Preliminary Study on Human Adipose Stem Cells Promoting Skin Wound Healing Through Notch1 Signaling Pathway

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Research Article

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Abstract

Background: Mesenchymal stem cells (MSCs) have been documented as possible candidates for wound healing treatment because their use could reinforce the regenerative capacity of many tissues. Human adipose stem cells (hADSCs) have the advantages of easy access, large quantity and easy operation. They can be fully applied in the treatment of skin wounds. In this study, we aim to explore the roles and potential mechanisms of hADSCs in cutaneous wound healing.

Methods: hADSCs were obtained from human subcutaneous fat. Adipocytes and osteocytes differentiated from hADSCs were determined by staining with Oil Red O and alkaline phosphatase (ALP), respectively. We assessed the effects of hADSCs and hADSC conditional medium (CM) on wound healing in an injury model of mice. Than, we investigated the biological effects of hADSCs on human keratinocytes HaCAT cells in vitro.

Results: The results showed that hADSCs could be successfully differentiated into osteogenic and lipogenic cells. hADSCs and hADSCs-CM significantly promote skin wound healing in vivo. hADSCs significantly promoted HaCAT cells proliferation and migration through activating Notch1 signaling pathway, and activated the AKT signaling pathway by Rps6kb1 kinase in HaCAT cells. In addition, we found that hADSCs-mediated activation of Rps6kb1/AKT signaling was dependent on the Notch1 signaling pathway.

Conclusion: We demonstrated that hADSCs can promote skin cell-HaCAT cells proliferation and migration via Notch1 pathway, suggesting that hADSCs may provide an alternative therapeutic approach for the treatment of skin injury.

Background

The skin is not only the largest tissue and organ of the human body, but also a barrier to protect our body from various physical, chemical and microbial invasions[1]. Wound healing is known as one of the most perplexing biological processes for injured skin caused by trauma, burns, or diabetic diseases, which causes a great psychological burden[2]. Therefore, it is crucial to speed up the healing of skin wounds to restore their function and aesthetics. Skin wound healing involves four precisely integrated and overlapping phases[3] .It requires a well-coordinated response of inflammation, cell proliferation, extracellular matrix formation, and re-epithelialization[4].It is a challenge for clinical treatment. Therefore, it is necessary to explore new treatment methods for skin wound healing.

MSCs are pluripotent stem cells with differentiation abilities, and have a significant promise for regenerative medicine because of their crucial role in improving various tissues to regenerate[5]. Some studies have reported the important role of MSCs in wound healing [6,7]. Quantitative studies have shown that MSCs promote skin wound healing by stimulating re-epithelialization[4,8], promoting angiogenesis[9,10], inhibiting inflammatory responses[11,12], regulating extracellular matrix remodeling[13],

promoting cell proliferation[14]and so on. As one of the MSCs, hADSCs have been widely investigated for their advantages of easy access, high volume and simple extraction. Indeed, applications of hADSCs for skin defects have shown some satisfactory results, such as modulating the inflammatory response[15-17], promoting the neovascularization[17-19] and enhancing the proliferation and migration of skin cells[20,21]. the present study aimed to elucidate the mechanism of hADSCs in wound healing.

Notch signaling pathway plays an important role in embryonic development, postnatal growth and development[22]. In mammals, the Notch signaling pathway has four main receptors (Notch1-4) and five ligands (Delta-like1 (Dll-1), Delta-like4 (Dll-4), Jagged1 (Jag1) and Jagged2 (Jag2), The receptor binds to the ligand at the membrane and releases the Notch intracellular domain (NICD) into the nucleus through the shearing action of γ-secretase, and binds to the corresponding transcription factors to form a transcriptional activation complex, thus regulating the expression of downstream target genes such as Hes1 (Hairy Enhancer of Split-1 , Hes1), etc[23]. Numerous studies have reported that the Notch signaling pathway plays a significant role in the wound healing of skin[24,25].

In the present study, we isolated hADSCs in human subcutaneous fat and characterized their morphology and pluripotency. Then, hADSCs were applied to treat the skin wounds in a mouse injury model and investigated whether hADSCs regulate wound healing through the Jagged1/Notch signaling pathway. We found that hADSCs and hADSCs-CM can promote skin wound healing through activation of the Jag1/Notch1 signaling pathway. In vitro experiments, we have observed that hADSCs significantly enhanced the proliferation and migration of human keratinocytes HaCAT via targeting Notch1 pathway. In addition, the AKT pathway was markedly activated in HaCAT cells by Rps6kb1 kinase.

Materials And Methods

Isolation, culture of hADSCs

Human subcutaneous adipose tissue samples were provided by the Nanjing Medical University Dental Hospital, and verbal consent was obtained by the participating patients. The adipose tissue was taken under aseptic conditions into pre-cooled PBS containing 10% penicillin/streptomycin at 4°C and quickly brought back to the laboratory for aseptic operation; the tissue was rinsed 3 times repeatedly with PBS containing 2% penicillin/streptomycin, the blood vessels and connective tissue were removed, and the adipose tissue was cut into pieces of approximately 1 mm*1 mm in size, then samples were digested with 0.1% type I collagenase (Sigma, USA) under gentle agitation for 60 minutes at 37°C, The pellet was filtered with a 70μm nylon mesh filter, and centrifuged at 1500 r/min for 10min; the lower cell precipitate was taken and cultured overnight at 37°C/5% CO2 in control medium (Dulbecco's modified Eagle's media (DMEM), 10% fetal bovine serum (FBS),(Sigma, USA), 100U/mL of penicillin, and 100μg/mL of streptomycin. 48 hours for the first fluid change and every two days for each experiment, only cells between the 3rd and 7th generation were used for subsequent experiments.

Preparation of hADSCs-CM

To collect hADSC-CM, hADSCs were seeded in 100 mm culture dishes. When the hADSCs reached confluence, the medium was changed to a DMEM/F12 serum-free medium and culture for 24h, then conditioned media of hADSCs were collected, centrifuged at 2000r/min for 10min, and keeped in 4° refrigerator.

In vitro multi-lineage differentiation of hADSCs

For differentiation, cells were grown to confluence before induction, Osteogenic Induction Solution (Gibco) and Lipogenic induction solution (Gibco) were changed every 3–4 days. After 21 days, cells were fixed for appropriate differentiation-specific staining: Oil Red O for adipogenesis, ALP for osteogenesis.

