scientific reports

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USP37-stabilized SALL4 promotes the keloid formation by PI3K/AKT pathway

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Spalt-like transcription factor 4 (SALL4) plays a vital role in the progression of many human diseases. However, the role and mechanism of SALL4 regulates in keloid formation remain unclear. The mRNA levels of SALL4 and ubiquitin-specific peptidase 37 (USP37) in keloid tissues and keloid fibroblasts were determined by quantitative real-time PCR. Western blot was performed to measure the protein levels of SALL4, USP4/10/37, collagen-related markers, and PI3K/AKT-related markers. The growth, invasion and migration of keloid fibroblasts were determined using CCK8 assay, EdU assay, flow cytometry, transwell assay and wound healing assay. Cell glycolysis was assessed by detecting glucose consumption and lactate production. The interaction between USP37 and SALL4 was confirmed by co-immunoprecipitation assay. SALL4 had increased expression in keloid tissues and keloid fibroblasts. Silencing of SALL4 inhibited the growth, invasion, migration, extracellular matrix (ECM) accumulate and glycolysis of keloid fibroblast, while its overexpression had the opposite effects. In terms of mechanism, USP37 stabilized SALL4 expression through deubiquitinating. Functional experiments suggested that SALL4 overexpression reversed the inhibitory effect of USP37 knockdown on keloid fibroblast functions. Moreover, USP37/SALL4 axis could increase the activity of PI3K/AKT pathway, and PI3K pathway inhibitor LY294002 abolished SALL4-mediated the promoting on keloid fibroblast functions. USP37-activated SALL4 might enhance keloid fibroblast growth, invasion, migration, ECM accumulation and glycolysis via activating PI3K/AKT pathway.

Keywords Keloid fibroblasts, SALL4, USP37, PI3K/AKT

Keloids are overgrowths of abnormal scar tissue secondary to traumatic skin injury or spontaneous formation, and are a common condition in the fields of plastic surgery, dermatology and burn trauma surgery[1–3]. The main symptoms of keloids are itching, burning pain, firm pink papules and thickening of the skin[4,5]. The symptoms caused by keloid may affect the normal life of patients, and its growth in the area of large tension may limit the patient's exercise ability, and in more severe cases may induce scar cancer[6]. Because of its high recurrence rate and treatment resistance, keloids have become one of the clinically intractable diseases in the field of surgery and dermatology, which place a serious physical and mental burden on patients[7,8]. Therefore, there is an urgent need to further understand the causes of keloids to find better treatments in clinical trials.

Spalt-like transcription factor 4 (SALL4) is an important transcription factor regulating the development of embryonic abdominal motor neurons, which abnormal expression usually indicates the possibility of neoplastic lesions in primordial germ cells[9,10]. Studies have shown that SALL4 is upregulated in many cancers and serves as oncogene to participate in tumorigenesis[11,12]. Furthermore, SALL4 has been found to promote cell migration, proliferation, and glycolysis[13–15]. Overexpression of SALL4 had an increasing effect on cell invasion and proliferation of colorectal cancer[16]. It has been reported that the pro-proliferation and pro-invasion effects of SALL4 was related to the activity of PI3K/AKT pathway[17,18]. Through GEO database (GSE212954) analyzing, we revealed a significantly high expression of SALL4 in keloid tissues. However, the role of SALL4 in keloid formation has not been reported.

Protein post-translational modification plays an important role in cell signal transduction. Ubiquitination is a widespread post-translational modification, which can affect cellular functions by regulating protein stability[19].

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Ubiquitin-specific peptidases (USPs) are key members of the deubiquitinating enzyme family and are involved in many biological functions by recognizing ubiquitination signals from specific proteins[20,21]. Previous studies demonstrated that USP37 promoted keloid formation by enhancing keloid fibroblast proliferation and collagen production[22]. Also, USP11 had been confirmed to accelerate proliferation, invasion and migration in keloid derived-fibroblasts[23]. Moreover, USP10 participated in the regulation of TRAF4 on fibroblast proliferation in keloids[24]. Knockdown of USP4 attenuated the formation of pathological scarring[25]. To identify the upstream deubiquitinating enzymes that regulate SALL4 expression, the effects of si-USP37, si-USP10, si-USP11 and si-USP4 on SALL4 expression were investigated. The results showed that only USP37 knockdown could reduce SALL4 expression. However, whether USP37 influences keloid formation through regulating SALL4 deubiquitination is unknown.

Here, we hypothesized and demonstrated that USP37 deubiquitinated and stabilized SALL4 to activate PI3K/AKT pathway, thereby promoting proliferation, invasion, migration, extracellular matrix (ECM) accumulation and glycolysis in keloid fibroblasts. The discovery of USP37/SALL4/PI3K/AKT axis provided a new idea for keloid treatment.

Materials and methods

Samples collection

Approval for our study was granted by the Ethics Committee of the First Affiliated Hospital of the Naval Medical University (No. 112012098) and all experimental procedures in this study were performed in accordance with the Declaration of Helsinki. Keloid tissues ($n=27$) and normal skin tissues (≥ 10 cm from keloid, $n=27$) were collected from 27 keloid patients (12 males and 15 females, mean age: 31.2 ± 9.1) in the First Affiliated Hospital of the Naval Medical University. All samples were preserved in −80 °C for subsequent studies. Each participant signed an informed consent form.

Primary fibroblasts isolation and culture

Keloid fibroblasts and normal fibroblasts were isolated from fresh removed keloid tissues and normal skin tissues using collagenase digestion method as previously described[22], respectively. The isolated fibroblasts were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin (15140122, Gibco, Carlsbad, CA, USA) at 37 °C with 5% CO_2 incubator. Primary fibroblasts were cultured for 3 generations and then used for experiments.

