proliferation and differentiation of HFSc. TGF-β1, Wnt and β-catenin expression levels were upregulated in HFSc following transfection of PlncRNA-1. Results demonstrated that TGF-β1 inhibitor LY2109761 blocked proliferation and differentiation of HFSc promoted by PlncRNA-1 transfection. In addition, TGF-β1 inhibitor LY2109761 led to decreased Wnt and β-catenin expression levels in HFSc. Furthermore, PlncRNA-1 transfection stimulated the cell cycle of HFSc, whereas TGF-β1 inhibitor LY2109761 inhibited the cell cycle of HFSc and decreased the acceleration of the cell cycle induced by PlncRNA-1 transfection. In conclusion, these findings suggest that PlncRNA-1 may promote proliferation and differentiation of HFSc through upregulation of TGF-β1-mediated Wnt/β-catenin signaling pathway.

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Introduction

Long non-coding RNAs (lncRNAs) are a class of small non-protein coding transcripts longer than 200 nucleotides (1). Molecular biology analyses have suggested that lncRNAs participate in variety of processes of cellular metabolism by regulation of different signal pathways in many kinds cells (2). In recent years, lncRNA have now become the new hotspots in an ocean of human diseases including metabolic diseases, hereditary disease and cancer (3-5). Evidences have indicated that lncRNAs are associated with stem cell pluripotency, indicating lncRNA may integrate into the pluripotency network, as well as prominent questions in this emerging field (6,7). Previous study has indicated that lncRNA can regulate cell proliferation, apoptosis and differentiation of P19 cells by regulating Mef2c gene (8). Systematically profiling and annotating lncRNAs have showed that lncRNAs provide valuable resources for both experimental biologists and bioinformaticians in various human embryonic stem cell (9).

Hair follicle stem cells (HFSc) belong to adult stem cell and show remarkable proliferation ability in the static state. Research has found that the HFSc have multidirectional differentiation potential, which can differentiate into skin, hair follicles, sebaceous glands and participates in skin wound healing process (10). Previous study has showed that generation of induced pluripotent stem cells can be differentiated from hair follicle bulge neural crest stem cells (11). Lien *et al* have indicated that *in vivo* transcriptional governance of HFSc can be regulated by Wnt regulators (12). Additionally, differentiation of human HFSc into endothelial cells induced by vascular endothelial and basic fibroblast growth factors has been clearly presented (13). Notably, current state of knowledge, the widening gap in translational research and future challenges of human epithelial HFSc and their progeny need to be further investigated in future.

In the present study, the potential signal pathways of proliferation and differentiation of HFSc were analyzed *in vitro*. Findings in this study demonstrated the importance of transforming growth factor (TGF)-β1-mediated Wnt/β-catenin signal pathways in the proliferation and differentiation of HFSc. We here show that the proliferation and differentiation of HFSc is based on the interaction of PlncRNA-1 with the

TGF-β1 expression levels thereby regulating the Wnt/β-catenin signal pathways in HFSc.

Materials and methods

Cells and reagents. HFSc and hair follicle cells (HFc) were purchased from Beijing JingMeng High-Tech Stem Cell Technology Co., LTD (Beijing, China). HFSc and hair follicle cells were cultured in MEM medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA, USA). All cells were cultured in a 37˚C humidified atmosphere of 5% CO_2.

Transfection of PlncRNA-1 assay. PlncRNA-1 and negative control lncRNA-vector (control) were obtained from GenePharma (Shanghai, USA). PlncRNA-1 was cloned into the pBabe vector to generate the PlncRNA-1 and further used to transfect HFSc to establish the PlncRNA-1 overexpression cell line. Transfection of PlncRNA-1 or negative control lncRNA-vector was performed using X-treme GENE RNA transfection reagent (Roche, Switzerland). Transfection concentrations were 100 nM for PlncRNA-1 and lncRNA-vector.