In vivo skin wound model and treatment

Fifteen 6-8 weeks size nude rats were purchased and housed at the Nanjing Medical University Animal Production Center under standard laboratory conditions. All animal procedures described here were reviewed and approved by the Animal Care and Use Committee of Nanjing Medical University. 15 mice were injected intraperitoneally with 1% Pentobarbital sodium and then artificial wounds were made using an 8-mm punch biopsy in the back of mice. The mice were randomly divided into experimental group I: treated with 1×10^6 hADSCs; experimental group II: treated with 100ul hADSCs-CM; control group: treated with phosphate buffered saline (PBS). The operation was repeatedly administered at 0, 3, 6 and 10 days after surgery, and wound healing was observed and recorded at 0, 4, 7, 14 and 21 d after surgery.

Real-time quantitative fluorescence PCR

Total RNA was extracted from hADSCs, HaCAT cells and nude mouse skin. cDNA was synthesized using 5 × PrimeScript RT Master Mix (Vazyme Biotech, Nanjing, China). Real-time quantitative PCR was performed using SYBR Premix Ex Taq Kit (Vazyme Biotech, Nanjing, China) and Q7 real-time PCR system. GAPDH served as endogenous control and the $2^{-\Delta\Delta CT}$ method was used for the quantitation of relative gene expression levels. The primers were listed as follows:

GAPDH: F:5’-GAAGGTGAAGGTCGGAGTC-3’, R:5’-GAGATGGTGATGGGATTTC-3’

JAG1: F:5’-GGGGCAACACCTTCAACCTC-3’R:5’-CCAGGCGAAACTGAAAGGC-3’

NOTCH1: 5’-GAGGCGTGGCAGACTATGC-3’R:5’- CTTGTACTCCGTCAGCGTGA -3’

NICD: 5’-TGGACCAGATTGGGGAGTTC-3’R:5’-GCACACTCGTCTGTGTTGAC-3’

Western blot

Total protein was extracted from hADSCs, HaCAT cells and nude mouse skin. All proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA). The membranes were blocked with 5% BSA and incubated for 2h at room temperature. The blots were probed using primary antibodies

and incubated overnight at 4℃: cyclin E1 (#4129, CST), cyclin D1 (#55506, CST), CDK2 (#2546, CST), CDK4 (#12790, CST), CDK6(#13331,CST),E-cadherin (#3195, CST), N-cadherin (ab18203, Abcam), Zeb1(21544-1-AP,PT), Zeb2(14026-1-AP,PT), β-catenin(51067-2-AP,PT), Snail(#3879,CST), Slug(#9585,CST), Vimentin(#5741, CST), Notch1(#3608,CST), Jag1(#70109,CST), Hes1(bs-23073R,Bioss), Hes2(bs-12383R,Bioss), Hey1(bs-165002R,Bioss), Rpbj-κ(SC-271128,Santa Cruz Biotechnology). Then the membrane was washed three times with TBST and incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (Proteintech Group, Wuhan, China) for 1h at room temperature. Finally, immunoreactive bands were detected using an Immobilon Western Chemiluminescent HRP Substrate (Millipore) and visualized using ImageQuantLAS 4000 mini-imaging system (General Electrics). The membranes were stripped and re-probed with a GAPDH (#5174S, CST) as a loading control.

HE staining and immunofluorescence

Histopathological tests and immunofluorescence skin specimens were performed using animal model skin. Tissues were fixed with 4% paraformaldehyde, paraffin embedded, and sectioned at a thickness of 4 μm. Sections were stained with hematoxylin and eosin. For immunofluorescence experiments, paraffin sections were dewaxed, rehydrated and antigen-retrieval was performed by heating in EDTA buffer (pH9) for 15 min in a microwave pressure cooker. After cooling at room temperature, 3% hydrogen peroxide solution was used to block endogenous peroxides. Paraffin sections were stained with primary Rabbit anti-Notch1 (#3608, 1:100, CST), Mouse anti-Jag1 (#70109,1:100, CST), Mouse anti-K14 (10143-1-AP,1:100, PT) and Rabbit anti-N-cad (ab76011, Abcam) overnight at 4°C. Following washing three times with PBS, the cells were incubated with FITC or Cy3-conjugated goat anti-rabbit IgG (1:200, Proteintech) or goat anti-mouse IgG (1:200, Proteintech) for 1h and then counterstained with 4’,6-di-amidino-2-phenylindole (DAPI, Sigma Chemicals). Plates were taken using a fluorescence microscope (DM4000B, Leica, Germany) and analyzed by ImageJ software.

Cell culture and plasmids

The hADSCs were obtained as previously described. The HaCAT cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Cellmax) and 1% penicillin/streptomycin (Cellmax) at 37℃ in 5% CO_2 atmosphere. The coding region of Jag1 and Notch1 were constructed by GenePharma (Shanghai, China) and the si-RNA targets sequences specific for the human Notch1, Jag1 were purchased from GenePharma (Shanghai, China). Cells were cultured in a 6-well culture with 50%-60% confluence, starved in medium without FBS for 16h and then transfections of hADSCs and HaCAT cells with the related plasmids were performed with Lipofectamine 2000(Invitrogen) according to the manufacturer’s instructions.

In vitro co-culture experiment

The transfected HaCAT cells and hADSCs were co-cultured in transwell chambers (4 μm pore size in a 24-well plate format and 6-well plate format), (BD Biosciences), the experiments were divided into four parts:

Part I: HaCAT cells co-culture with hADSCs or not; Part II: HaCAT cells transfected with Notch1 plasmid or empty plasmid, hADSCs were transfected with Jag1 plasmid or empty plasmid, and then co-culture; Part III: PF-03084014 (Selleck Chem, Houston) were added into hADSCs-HaCAT co-culture systems. Part IV: HaCAT cells transfected with si-Notch1 or empty plasmid, hADSCs were transfected with si-Jag1 or empty plasmid, and then co-culture.

Wound healing and cell migration assays

6-well plate co-culture system: hADSCs were located in the upper chamber and HaCAT cells were located in the lower chamber, then the HaCAT cells were scratched vertically with a 10ul sterile pipette tip, the area of the scratch was observed by photographing under an inverted microscope at 0h, 12h and 24h, respectively. The transfected HaCAT cells were resuspended in serum-free culture medium and then inoculated at a density of 8×10^4 cells/ml in the upper chamber. The transfected hADSCs were spread in the lower chamber, fixed in 4% paraformaldehyde for 30 min after 36h, and stained with crystal violet for 15 min. Three fields of view were randomly selected under an inverted microscope to observe the cells.