Fibroblast transfection and treatment

Keloid fibroblasts were seeded in 6-well plate and cultured until 50% confluences. Keloid fibroblasts were transfected with siRNA of SALL4/USP37/USP4/USP10/USP11 (si-SALL4: F 5'-UGCAAUUUUUCUUAUGUUCCA-3', R 5'-GAACAUAAGAAAAAUUGCACU-3'; si-USP37: F 5'-UAUGUAAUAGCAAUUCACCUU-3', R 5'-GGUGAAUUGCUAUUACAUAGG-3'; si-USP4: F 5'-AUUAAGUGUUCUUUCAAGGUC-3', R 5'-CCUUGAAAGAACACUUAAUUG-3'; si-USP10: F 5'-AUAUACUGCCCUAUUCCAGGA-3', R 5'-CUGGAAUAGGGCAGUAUAUUU-3'; si-USP11: F 5'-GUGUGUGUGUAUAUAUAAAGC-3', R 5'-UUUAUAUAUACACACACACAG-3'), the pcDNA SALL4 overexpression vector, and their controls (si-NC and pcDNA) by Lipofectamine 3000 (L3000075, Invitrogen, Carlsbad, CA, USA). For co-transfection, keloid fibroblasts were co-transfected with 50 nM si-USP37 and 4.0 μg pcDNA SALL4 overexpression vector using Lipofectamine 3000 according to the manufacturer's protocol. Keloid fibroblasts transfected with si-USP37 were treated with 10 μM proteasome inhibitor MG132 (HY-13259, MedChemExpress, Monmouth Junction, NI, USA) for 24 h to reveal the effect of USP37 on SALL4 ubiquitination and expression. Besides, keloid fibroblasts transfected with SALL4 overexpression vector were treated with LY294002 (25 μM; HY-10108, MedChemExpress) for 24 h to explore the role of PI3K/AKT pathway in SALL4-mediated keloid fibroblast functions.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from fibroblasts and tissues (ground into powder in liquid nitrogen) by TRIzol reagent (15596026CN, Invitrogen) and then used for synthesizing cDNA with PrimeScript RT Reagent Kit (RR716, Takara, Tokyo, Japan). After that, cDNA was mixed with SYBR Green (RR820A, Takara) and specific primers (Table 1) for PCR amplification. Relative expression was calculated by $2^{-\Delta\Delta Ct}$ method with GAPDH as internal control.

Western blot (WB)

Keloid fibroblasts were lysed with RIPA lysis buffer (P0013B, Beyotime, Shanghai, China). The total proteins were extracted in cell lysates, separated by SDS-PAGE and transferred to PVDF membranes. Membrane was closed with blocking buffer (P0023B, Beyotime), and incubated with anti-SALL4 (ab29112, 1:1000, Abcam, Cambridge, MA, USA), anti-collagen I (ab34710, 1:1000, Abcam), anti-fibronectin (ab2413, 1:1000, Abcam), anti-USP37 (ab72199, 1:5000, Abcam), anti-USP4 (ab181105, 1:1000, Abcam), anti-USP10 (ab109219, 1:1000, Abcam), anti-PI3K (ab191606, 1:1000, Abcam), anti-p-PI3K (ab182651, 1:500, Abcam), anti-AKT (ab8805, 1:500, Abcam), anti-p-AKT (ab18206, 1:1,000, Abcam), anti-GAPDH (ab9485, 1:2500, Abcam) and secondary antibody (ab205718, 1:50000, Abcam). After that, membrane was incubated with BeyoECL Plus solution (P0018AS, Beyotime) to detect protein signals.

Cell proliferation assay

Keloid fibroblasts were seeded in 96-well plates (2000 cells/well) cultured for 48 h. Then, cells were incubated with CCK8 reagent (C0041, Beyotime) for 2 h. After that, the microplate reader was used to measure cell viability at 450 nm.

Name		Primers for PCR (5'–3')
SALL4	Forward	TGCCTTGAAACAAGCCAAGC
	Reverse	GGAGACTGCTCCGACACTT
USP37	Forward	CCTACTTCCATTACCCTCGGC
	Reverse	TCCGGAAGCCATCAACTCAG
LHX9	Forward	GCCAGTGCAACCACCATTAC
	Reverse	GATCTGCGCTCCATCTCCTC
CILP2	Forward	GAAACACCCTGAGTCCCGAG
	Reverse	CCATGCCTTGCAGTGGTAGA
RGS8	Forward	CTGTGGCCGGGTCTTGAAT
	Reverse	CTGCGGGGCTCAGGAATTTA
SLC24A2	Forward	CACCTGACAAGGGCCATCAT
	Reverse	AGCTGCGTTTTGCTGAAAGG
CYP24A1	Forward	TTGCCAGCGATAATACGCCT
	Reverse	AAGTATCAACACCTGGGCCT
GAPDH	Forward	GACCACAGTCCATGCCATCAC
	Reverse	ACGCCTGCTTCACCACCTT

Table 1. Primer sequences used for qRT-PCR.

For EdU assay, EdU Cell Proliferation Kit (C0078L, Beyotime) was used according to the operating instructions. Briefly, keloid fibroblasts in 96-well plates were labelled with EdU solution and DAPI solution one by one. Fluorescence microscope was used to observe the rate of EdU positive cells.

Flow cytometry

After transfection, keloid fibroblasts were collected, rinsed with PBS and resuspended with binding buffer. Cells were incubated with Annexin V-FITC at room temperature away from light for 15 min. Afterwards, cells were rinsed, resuspended and incubated with PI dyeing solution (APOAF, Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Apoptosis rate was analyzed under flow cytometry.