Cells proliferation and differentiation. HFSc or PlncRNA-1 overexpression HFSc were cultured and treated by TGF-β1 inhibitor LY2109761 (0.5 mg/ml) for 24 h at a 37˚C humidified atmosphere of 5% CO_2. Cells proliferation was determined by ^{3}H-Thymidine incorporation and differentiation was analyzed by flow cytometry referencing previous report (14-16). Briefly, HFSc (250,000 cells/well) were seeded on TGF-β1 inhibitor LY2109761 (0.5 mg/ml) coated plates and cultured until they reached 70-80% confluence. For HFSc differentiation analysis, HFSc were incubated with FITC-labeled anti-CD34 antibody (1: 500; ab81289; Abcam, Cambridge, UK) for 2 h at a 4˚C. Cells were then washed PBS and analyzed the percentage of FITC-positive HFSc using flow cytometry.

Analysis cells cycle. To analyze the effects of PlncRNA-1 and/or LY2109761on the cell cycle stage of HFSc, flow cytometry was performed. Exponentially, culturing HFSc or PlncRNA-1 overexpression hair were treated with LY2109761 (0.5 mg/ml) for 24 h. Cells were washed and trypsinized and rinsed with phosphate-buffered saline (PBS). All cells were fixed in 75% ice-cold ethanol for 5 min and then washed with PBS three times. The fixed cells were washed with RNase A (20 μg ml/l, Fermentas) and stained propidium iodide (20 μg ml/l, Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37˚C. The percentages of cells in G1 phase were analyzed using BD FACS Calibur (Becton Dickinson, NJ, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from HFSc by using RNAeasy Mini kit (QIAGEN, Gaithersburg, MD). 1 μg total RNA was used to transcribe into cDNA by using the reverse transcription kit (QIAGEN, Gaithersburg, MD) and confirmed quality by electrophoresis. The cDNA (10 ng) was subjected to quantitative PCR (Bio-Rad, Laboratories, Inc., Hercules, CA, USA) with SYBR-Green Master Mix system (50 ng of genomic DNA, 200 μM dNTP, 2.5 units of Taq DNA polymerase, and 200 μM primers) according to manufacturers' protocols, followed by preliminary denaturation at 94˚C for 2 min, 35 cycles at 94˚C for 30 sec, annealing temperature reduced to 64˚C for 30 sec and 72˚C for 10 min. All the forward and reverse primers were synthesized by Invitrogen (PlncRNA-1, NR_038892.1; CD200, KJ897199.1; CD133, HQ628627.1; OCT4, HQ907734.1; SOX2, AH011668.2; TGF-β1, NM_001311325.1; Wnt3, DQ658158.1; β-catenin, M77013.1; Axin2, AF205888.1; CyclinD1, NM_001086776.1 and Myc: NM_002467.4). Relative mRNA expression changes were calculated by $2^{-\Delta\Delta Ct}$ (17). The results are expressed as the n-fold way compared to control.

Western blot analysis. HFSc were homogenized in lysate buffer containing protease-inhibitor and were centrifuged at 8000 rpm/min at 4˚C for 10 min. The supernatant of mixture were used for analysis of purpose protein. The primary antibodies used in the immunoblotting assays were: TGF-β1 (1:500; ab92486), Wnt (1:500; ab28472), β-catenin (1:500; ab32572), and β-catenin (1:500; ab8227; all Abcam). Horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Laboratories, Inc.) was used at a 1:5,000 dilution and detected using a Western Blotting Luminol Reagent. The results were visualized by using chemi-luminescence detection system (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis. All date were expressed as mean ± SD of triplicate dependent experiments and analyzed by using student t-tests or one-way ANOVA (Tukey HSD test). Significance was established with the SPSS statistical (SPSS, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA USA). $^*P<0.05$ and $^{**}P<0.01$ was considered to indicate a statistically significant difference.