Cell proliferation assay

HaCAT cells in logarithmic growth phase were inoculated in the lower chamber of 24-well plates with 3×10^4 cells/well, 100μl medium/well, and 5 replicate wells were set for each group. After hADSCs were plated in the upper chamber of the co-culture, the cells were incubated in a constant temperature incubator at 37℃ and 5% CO2 for 6-8 h. The day 0 was set. CCK8 solution 10 μl and 90 μl serum-free medium were added to each well on days 0, 1, 2, 3, 4 and 5 after plating respectively, the cells were incubated in the incubator for 1-2 h and then measured by enzyme marker at 450 nm. The absorbance at the optical density value (OD) was measured by an enzyme marker.

Statistical analysis

ImageJ was used to analyze the image results, and the data were statistically analyzed using Graphpad prism8.0.1. The experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and t-test was used for comparison between two groups, and one-way ANOVA was used for comparison between multiple groups for single-factor changes, and $p<0.05$ was considered as statistically different.

Results

Culture and identification of hADSCs

A small number of spindle cells were visible 48h after inoculation, and swirling arranged cells were seen after 7 days of culture (Fig.1A). To identify the multipotency of hADSCs, we performed adipogenic and osteogenic differentiation. Under adipogenic and osteogenic differentiation conditions, most hADSCs could differentiate into adipocytes and osteocytes, respectively (Fig.1B, C).

Both hADSCs and hADSCs-CM promoted healing of skin injury

To investigate the therapeutic potential of hADSCs and hADSCs-CM on wound healing, we first established a mouse skin wound model and treated with 1×10^6 hADSCs, or hADSC-CM. Wounds treated with PBS served as controls. Wounds were carefully measured at 0, 4, 7, 14 and 21 days and the results showed that hADSCs and hADSC-CM significantly accelerated wound closure compared to PBS group (Fig.2A, B). On postoperative day 14, both experimental and control groups shed scabs, but the scar area in the control group was significantly larger than the hADSCs and hADSCs-CM groups (Fig.2A). On the 4th and 7th day after moulding, HE staining showed that the regenerated epithelial length of wounds treated with hAMSCs and hAMSC-CM was obviously longer in the PBS group (Fig,2C, D), indicating an enhanced ability to re-epithelialize compared to PBS group. In vitro, HaCAT cell were treated with hADSCs or normal medium (NM). Wound healing and cell migration assays showed that HaCAT cells in hADSCs group migrated obviously when compared with NM group (Fig.2E, F). CCK8 assays showed that HaCAT cells in hADSCs group sharply increased when compared with NM group (Fig.2G).

Notch1 signaling pathway was activated during skin wound healing promoted by hADSCs

Some scholars have found higher expression of Jag1 in ADSCs [26], but no study has shown the role of higher Jag1-expressing ADSCs in skin wound healing. In vivo, we first detected the expression of Jag1 and Notch1 through immunofluorescence assay and found that the expression was significantly higher in the hADSCs group and hADSCs-CM group (Fig.3A). We also examined the expression of EMT-related index N-cad, which was higher in the hADSCs and hADSCs-CM groups than the PBS group (Fig.3A). We further examined the expression of Jag1, Notch1 pathway-related genes in skin tissue on postorative days 4, 7 and 14 by qRT-pcr and western blot assay, the results showed that the expression of Notch1 pathway-related factors in the hADSCs and hADSCs-CM groups compared with the PBS group was significantly increased (Fig.3B, C). To verify the results of animal experiments, we first examined the background expression of Notch1 pathway-related factors in hADSCs and HaCAT cells, and found that the expression of Jag1 in hADSCs was higher than HaCAT cells (Fig,3D), the expression of Notch1 in HaCAT cells was higher than hADSCs (Fig,3D). We further treated hADSCs and HaCAT cells with Jag1 and Notch1 overexpressing plasmids respectively , The transfection efficiency was confirmed by qRT-PCR (Fig,3D). The treated cells were co-cultured to investigate the biological effects of hADSCs on the proliferation and migration of HaCAT cells. Co-cultures transfected with empty plasmids were as control. In scratching experiment, we found that the closed area of hADSCs(Jag1)+HaCAT(N1) group and hADSCs(N1)+HaCAT(Notch1) group did not decrease significantly compared to the control hADSCs(N1)+HaCAT(N1) group. The closed area of hADSCs(Jag1)+HaCAT(Notch1) group decreased compared to the other three groups (Fig,3E). Which was further confirmed by transwell migration assay (Fig,3F). Considering the fact that EMT is a key process of migration, we analyzed related EMT proteins by western blot to determine whether hADSCs enhanced HaCAT cells migration via alteration of the EMT (Fig,3I). Next, we tested cell proliferative potential in HaCAT cells. The CCK-8 assays revealed that hADSCs(Jag1)+HaCAT(Notch1) group promoted cell growth and viability compared with the control group (Fig,3G). In addition, we analyzed related cell-cycle proteins by western blot to determine whether hADSCs enhanced HaCAT cells proliferation via alteration of the cell cycle, the expression of CDK4 and

cyclinE1 in hADSCs(Jag1)+HaCAT(Notch1) group were significantly higher (Fig,3I). Afterwards, we examined the expression of Notch1 pathway-related factors in HaCAT cells, and found that the expression of Notch1 pathway downstream genes Hes1, Hes2, and Hey1 was significantly increased only in hADSCs(Jag1) +HaCAT(Notch1) group (Fig,3H), indicating that the Notch1 pathway was activated in hADSCs(Jag1)+HaCAT(Notch1) group. These results suggest that hADSCs with higher expression of Jag1 can activate the Notch1 signaling pathway in HaCAT cells, thereby promoting their migratory and proliferative abilities.

Decreased proliferation and migration of HaCAT cells after inhibition of Notch1 pathway

To further clarify the result of in vitro experiment, we added the γ-shearase inhibitor PF-03084014, which is involved in the activation of Notch1 pathway, to the co-culture system and detected the protein levels of the downstream genes of the Notch1 pathway by western blot assay (Fig,4D). We found that the Notch1 pathway was obviously inhibited by the addition of the inhibitor. We then performed co-culture experiments and found that after the addition of inhibitors, the migration ability of hADSCs(Jag1)+HaCAT(Notch1)+PF group was significantly reduced in scratch and transwell assays (Fig,4A, B), the expression of EMT-related protein was lower than hADSCs(Jag1)+HaCAT(Notch1) group (Fig,4D). The proliferative capacity of HaCAT cells was also significantly decreased in the hADSCs(Jag1)+HaCAT(Notch1)+PF group in the CCK8 experiment (Fig,4C).The expression of cell cycle proteins was also significantly lower in the hADSCs(Jag1)+HaCAT(Notch1)+PF group (Fig,4D). The results indicated that inhibition of the Notch1 pathway could reduce the effect of hADSCs on the proliferation and migration ability of HaCAT cells.