Transwell assay

Diluted Matrigel was pre-coated into the upper of transwell chamber (354480, BD Biosciences, San Jose, CA, USA), and complete medium was filled into lower chamber. After keloid fibroblasts were inoculated for 24 h in the upper chamber with serum-free medium, the upper chamber was removed and cells were fixed by 4% paraformaldehyde and stained with 0.1% crystal violet, followed by the invasive cells were counted under a microscope.

Wound healing assay

Keloid fibroblasts were inoculated and cultured in 24-well plates. Using the ruler as a reference, cell layer was created a wound with a 10 μL pipette. After cultured with serum-free medium for 24 h, cell wound area was photographed and the migration distance was counted by detecting wound area at 0 h and 24 h.

Cell Glycolysis evaluation

According to the product instructions, glucose Assay Kit (ab65333, Abcam) and L-Lactate Assay Kit (ab65331, Abcam) were used to measure glucose consumption and lactate production in keloid fibroblasts, respectively.

Co-immunoprecipitation (Co-IP) assay

Keloid fibroblasts were lysed with RIPA buffer to obtain cell lysates. Then cell lysates were extracted into 2 parts: input group and IP group. The cell lysate was incubated with anti-IgG, anti-SALL4 and anti-USP37 for 2 h at room temperature, and then treated with protein A/G agarose (HY-K0230, MedChemExpress) for 1 h. Immunoprecipitates were eluted and then used for WB analysis to assess the interaction between USP37 and SALL4.

Ubiquitination assay

According to the instructions of VIVAlink ubiquitin kit (VB2952-50, Amyjet Scientific, Wuhan, China), keloid fibroblasts were transfected with HA-Ub, Flag-SALL4, and si-NC/si-USP37, and then cell lysates were incubated with anti-flag, anti-HA, and protein A/G agarose overnight. After that, the immunoprecipitated protein was eluted and collected for WB analysis to measure the effect of USP37 knockdown on the ubiquitination of SALL4.

Statistical analysis

All analyses were performed using GraphPad Prism 7.0 software and data were presented as mean ± SD. Shapiro-Wilk normality test was used for normality analysis. Results were compared between different groups using Student's *t*-test or ANOVA. $P < 0.05$ was considered as significant.

Results

SALL4 was highly expressed in keloid tissues and keloid fibroblasts

As shown in Fig. 1A-B, GEO database (GSE212954) revealed the top 6 genes differentially expressed in keloid tissues and normal skin tissues, among which SALL4 was significantly highly expressed in keloid tissues. Through qRT-PCR, we confirmed that SALL4 mRNA expression was higher in keloid tissues than the other 5 genes (Fig. 1C), so it was used for follow-up studies. In addition, we examined SALL4 mRNA expression in 27 paired normal skin tissues and keloid tissues, and confirmed the upregulated SALL4 in keloid tissues (Fig. 1D). Besides, we found that SALL4 protein expression was elevated in keloid fibroblasts (Fig. 1E).

SALL4 regulated keloid fibroblast functions

Therefore, we investigated the role of SALL4 in keloid formation. After transfected with si-SALL4 into keloid fibroblasts, SALL4 protein expression was markedly reduced (Fig. 2A). SALL4 knockdown repressed viability and EdU positive cell rate, while promoted apoptosis rate in keloid fibroblasts (Fig. 2B-D). Also, SALL4 silencing reduced invaded cell numbers, migration distance, and the protein levels of collagen-related markers (collagen I and fibronectin) (Fig. 2E-G). Moreover, downregulation of SALL1 inhibited glucose consumption and lactate production (Fig. 2H-I). Furthermore, keloid fibroblasts were transfected with SALL4 overexpression vector to enhance the protein expression of SALL4 (Fig. 3A). SALL4 upregulation facilitated viability, EdU positive cell rate, invaded cell numbers and migration distance of keloid fibroblasts (Fig. 3B-E). Besides, SALL4 upregulation enhanced ECM accumulation and glycolysis in keloid fibroblasts (Fig. 3F-I). Above all, SALL4 might promote keloid fibroblast functions.

USP37 stabilized SALL4 expression

After transfected with si-USP37/si-USP4/si-USP10/si-USP11 into keloid fibroblasts, the protein levels of USP37, USP4 and USP10 were markedly decreased, respectively (Fig. 4A-C and Supplementary Fig. 1A). By detecting SALL4 protein expression in keloid fibroblasts transfected with si-USP37/si-USP4/si-USP10/si-USP11, we found that it could be only reduced by si-USP37 (Fig. 4D and Supplementary Fig. 1B). Therefore, USP37 was used as the upstream target of SALL4 in follow-up study. USP37 mRNA and protein expression was confirmed to be higher in keloid tissues (Fig. 4E-F). Through qRT-PCR, SALL4 mRNA expression was not affected by USP37 knockdown (Fig. 4G). Besides, SALL4 level was enriched in anti-USP37, indicating that USP37 could bind to SALL4 protein (Fig. 4H). Further analysis revealed that proteasome inhibitor MG132 reverted the reducing effect of si-USP37 on SALL4 protein expression (Fig. 4I), and knockdown of USP37 promoted the ubiquitination