Results

Effects of PlncRNA-1 expression on cells cycle, proliferation and differentiation of HFSc. Previous studies have indicated that CD200 and CD133 are expressed in HFSc and OCT4 and SOX2 genes are representative differentiation genes for HFSc (18-21). We compared these gene expression levels between HFSc and HFc. We showed that gene expression levels of CD200 and CD133 were higher in HFSc than HFc (Fig. 1A). Differentiation genes of OCT4 and SOX2 were also overexpressed in HFSc compared to HFc (Fig. 1B). PlncRNA-1 expression was investigated in HFSc. We found that PlncRNA-1 expression was downregulated in HFSc compared to HFc determined by RT-qPCR (Fig. 1C). Results showed that PlncRNA-1 transfection promoted cells cycle of HFSc (Fig. 1D). We also found that proliferation and differentiation of HFSc were stimulated by PlncRNA-1 transfection (Fig. 1E and F). PlncRNA-1 transfection also promoted growth of HFSc (Fig. 1G). These results indicate PlncRNA-1 regulates cells cycle, proliferation and differentiation of HFSc.

Effects of PlncRNA-1 expression on TGF-β1, Wnt and β-catenin expression levels in HFSc. We analyzed TGF-β1, Wnt and β-catenin expression levels in HFSc after PlncRNA-1 transfection. We confirmed PlncRNA-1 transfection increased PlncRNA-1 expression in HFSc (Fig. 2A). Results showed that TGF-β1, Wnt3 and β-catenin expression levels were upregulated

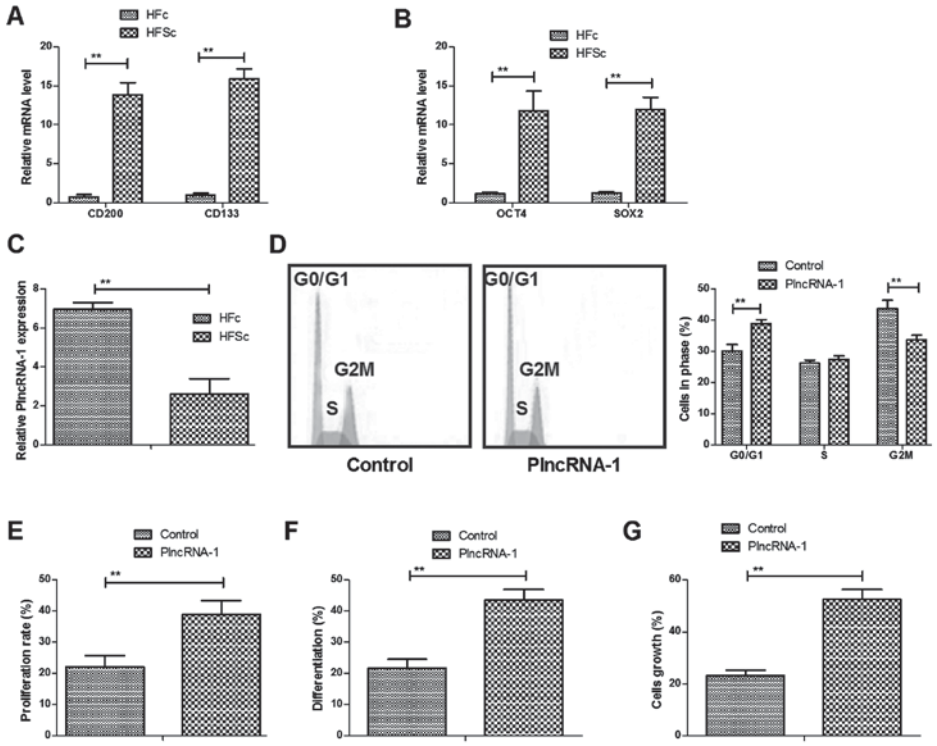

Figure 1. Effects of PlncRNA-1 expression on cells cycle, proliferation and differentiation of HFSc. (A) Gene expression levels of CD200 and CD133 between HFSc and HFc. (B) Gene expression levels of OCT4 and SOX2 between HFSc and HFc. (C) PlncRNA-1 expression between HFSc and HFc determined by RT-qPCR. (D) PlncRNA-1 transfection promotes cells cycle of HFSc. (E and F) PlncRNA-1 transfection promotes proliferation (E) and differentiation (F) of HFSc. (G) PlncRNA-1 transfection promotes growth of HFSc. *P<0.05, **P<0.01 for differences between PlncRNA-1 and control groups. HFSc, hair follicle stem cells; HFc, hair follicle cells.