Notch1 signaling pathway in HaCAT cells activated AKT pathway via Rps6kb1 kinase

A recent report found that Knockdown of Notch1 deactivated Akt phosphorylation in Laryngeal squamous cell carcinoma[27]. However, no associated published articles have reported the relationship of Notch1 and AKT pathway in wound healing. To further clarify the effect of the Notch1 pathway activation on the biological function of HaCAT cells, we examined the expression of AKT pathway by western blot assay (Fig,5A, B). We found that Rps6kb1 and Pdk1 kinase among AKT pathway are closely related to the Notch1 pathway (Supplementary Material 1,2), so we conjectured that activation of Notch1 pathway in HaCAT cells could increase the expression of Rps6kb1or Pdk1 and activate AKT pathway. The expression of Rps6kb1 kinase was also increased in the Notch1 pathway activated hADSCs(Jag1)+HaCAT(Notch1) group in the western blot and pcr assays, but there was no difference in the protein expression level of Pdk1(Fig,5D). To further test the conjecture, we transfected si-jag1 and si-notch1 in hADSCs and HaCAT cells respectively to suppress intracellular protein expression (Fig,5E, F). It was found that the expression of Rps6kb1 was significantly decreased in HaCAT cells after inhibition of Notch1 expression (Fig,5J, K), and the proliferation and migration ability of HaCAT cells were also down-regulated after transfection with si-jag1 and si-notch1 in the cell phenotype (Fig,5G, H, I).

Discussion

In the last decades, a number of studies have demonstrated that MSCs play the regulation and stimulation roles of skin tissue trauma and participate in the regulation of skin homeostasis and healing processes by interacting with cells in the skin. In cell therapy, literatures have proved the positive effect of ADSCs on tissue regeneration. Adipose-derived MSCs are easier and more abundant to obtain than MSCs from other sources. ADSCs also have a higher proliferation capacity with reduced donor pain [28,29].

The role and mechanism of ADSCs in skin wound healing have also been partially reported. During the inflammatory phase, ADSCs secrete TGF-β together with IL-1β and IL-6, increasing the recruitment of macrophages and polarization from M1 to M2[18]. Sanja et al[30]demonstrated that ADSCs-CM stimulated macrophages and increased the secretion of TNF and IL-10 anti-inflammatory cytokines, thereby stimulating wound healing. During angiogenesis, it has been reported that ADSCs can regulate the expression of VEGF genes in endothelial cells by secreting VEGF factors themselves or through the expression of HIF-1α, thereby regulating angiogenesis[31]. During the proliferative remodeling phase, fibroblasts transform into myofibroblasts, synthesizing and secreting ECM in large quantities to form scar tissue[32]. Wang et al[33]. found that fibroblasts extracted from scar tissue cultured with ADSCs-CM had reduced proliferation and down-regulated expression of ECM-related genes and proteins.

Mice are the most commonly used animal model for their rapid reproduction and economical[34,35]. However, the physiology of mice differs from humans and can't effectively replicate the entire process of certain diseases in humans[36,37]. Human and mouse skin have the same number of cell layers in the dermis and epidermis, but not the same thickness[35,38,39]. In addition, the subcutaneous tissue of mice contains a sarcolemma that is almost non-existent in humans, which can give the skin a large contraction potential. However, the dermis of human skin is firmly attached to the subcutaneous tissue and shrinks much less than mice during the progress ofwound healing[35,40,41]. Therefore, trying to be more close to the healing process of human skin wounds, some scholars suggest using silicone rings to immobilize the wound and reduce its contraction [41,42]. Although mouse skin shrinkage makes it difficult to study, it is still a commonly used model for skin wound healing because of its 95% genetic identity with humans [39] and the 30.2% identity between murine and human skin [35]. In this study, we preliminarily verified that hADSCs and hADSCS-CM can accelerate wound healing in the injury models of mice.

Notch signaling pathway is closely related to cell proliferation, differentiation, development and homeostasis[22]. For skin wound healing, Xu et al[43]. demonstrated that far infrared can promote full-thickness skin wound healing in rats through the Notch1/Twist1 axis. It has also been reported that platelet rich plasma improves the proliferation and migration of endothelial cells and increases the expression of Notch pathway related genes in diabetic skin trauma mice[44]. As one of the significant Notch pathway ligands, Jag1 can regulate maturation of the human epidermis by activating Notch pathway[45]. However, the role of Jag1 in hADSCs in wound healing is largely unknown. Furthermore, Stem cells themselves, conditioned media and exosomes can participate in skin wound healing by regulating various cytokines. The results of our in vivo experimentin suggest that Jag1 / Notch1 pathway also plays a certain role in promoting skin wound healing by hADSCs and hADSCs-CM.

Initially, Notch1 signaling pathway was thought to be activated by physical contact between adjacent cells that binds corresponding ligands to receptors. Some researchers also found that non-physical contact co-culture of two kinds of cells can also activate the Notch1 pathway. Wang et al[46]. found that MSCs affect the proliferation and differentiation of neural stem cells by activating Notch1 signaling through transwell co-culture. Other studies have also demonstrated that transwell co-culture can affect the transduction of Notch1 signaling between the two cell types[47-49]. Therefore, in order to verify the underlying mechanism of hADSCs in vivo, transwell co-culture system was selected for further study and verification in the subsequent in vitro experiments.

One of the main signs of wound healing is wound contraction, which reduces the surface area of the wound that must be re-epithelialized[1]. The epidermis is the most superficial layer of skin tissue and contains mainly keratin-forming cells, which protect the body from UV radiation, microorganisms and extreme temperatures[7]. Following wounding, keratinocytes at the wound edge loosen their adhesions to each other and to the basal lamina to close the defect, forming the migrating epithelial tongue. Keratinocytes in the wound are also actively interacting with fibroblasts, endothelial cells, and immune cells in the wound[1]. Therefore, in our in vitro experiments we mainly investigated the effect of hADSCs on HaCAT cells' migration and proliferation ability. The results showed that the expression of genes related to Notch1 pathway were significantly increased after the co-culture of treated hADSCs and treated HaCAT cells. At the same time, the increased expression of EMT and cycle-related proteins was consistent with our cell phenotype, suggesting that hADSCs can activate the Notch1 pathway in HaCAT cells and promote the proliferation and migration of HaCAT cells. In addition, our results demonstrate that activation of Notch1 pathway in non-physical contact co-culture system also depends on ligand-receptor binding. The expression of downstream genes of Notch1 pathway in HaCAT cells was inhibited when γ -secretase inhibitors, the migration and proliferation of HaCAT cells were significantly reduced, as well as the expression of EMT and cell cycle-related proteins.