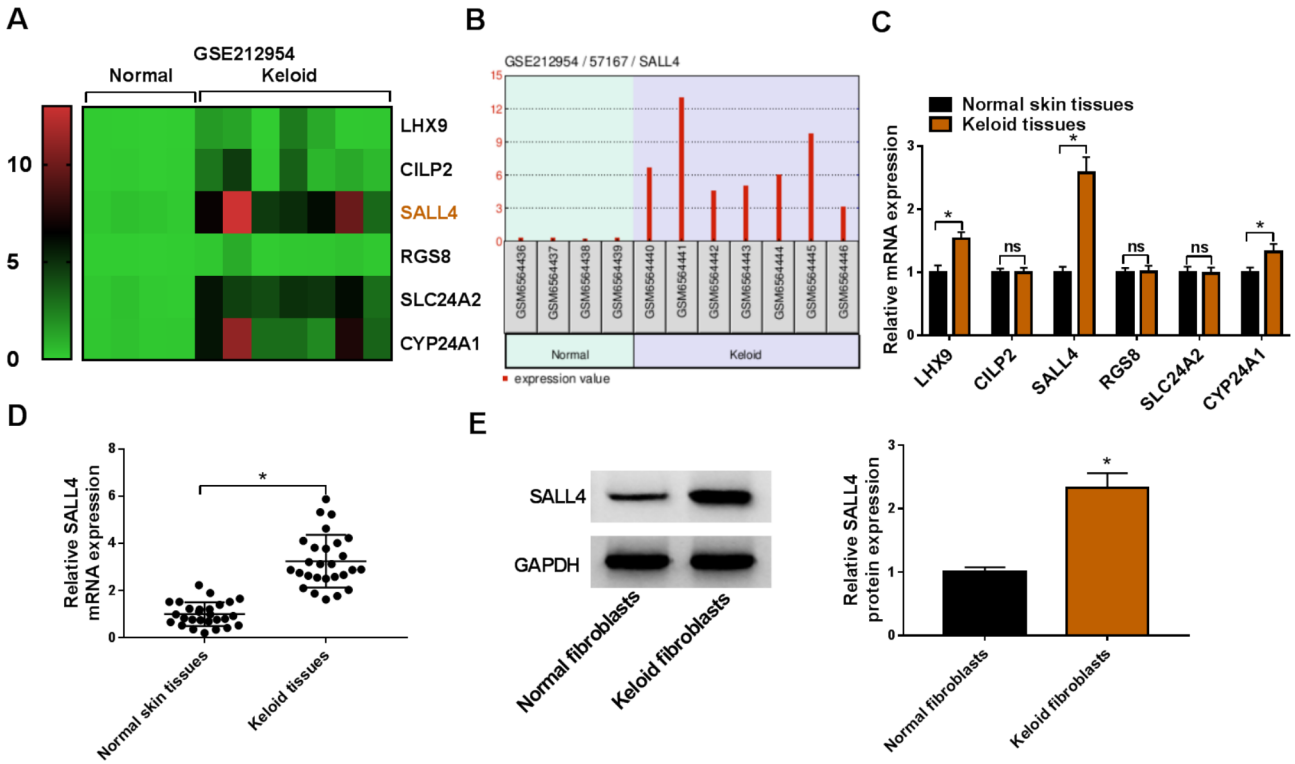

Fig. 1. SALL4 expression in keloid tissues and keloid fibroblasts. (A-B) GEO database (GSE212954) analyzed SALL4 levels in normal skin tissues ($n = 4$) and keloid tissues ($n = 7$). (C) Levels of related genes were assayed by qRT-PCR in normal skin tissues and keloid tissues ($n = 3$). (D) qRT-PCR was used to test SALL4 mRNA levels in normal skin tissues ($n = 27$) and keloid tissues ($n = 27$). (E) SALL4 protein level in normal fibroblasts and keloid fibroblasts was measured by WB analysis ($n = 3$). C, one-way ANOVA; D-E, Unpaired t test. $^*P < 0.05$.