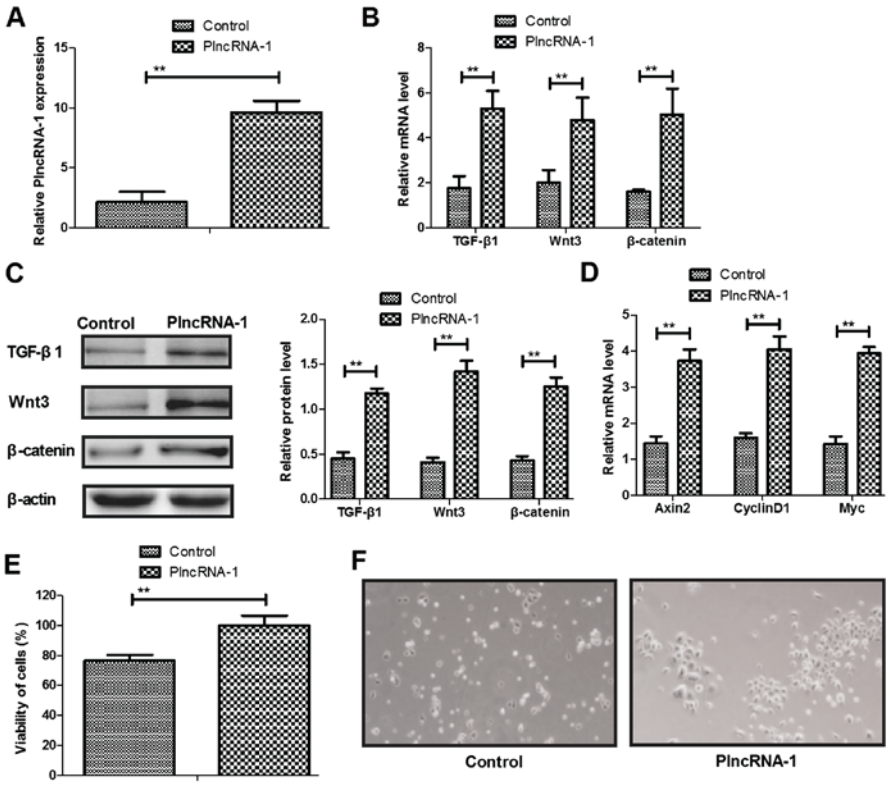

Figure 2. Effects of PlncRNA-1 expression on TGF-β1, Wnt and β-catenin expression levels in HFSc. (A) PlncRNA-1 transfection increases PlncRNA-1 expression in HFSc. (B) PlncRNA-1 transfection upregulates TGF-β1, Wnt3 and β-catenin mRNA expression levels in HFSc. (C) PlncRNA-1 transfection upregulates TGF-β1, Wnt and β-catenin protein expression levels in HFSc. (D) PlncRNA-1 transfection increases Wnt signaling pathway downstream effectors Axin2, CyclinD1 and Myc gene expression in HFSc. (E) PlncRNA-1 overexpression improves viability of HFSc. (F) PlncRNA-1 transfection does not change the stemness of HFSc. *P<0.05, **P<0.01 for differences between PlncRNA-1 and control groups. TGF, transforming growth factor; HFSc, hair follicle stem cells; HFc, hair follicle cells.