The AKT pathway controls many cellular functions, including cell growth, survival, and cell metabolism[50]. It has been previously reported that MSCs can activate the AKT pathway of keratinocytes and fibroblasts, thus promoting skin wound healing[51-54]. In our results, it was found that the AKT pathway was activated in HaCAT cells. When the Notch1 pathway was inhibited, the AKT pathway was also suppressed. To determine how the Notch1 pathway affects the AKT pathway, we predicted from the Jaspar database that Notch1 pathway downstream of hes1, hes2, hes5-7 and the kinase Rps6kb1, Pdk1 of the AKT pathway are closely related (Supplementary Material 1,2). Further research has found that the expression of Rps6kb1 kinase in pcr and western blot experiments was consistent with the trend of AKT/p-AKT expression, while the expression trends of hes5-7 and Pdk1 were inconsistent. Reduced expression of Rps6kb1 kinase was also found in the group with the addition of γ-secretase inhibitor, consistent with the trend of Notch1 and AKT/p-AKT expression. To further verify the relationship between Notch1 and AKT pathway, we detected the reduced expression of Rps6kb1 kinase in HaCAT cells transfected with si-Notch1. As far as we know, this is the first report that hADSCs can activate the Notch1

pathway in HaCAT cells by co-culture in a non-contact manner and can affect the expression of Rps6kb1 kinase via Notch1 signaling pathway to activate the AKT pathway.

Our in vivo experiments demonstrated that hADSCs can promote skin wound healing. Furthermore, we found that hADSCs can activate the Notch1 signaling pathway during the skin wound healing process. In Vitro experiments revealed increased expression of Notch1 pathway-related factors in HaCAT cells, and this effect was attenuated by the addition of PF-03084014 inhibitor. These results emphasize that interactions between hADSCs and HaCAT cells mediated at least in part by Notch1 signaling promote the proliferation and migration of HaCAT cells.

The healing of skin wounds is a highly complex process, in which the microenvironment of the wounds and the biological roles of multiple cells are involved. We should further investigate the role and molecular mechanism of hADSCs in promoting skin wound healing, so that they can be more easily and quickly used in clinical treatment in the future.

Conclusion

This was a signifcant report on the mechanism of hADSCs in skin wound healing. We explained that hADSCs can promote skin cell-HaCAT cells proliferation and migration via Notch1 pathway. In the clinic, hADSCs may provide an alternative therapeutic approach for the treatment of skin injury.

Abbreviations

MSCs: Mesenchymal stem cells; hADSCs:Human adipose stem cells; ALP: alkaline phosphatase; hADSCs-CM:Human adipose stem cells conditional medium; NICD: Notch intracellular domain; Hes1:Hairy Enhancer of Split-1; EMT:Epithelial-mesenchymal transformation; TGF-β□transforming growth factor-β□ IL-1β□Interleukin-1β□IL-6□Interleukin-6□ TNF□tumor necrosis factor□VEGF□vascular endothelial growth factor□HIF-1α□hypoxia inducible factor-1α□ ECM: extracellular matrix.

Declarations

Ethics approval and consent to participate

All the protocols involved in our experiments were approved by the Ethics Committee of Nanjing Medical University and study methodologies conformed to the standards set by the Declaration of Helsinki.

Consent for publication

All the co-authors consent to publish the work in Cell Communication & Signaling.

Competing interests

The authors declare no competing interests.

Acknowledgements

Not applicable.

Authors' contributions

Y.W., Q.W. and Y.Z. conceived the study design and drafted the article together. C.W. proofread the manuscript. X.D and H.W. performed the experiments and analyzed the data. Y.W., W.Z. and X.S. sought approval from the ethics committee, and provided funding. All authors read and approved the final manuscript.

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Availability of supporting data

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

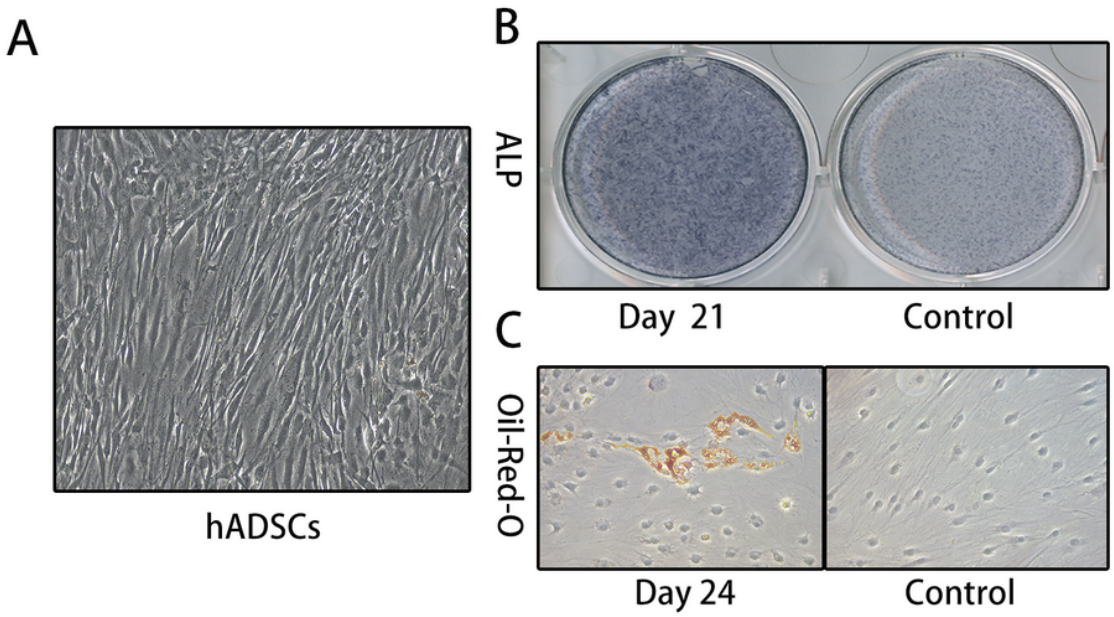

Figure 1

Identification of hADSCs. **A** Representative photomicrograph of adherent hADSCs with spindle shapes on plastic cell culture dish. **B, C** Multiple differentiation potential of hADSCs. The hADSCs were differentiated into matured adipocytes and osteocytes after incubation with adipogenic or osteogenic differentiation medium at the times indicated, respectively. Adipocytes and osteocytes differentiated from hADSCs were determined by staining with Oil Red O and ALP, respectively.