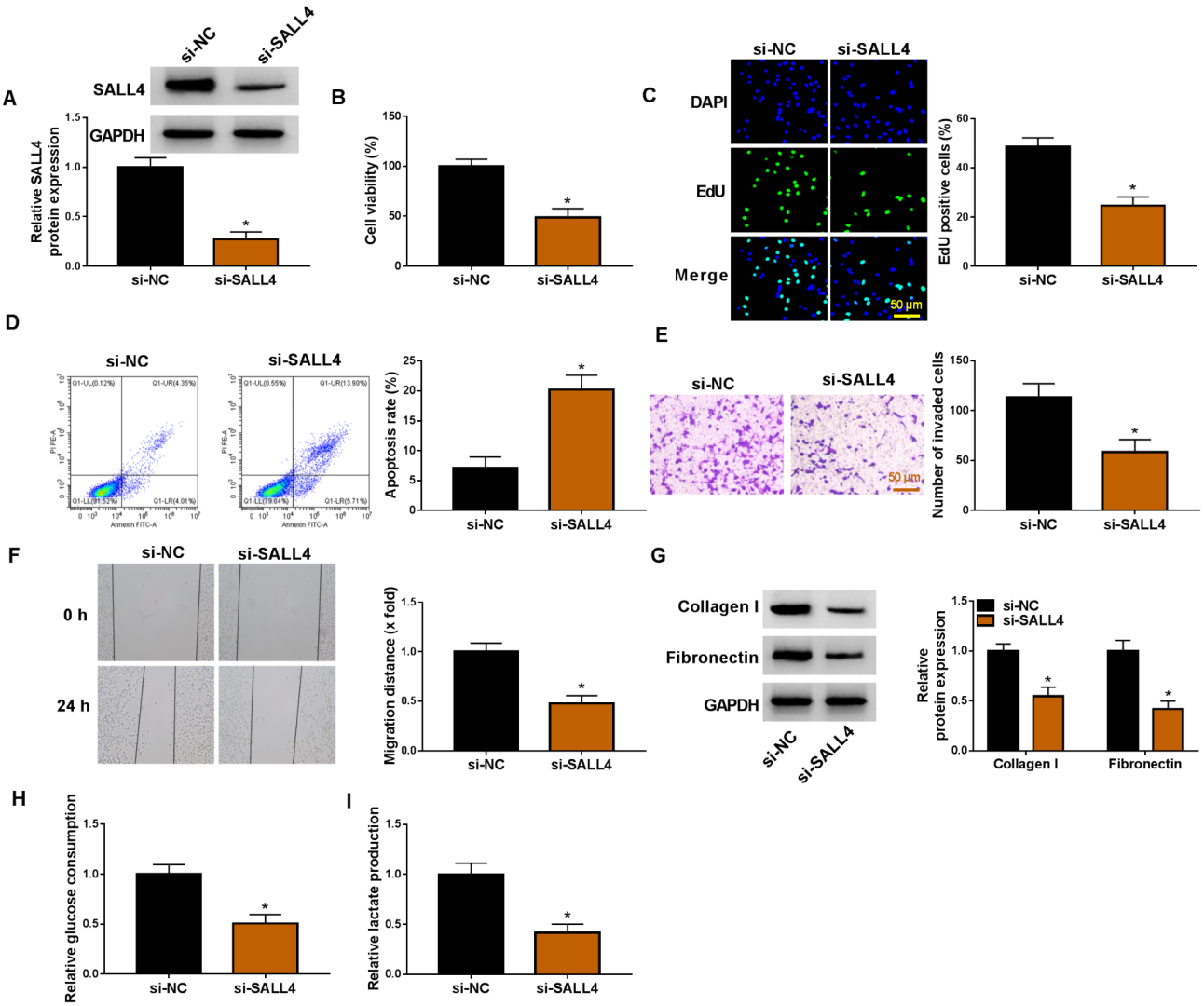

Fig. 2. The effect of SALL4 knockdown on keloid fibroblast functions. Keloid fibroblasts were transfected with si-NC/si-SALL4 ($n = 3$). (A) WB analysis was used to test the SALL4 protein level in each group. Cell viability, EdU positive rate, apoptosis, invasion and migration were measured by CCK8 assay (B), EdU assay (magnification 200×) (C), flow cytometry (D), transwell assay (magnification 200×) (E) and wound healing assay (F). (G) Collagen I and fibronectin levels were detected by WB analysis. (H-I) Glucose consumption and lactate production were ensured using the corresponding kits. A-F, H and I, Unpaired *t* test; G, one-way ANOVA. $^*P < 0.05$.

of SALL4 (Fig. 4J). Besides, IF staining suggested that USP37 and SALL4 were co-localized in the cytoplasm, and USP37 knockdown reduced their fluorescence activity (Fig. 4K). The experimental results indicated that USP37 deubiquitinated SALL4 to stabilize its expression.

SALL4 reverted the effects of USP37 downregulation on keloid fibroblast functions

To examine whether USP37 regulated SALL4 to mediate keloid formation, keloid fibroblasts were co-transfected with si-USP37 and SALL4 overexpression vector. SALL4 overexpression vector abolished si-USP37-mediated the decreasing effect on SALL4 protein expression (Fig. 5A). USP37 knockdown repressed viability, EdU positive cell rate, promoted apoptosis rate in keloid fibroblasts, but these effects were reverted by SALL4 upregulation (Fig. 5B-D). The inhibitory effect of si-USP37 on invaded cell numbers, migration distance, collagen I level, fibronectin level, glucose consumption and lactate production could be eliminated by overexpressing SALL4 (Fig. 5E-I and Supplementary Fig. 2). All results indicated that USP37 stabilized SALL4 to enhance keloid fibroblast growth, invasion, migration, ECM accumulation and glycolysis.

USP37/SALL4 axis mediated the PI3K/AKT pathway

Given the important role of PI3K/AKT pathway in keloid formation, we further investigated the effect of USP37/SALL4 axis on the activity of PI3K/AKT pathway. Through WB analysis, we found that USP37 knockdown