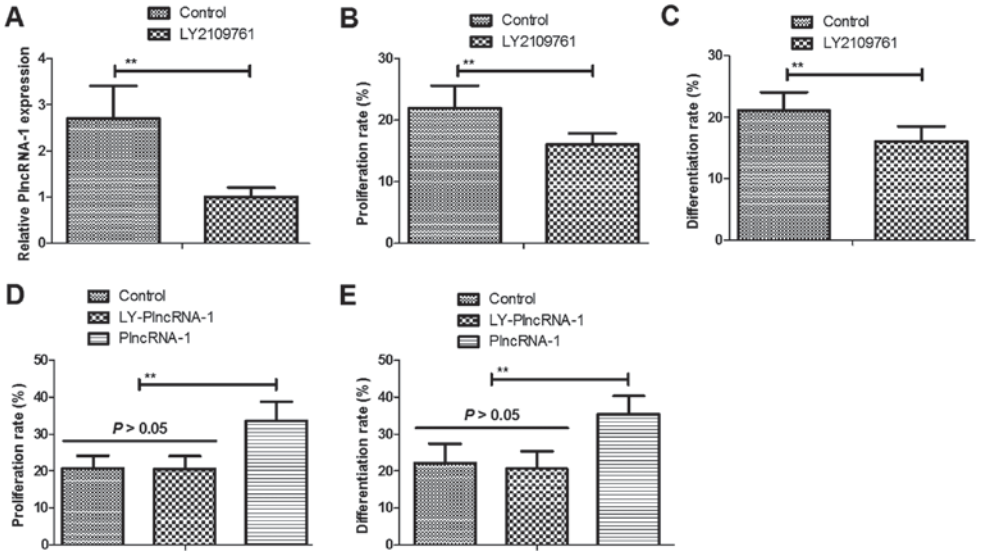

Figure 3. Effects of TGF-β1 inhibitor LY2109761 on proliferation and differentiation of HFSc. (A) TGF-β1 inhibitor LY2109761 decreases PlncRNA-1 expression in HFSc. (B and C) LY2109761 treatment significantly inhibits proliferation (B) and differentiation (C) of HFSc. (D and E) TGF-β1 inhibitor LY2109761 abolished PlncRNA-1 (LY-PlncRNA-1) transfection-induced proliferation (D) and differentiation (E) of HFSc. **P<0.01 for differences between PlncRNA-1 and control groups. TGF, transforming growth factor; HFSc, hair follicle stem cells; HFc, hair follicle cells.