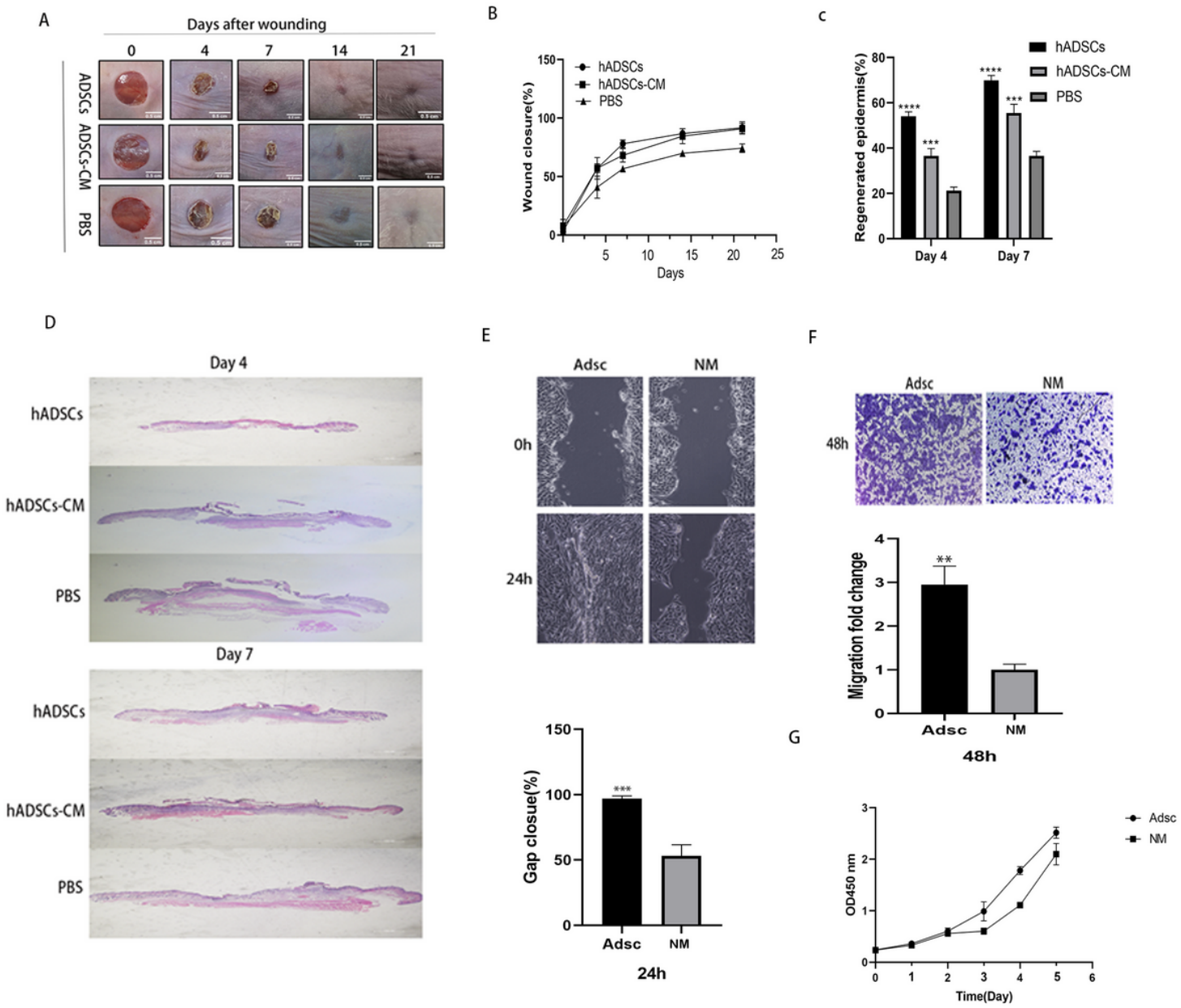

Figure 2

hADSCs or hADSC-CM accelerated wound closure. **A** Representative images of wounds after transplantations of PBS, hADSCs and hAMSC-CM at day 0, day 7, day 14, and day 21. **B** Measurement of wound closure at different time points treated with PBS, hADSCs, or hADSC-CM. The results showed that the wound closure was significantly increased in response to hADSCs and hADSC-CM (n = 5). The percentage of wound closure was calculated as (area of original wound−area of measured wound)/area of original wound×100. **C** Measurement of regenerated epidermis at different time treated with PBS,

hADSCs, or hADSC-CM. The results showed that the wound closure was significantly increased in response to hADSCs and hADSC-CM (n=5). The percentage of regenerated epidermis was calculated as (length of regenerated epidermis−length of original wound length)/length of original wound ×100. **D** Representative photomicrographs of H&E stained sections from day-4 and day-7 wounds injected with PBS, hADSCs, or hADSC-CM. The dotted line indicated the regenerated epidermis. PBS group was used as a control. **E, F** hADSCs promoted the migration and wound closure of HaCAT cells in a wound scratch assay. Representative images of HaCAT scratch assays and transwell migration assays. **G** hADSCs promoted the proliferation of HaCAT cells in CCK8 assay. The in vitro wound-healing assay showed that hADSCs strongly improved the HaCAT wound closure compared with the PBS group. Significance was measured using a two-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$. $^{****}P < 0.0001$, ns, not significant.