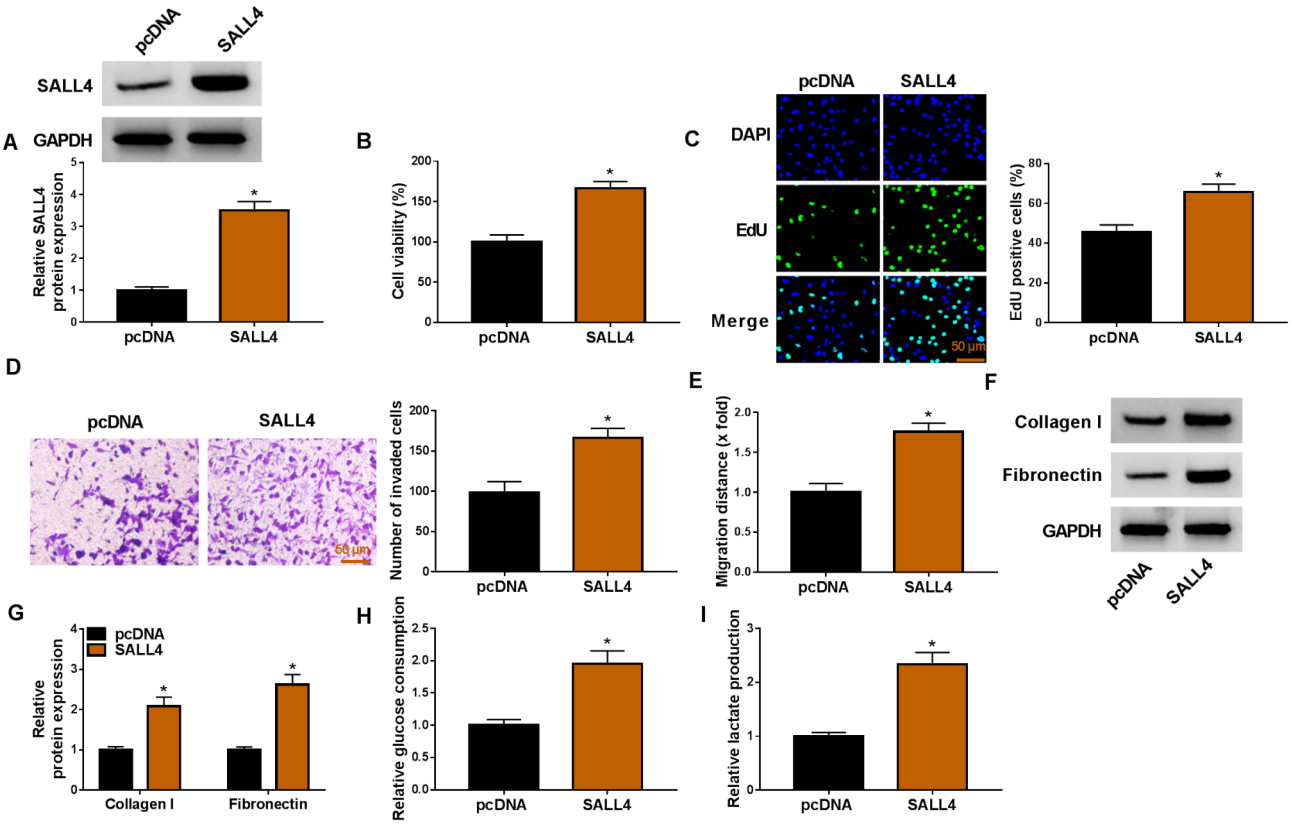

Fig. 3. The effect of SALL4 overexpression on keloid fibroblast functions. Keloid fibroblasts were transfected with pcDNA/SALL4 overexpression vector ($n = 3$). (A) SALL4 protein level was detected by WB analysis. CCK8 assay (B), EdU assay (magnification 200×) (C), transwell assay (magnification 200×) (D) and wound healing assay (E) were used to test the cell viability, EdU positive rate, invasion and migration. (F-G) WB analysis was performed to measure collagen I and fibronectin levels. (H-I) Glucose consumption and lactate production were assessed by the appropriate kits. A-E, H and I, Unpaired *t* test; G, one-way ANOVA. $^*P < 0.05$.

reduced the protein expression of p-PI3K/PI3K and p-AKT/AKT, whereas SALL4 reversed this effect (Fig. 6). These data showed that USP37/SALL4 axis might activate PI3K/AKT pathway.

PI3K pathway inhibitor reverted the effects of SALL4 upregulation on keloid fibroblast functions

To investigate whether SALL4 regulated keloid formation through PI3K/AKT pathway, keloid fibroblasts were transfected with SALL4 overexpression vector and treated with PI3K pathway inhibitor LY294002. LY294002 reversed the facilitation effect of SALL4 on keloid fibroblast viability, EdU positive cells, invaded cell numbers and migration distance (Fig. 7A-D). Besides, the promotion effect of SALL4 upregulation on the levels of collagen I, fibronectin, glucose consumption and lactate production could be reversed by LY294002 (Fig. 7E-G). These data suggested that SALL4 activated PI3K/AKT pathway to promote keloid fibroblast functions.

Discussion

Keloids, a scar characterized by a persistent and powerful proliferative force, are essentially a connective tissue tumor of the skin, often presenting a crabbed infiltration into the surrounding sound skin[26,27]. Despite significant improvements in the treatment, keloids still recur frequently and are difficult to treat[2]. It is necessary to reveal the molecular mechanism of keloid formation and provide new ideas for its treatment. The present study found that USP37-stabilized SALL4 could promote the biological function of keloid fibroblasts by activating the PI3K/AKT pathway, suggesting that the USP37/SALL4/PI3K/AKT axis might be an important potential target for keloid treatment.

SALL4, a member of the SALL family, is a major regulator that promotes cell stemness during biological development and tumor growth[9,28,29]. Previous studies revealed that SALL4 was upregulated in breast cancer, and its knockdown inhibited cell proliferation and migration in vitro[30]. Besides, SALL4 was highly expressed in lung cancer, and its downregulation reduced cell proliferation, invasion and metastasis[14]. Furthermore, SALL4 facilitated gastric cancer cell growth and glycolysis[31]. Thus, SALL4 is also an important regulator of cellular functions. A recent study indicated that SALL4 promoted cardiac fibroblast proliferation and migration to facilitate myocardial infarction[32]. Although SALL4 plays an important role in regulating fibroblast function, whether SALL4 regulates keloid fibroblast function to mediate keloid progression remains unclear. Here, we