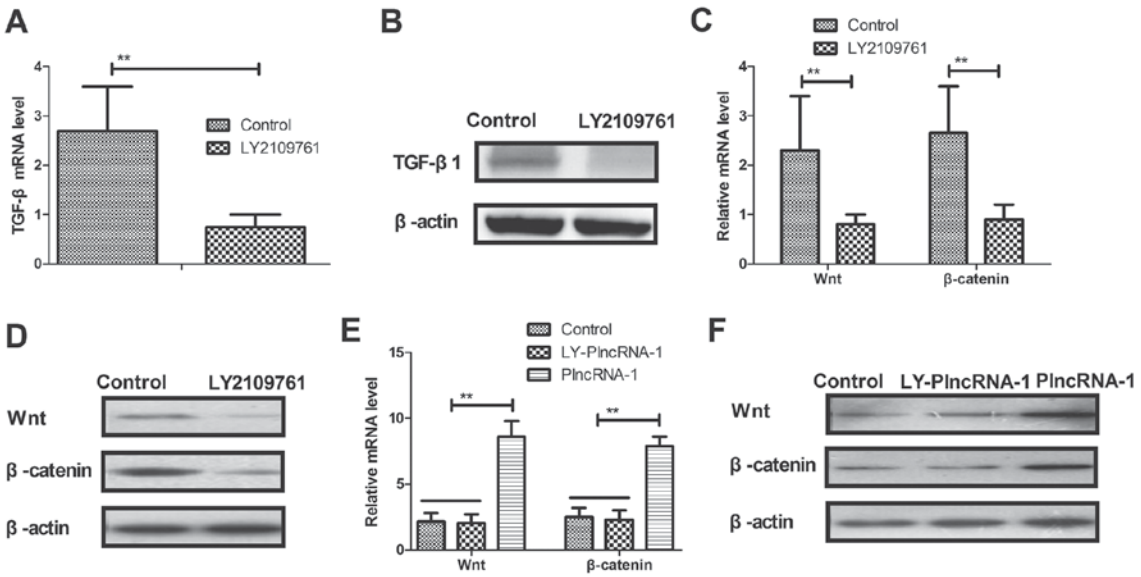

Figure 4. Effects of TGF-β1 inhibitor LY2109761 on Wnt and β-catenin expression levels in HFSc. (A and B) TGF-β1 inhibitor LY2109761 significantly decreases TGF-β1 mRNA (A) and protein (B) expression levels in HFSc. (C and D) TGF-β1 inhibitor LY2109761 significantly downregulates Wnt and β-catenin mRNA (C) and protein (D) expression levels in HFSc. (E and F) TGF-β1 inhibitor LY2109761 abolishes PlncRNA-1 transfection-increased Wnt and β-catenin mRNA (E) and protein (F) expression levels in HFSc. **P<0.01 for differences between PlncRNA-1 and control groups. TGF, transforming growth factor; HFSc, hair follicle stem cells.

by PlncRNA-1 transfection in HFSc (Fig. 2B and C). We demonstrated that PlncRNA-1 transfection increased Wnt signaling pathway downstream effectors Axin2, CyclinD1 and Myc gene expression in HFSc compared to control (Fig. 2D). We found that PlncRNA-1 expression upregulation led to increasing of viability of HFSc (Fig. 2E). The identity of the stem cells demonstrated small colonies and the cells were round and small, with uniform morphology (Fig. 2F), which showed that PlncRNA-1 transfection did not change the stemness of HFSc. These results indicate that PlncRNA-1 promotes TGF-β1, Wnt and β-catenin expression levels in HFSc.

Effects of TGF-β1 inhibitor LY2109761 on proliferation and differentiation of HFSc. We next analyzed the effects of TGF-β1 inhibitor LY2109761 on proliferation and differentiation of HFSc. We observed that TGF-β1 inhibitor LY2109761 decreased PlncRNA-1 expression in HFSc (Fig. 3A). As shown in Fig. 3B and C, LY2109761 inhibited proliferation and differentiation of HFSc. We also showed that TGF-β1 inhibitor LY2109761 abolished PlncRNA-1 (LY-PlncRNA-1) transfection-induced proliferation and differentiation of HFSc (Fig. 3D and E). These results indicate that TGF-β1 inhibitor LY2109761 abolishes PlncRNA-1-regulated proliferation and differentiation of HFSc.