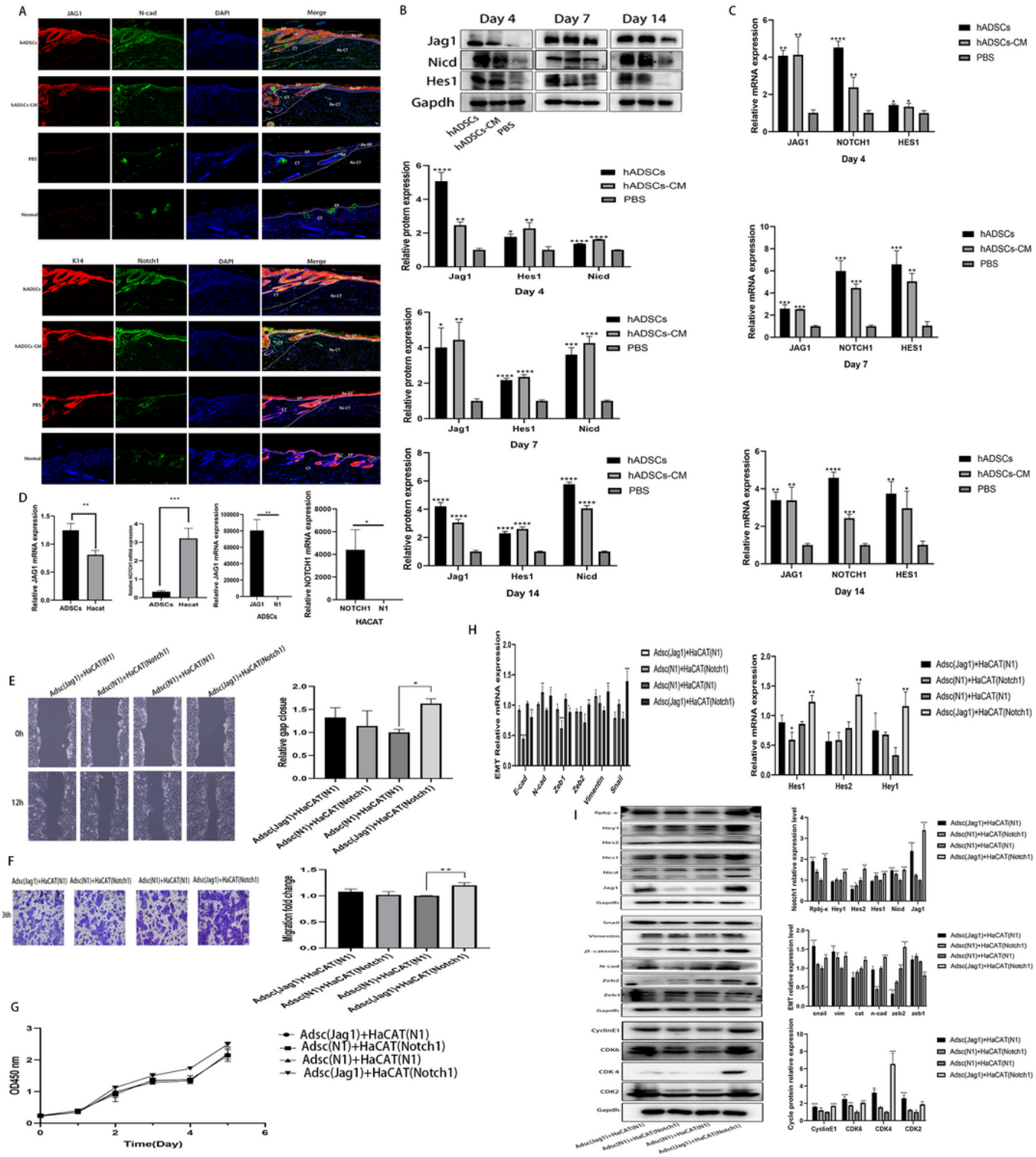

Figure 3

hADSCs may promote healing of skin injury via the Notch1 pathway. **A** Representative immunofluorescence images of Jag1, Notch1, N-cad and K14 expression for re-epithelialization in skin tissues. **B** Western blot assay for Jag1, Notch1, Nicd and Hes1 expression in skin tissues at 4 days,7 days and 14 days after treatment. **C** qRT-pcr assay for Jag1, Notch1, Nicd and Hes1 expression in skin tissues at 4 days,7 days and 14 days after treatment. **D** qRT-pcr assay for Jag1 or Notch1 original expression in

hADSCs or HaCAT cells respectively and the efficiency of transfection. **E, F** hADSCs treated with Jag1 overexpression promoted migration and wound closure of HaCAT cells treated with notch1 overexpression. **G** hADSCs treated with Jag1 overexpression promoted prolification of HaCAT cells treated with notch1 overexpression. **H, I** qRT-pcr assay and western blot assay for Notch1, EMT and cell cycle associated-proteins expression in HaCAT cells after transfection and co-culture. Data were presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *****P* < 0.0001, ns, not significant.