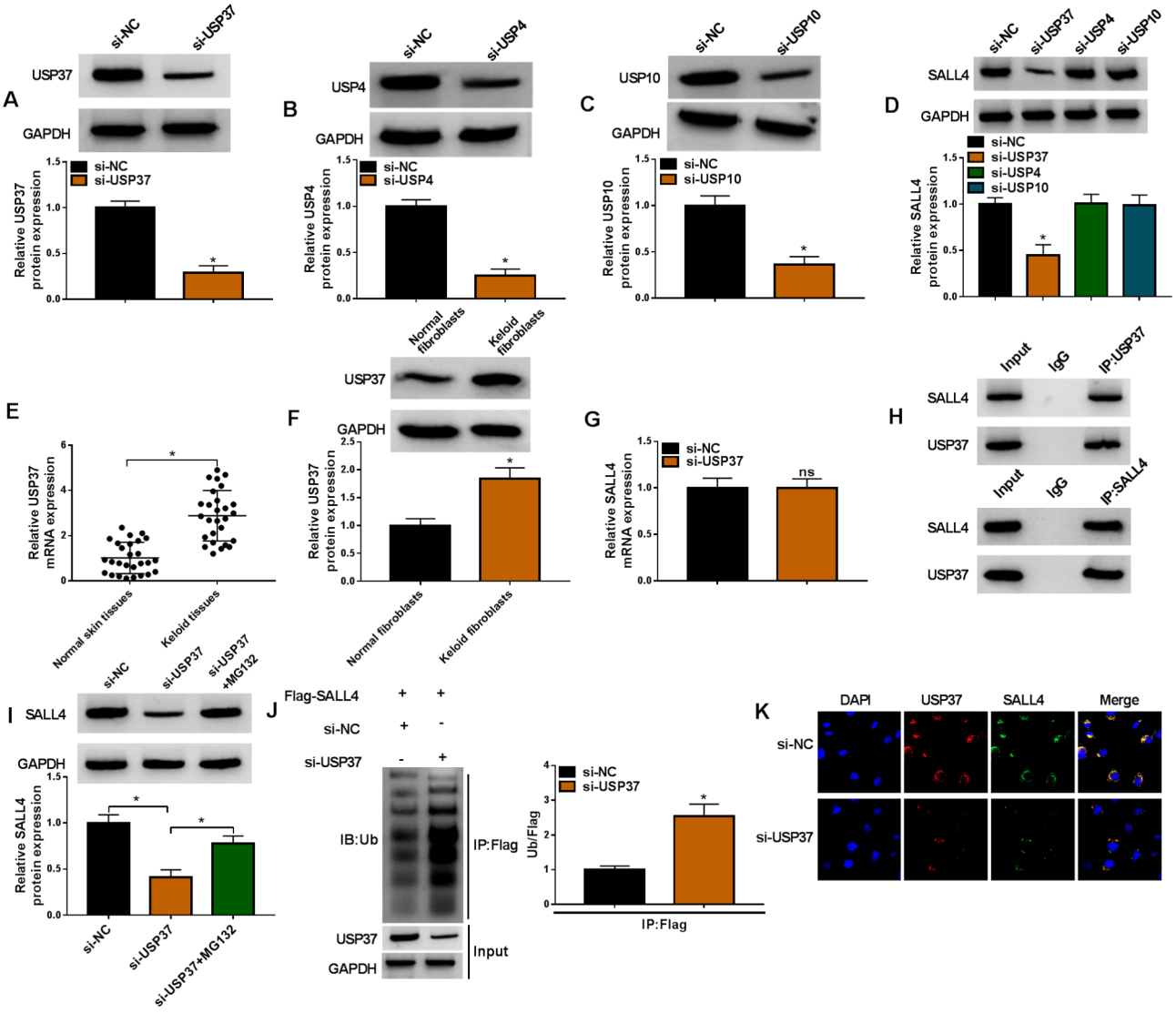

Fig. 4. Effects of USP37 on SALL4 expression. (A-C) The transfection efficiencies of si-USP37/si-USP4/si-USP10 were confirmed by WB analysis ($n = 3$). (D) SALL4 protein level was detected by WB analysis in keloid fibroblasts transfected with si-NC/si-USP37/si-USP4/si-USP10 ($n = 3$). (E-F) qRT-PCR ($n = 27$) and WB ($n = 3$) were used to test USP37 mRNA and protein levels in normal skin tissues and keloid tissues. (G) SALL4 mRNA level was tested through qRT-PCR in keloid fibroblasts transfected with si-NC/si-USP37 ($n = 3$). (H) Co-IP was used to assess the interaction between USP37 and SALL4. (I) SALL4 protein level was measured using WB analysis in keloid fibroblast transfected with si-NC/si-USP37 and treated with MG132. (J) Ubiquitination assay was performed in keloid fibroblast transfected with or without si-NC/si-USP37. (K) IF staining confirmed the co-localization of USP37 and SALL4. A-C, E-G and J, Unpaired *t* test; D and I, one-way ANOVA. $^*P < 0.05$.

found the upregulated SALL4 in keloid tissues through GEO database, and further analysis confirmed the high SALL4 expression in keloid tissues and keloid fibroblasts. Functional studies showed that SALL4 downregulation inhibited keloid fibroblast growth, invasion, migration, ECM accumulation and glycolysis, while its upregulation had the opposite effect, suggesting that SALL4 might promote fibroblast functions to accelerate keloid formation.

Deubiquitination is an important intracellular physiological process associated with protein-specific degradation, as well as an important modality for regulating cell signaling pathways[33,34]. Many deubiquitinating enzymes have been found to play important roles in keloid formation, such as USP37[22], USP4[25], USP10[24]and USP11[23]. Previous study revealed that USP37 deubiquitinated and stabilized BLM to maintain the DNA damage response[35]. Besides, USP37 deubiquitination-stabilized snails promoted lung cancer cell migration[36]. Importantly, USP37 had been confirmed to accelerate keloid fibroblast proliferation and collagen production by mediating the deubiquitination and stabilization of c-Myc[22], suggesting that USP37 played positive role in keloid formation. To determine the underlying molecular mechanism of SALL4 in the regulation of keloid formation, we examined the regulation of SALL4 expression by 3 deubiquitinating enzymes (USP37, USP4 and USP10). By screening, we determined that USP37 knockdown could reduce SALL4 expression, and further analysis