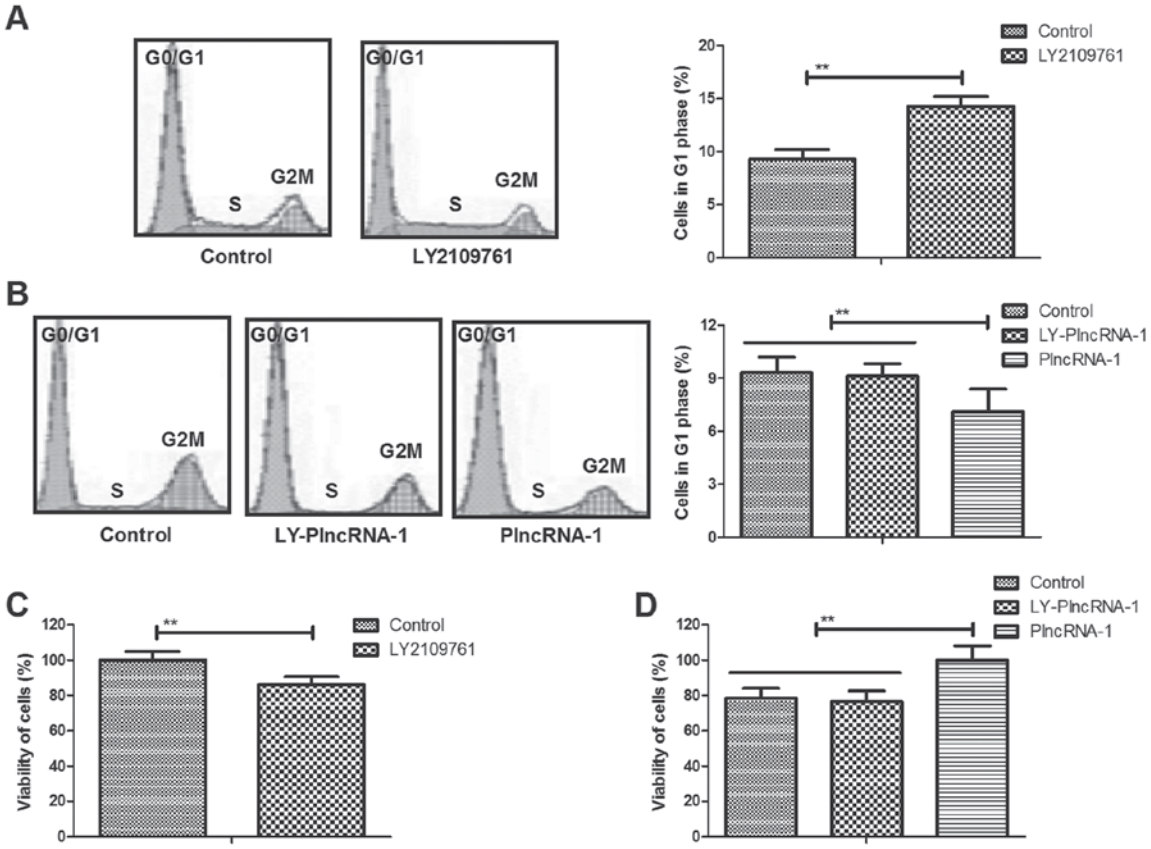

Figure 5. Effects of TGF-β1 inhibitor LY2109761 on cells cycle of HFSc. (A) TGF-β1 inhibitor LY2109761 inhibits cells cycle of HFSc. (B) TGF-β1 inhibitor LY2109761 canceled PlncRNA-1 transfection-promoted cells cycle of HFSc. (C) TGF-β1 inhibitor LY2109761 leads to decreasing of viability of HFSc. (D) TGF-β1 inhibitor LY2109761 abolishes PlncRNA-1 transfection-increased viability of HFSc. **P<0.01 for differences between PlncRNA-1 and control groups. TGF, transforming growth factor; HFSc, hair follicle stem cells.

Effects of TGF-β1 inhibitor LY2109761 on Wnt and β-catenin expression levels in HFSc. The effects of TGF-β1 inhibitor LY2109761 on Wnt and β-catenin expression were investigated in HFSc. As shown in Fig. 4A and B, TGF-β1 inhibitor LY2109761 significantly decreased TGF-β1 expression in HFSc. Results demonstrated that TGF-β1 inhibitor LY2109761 significantly downregulated Wnt and β-catenin expression levels in HFSc (Fig. 4C and D). We observed that TGF-β1 inhibitor LY2109761 abolished PlncRNA-1 transfection-promoted Wnt and β-catenin expression levels in HFSc (Fig. 4E and F). These results indicate that TGF-β1 inhibitor LY2109761 downregulates Wnt and β-catenin expression levels in HFSc.

Effects of TGF-β1 inhibitor LY2109761 on cells cycle of HFSc. We further analyzed effects of TGF-β1 inhibitor LY2109761 on cells cycle of HFSc. As shown in Fig. 5A, TGF-β1 inhibitor LY2109761 inhibited cells cycle of HFSc compared to non-treated cells. Results showed that TGF-β1 inhibitor LY2109761 abolished PlncRNA-1 transfection-promoted cells cycle of HFSc (Fig. 5B). We observed that TGF-β1 inhibitor LY2109761 also led to decreasing of viability of HFSc (Fig. 5C). We also found that TGF-β1 inhibitor LY2109761 abolished PlncRNA-1 transfection-increased viability of HFSc (Fig. 5D). These results suggest TGF-β1 inhibitor (LY2109761) canceled cells cycle of HFSc promoted by PlncRNA-1 transfection.