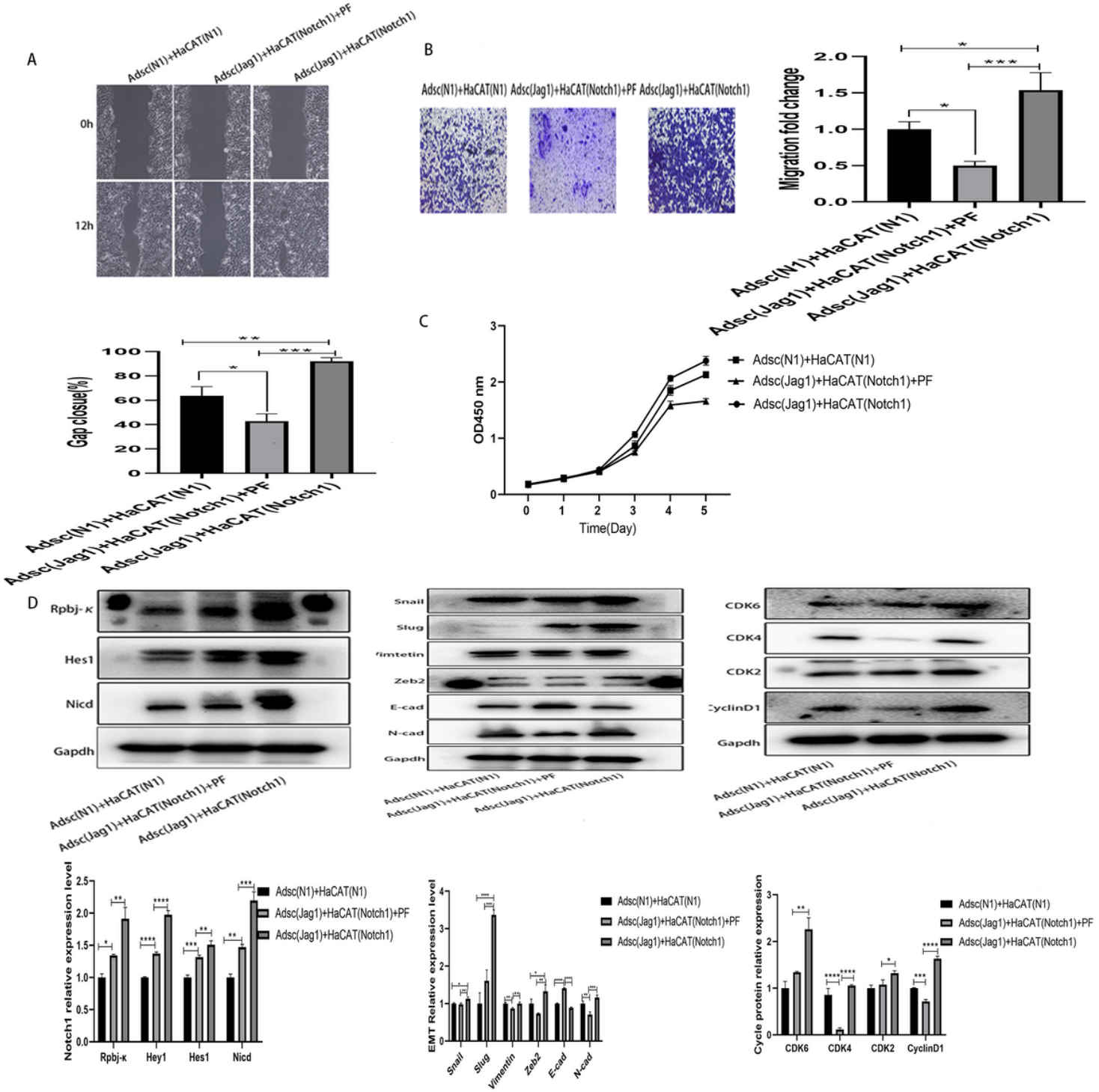

Figure 4

Decreased proliferation and migration of Hacat cells after inhibition of Notch1 pathway. **A, B, C** Notch1 inhibition supressived the migration and proliferation ability of HaCAT cells. **D** Western blot assay for Notch1, EMT and cell cycle associated-proteins in HaCAT cells treated with hADSCs in the presence or absence of PF-03084014 (3mmol/ml). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *****P* < 0.0001, ns, not significant.

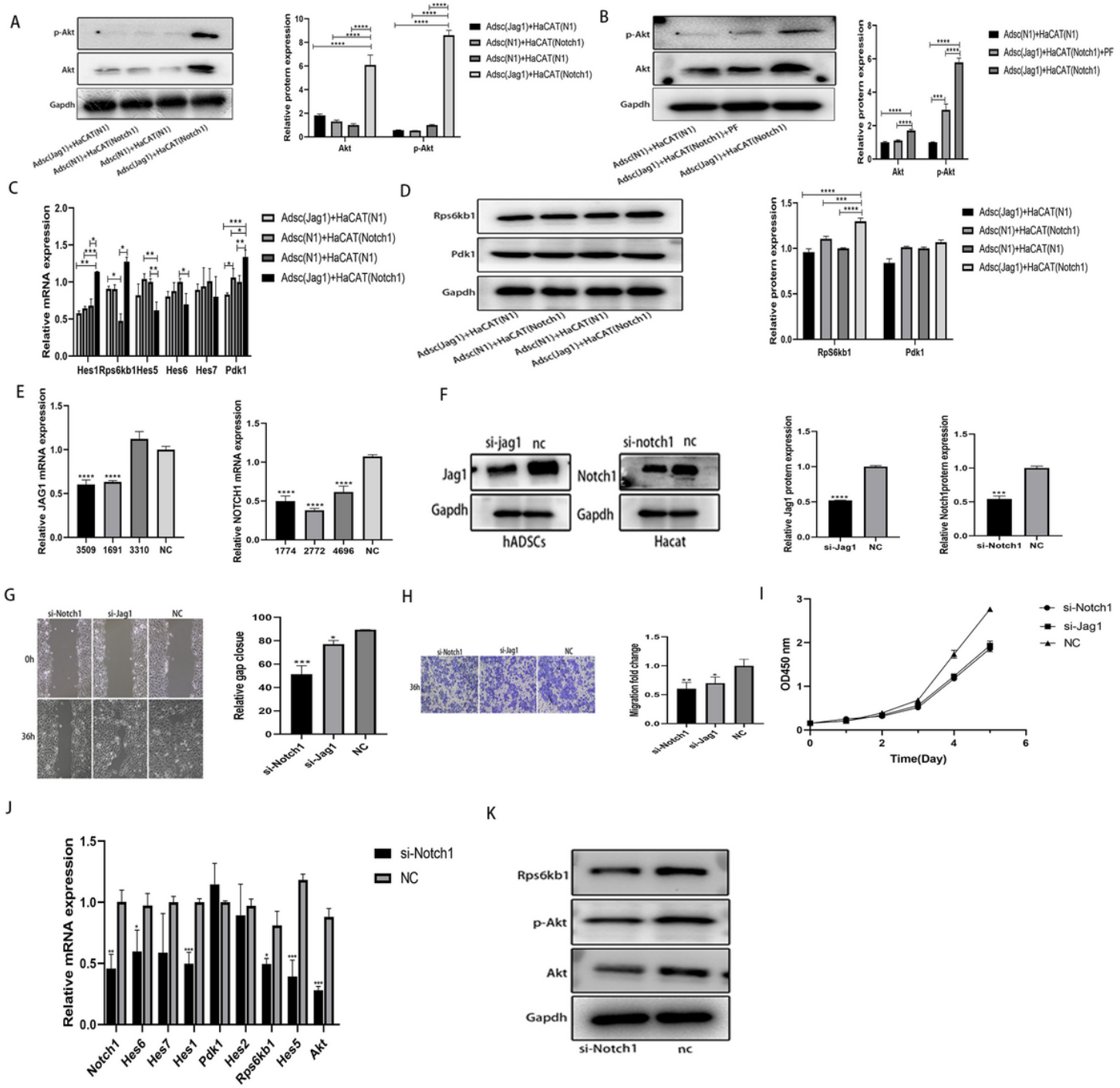

Figure 5

The activation of Notch1 pathway in HaCAT cells can activate AKT pathway via Rps6kb1 kinase. **A** western blot assay showed Notch1 activation up-regulated AKT and p-AKT expression of HaCAT cells and (**B**)Notch1 inhibition downregulated AKT and p-AKT expression of HaCAT cells. Notch1 downstream target genes(**C**) and AKT relative kinase (**C, D**) were measured in HaCAT cells after the same transfection. The efficiency of transfection was validated by qRT-PCR (**E**) and Western blot (**F**). (**G, H and I**) migration assay and transwell assay showed Notch1 knockdown inhibited the migration and proliferative ability of HaCAT cells. **J** The Notch1 downstream target genes, AKT and AKT relative kinase were measured by qRT-pcr. **K** Western blot assay for Rps6kb1, p-AKT, and AKT in HaCAT cells treated with si-Notch1. Data were presented as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ****$P < 0.0001$, ns, not significant

Supplementary Files

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