Discussion

HFSc present more potential in the progression of multilineage differentiation, which can be induced to differentiate into neurons and glial cells, smooth muscle cells and melanocytes, as well as melanocytes and keratinocytes (22,23). Previous reports have investigated the role of PlncRNA-1 in inducing apoptosis of human cancer cells (24-26). However, no reports analyzed the regulatory effects of PlncRNA-1 in the progression of HFSc. In this study, we investigated the role of PlncRNA-1 on proliferation and differentiation of HFSc *in vitro*. Results showed that PlncRNA-1 transfection significantly promoted cells cycle, proliferation and differentiation of HFSc. Previous reports have found that Wnt/β-catenin pathway involves in the regulation of stem cells proliferation and differentiation (27,28). Therefore, we assumed that PlncRNA-1 may regulate HFSc differentiation. Findings have found that PlncRNA-1 can promote proliferation and differentiation of HFSc through upregulation of TGF-β1-mediated Wnt/β-catenin signal pathway.

Previous study has revealed that the lncRNA GAS5 could regulate TGF-β-induced smooth muscle cell differentiation via RNA-Smad binding element (29). In this study, we demonstrated PlncRNA-1 transfection stimulated TGF-β1 expression, which further stimulated proliferation and differentiation of HFSc. However, TGF-β1 inhibitor LY2109761 blocked cells cycle of HFSc promoted by PlncRNA-1 transfection. Evidences

have showed that lncRNA can influence colorectal carcinoma cell lines proliferation by regulating cyclin D1 expression (30). Liu *et al* have indicated that miR-18b inhibits TGF-β1-induced HFSc differentiation into smooth muscle cells by targeting SMAD2, which provided novel insights into the regulatory mechanisms of TGF-β-induced differentiation of HFSc (31). In addition, the lncRNA PARROT is regarded as a regulator of c-Myc and upregulates proliferation and translation of human mammary epithelial cells (32). We reported that PlncRNA-1 transfection increased Wnt signaling pathway downstream effectors Axin2, CyclinD1 and Myc gene expression in HFSc compared to control. Overall, our findings in this analysis showed that PlncRNA-1 transfection led to upregulation of TGF-β1 that further contributed to promotion of cells cycle for HFSc.

Proliferation and differentiation of HFSc are essential for the trauma repair. Previous review has summarized the Wnt signal transduction pathway in controlling the proliferation and differentiation of HFSc (33). Leiros *et al* also indicated that HFSc differentiation is inhibited through cross-talk between Wnt/β-catenin and androgen signaling in dermal papilla cells from patients with androgenetic alopecia (34). Watabe *et al* has showed that stimulation of embryonic stem cell-derived endothelial cells with TGF-β resulted in phosphorylation of both Smad2 and Smadl/5 (35). Our results have indicated that inhibition of TGF-β1 expression inhibited Wnt/β-catenin expression levels in HFSc, which further resulted in inhibition of proliferation and differentiation of HFSc. Notably, results demonstrated that TGF-β1 inhibitor LY2109761 blocked proliferation and differentiation of HFSc promoted by PlncRNA-1 transfection, which provided potential insights to understand molecular mechanism mediated by PlncRNA-1 in HFSc.

In conclusion, results indicate that TGF-β1 inhibitor LY2109761 could canceled PlncRNA-1 transfection-promoted cells cycle, proliferation and differentiation of HFSc. Findings in the current study indicate that PlncRNA-1 could regulate cells cycle, proliferation and differentiation of HFSc via TGF-β1-mediated Wnt/β-catenin signal pathway. Our findings will help to understand the potential molecular mechanisms of differentiative capacity of HFSc and show PlncRNA-1 may contribute to the signal pathway about proliferation and differentiation of HFSc.

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