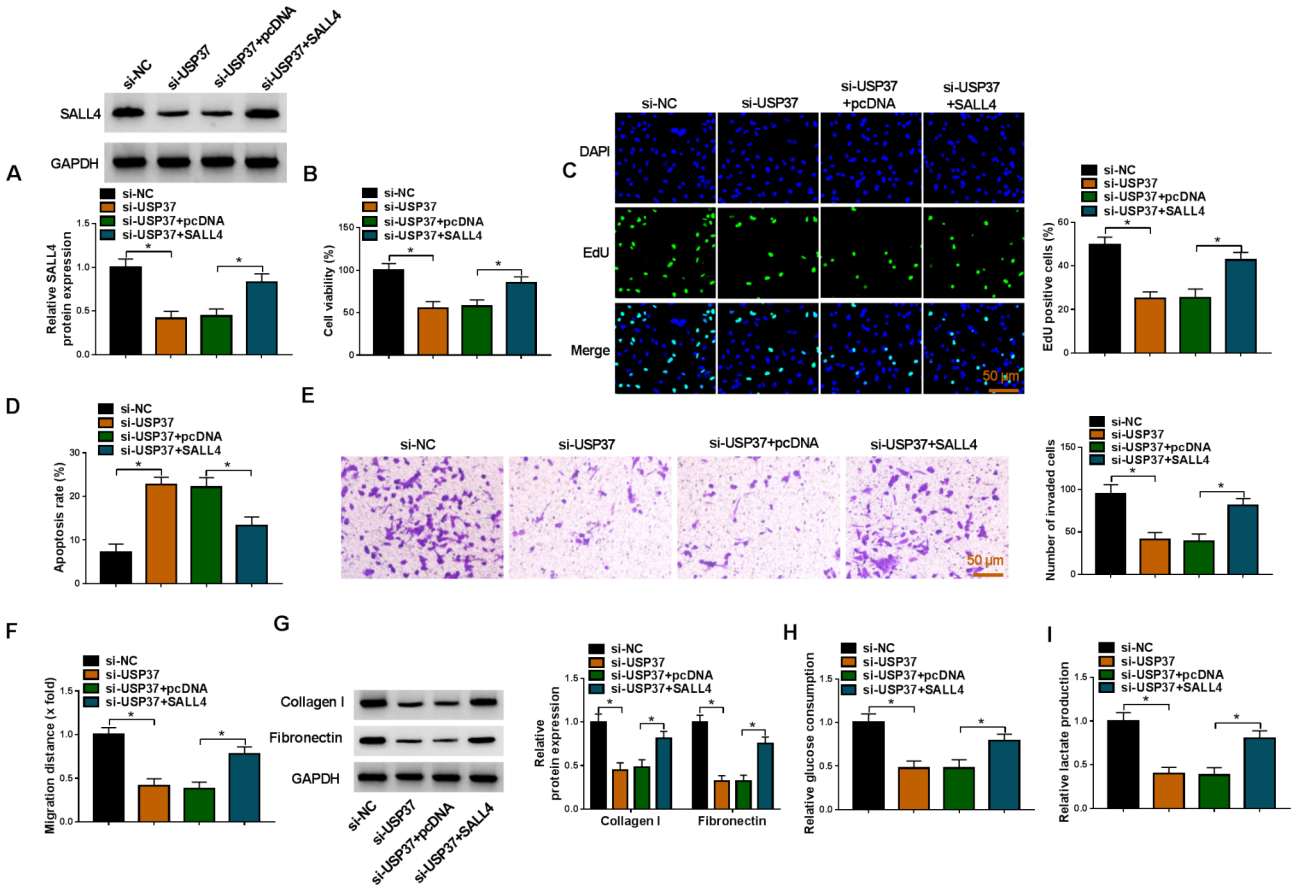

Fig. 5. Effect of USP37 and SALL4 on keloid fibroblast functions. Keloid fibroblasts were co-transfected with si-USP37 and SALL4 overexpression vector ($n = 3$). (A) SALL4 protein level was detected by WB analysis. CCK8 assay (B), EdU assay (magnification 200×) (C), flow cytometry (D), transwell assay (magnification 200×) (E) and wound healing assay (F) were performed to test cell viability, EdU positive rate, apoptosis, invasion and migration. (G) WB analysis was used to measure collagen I and fibronectin levels. (H-I) The corresponding kits were used to evaluate glucose consumption and lactate production. One-way ANOVA. $^*P < 0.05$.

confirmed that USP37 could stabilize SALL4 expression by deubiquitinating. Furthermore, SALL4 reversed si-USP37-mediated the inhibition on keloid fibroblast growth, invasion, migration, ECM accumulation and glycolysis, verifying that USP37 stabilized SALL4 to enhance fibroblast functions and facilitate keloid formation.

Activation of PI3K/AKT pathway may be a key step in keloid formation. Previously study showed that treatment of PI3K/AKT pathway inhibitor LY294002 suppressed keloid fibroblast proliferation, migration and invasion[37]. Besides, lncRNA uc003jox.1 activated PI3K/AKT pathway to promote keloid fibroblast proliferation and invasion[38]. These data confirmed that PI3K/AKT pathway could be a therapeutic target for keloids. Importantly, SALL4 contributes to the regulation of PI3K/AKT pathway activity in many human diseases. For example, SALL4 regulated the PTEN/PI3K/AKT pathway to facilitate glioma cell proliferation[18]. SALL4 modulated PI3K/AKT signaling through PTEN and BCL2, thereby increasing the survival and proliferation of HER2 + cell lines[39]. Consistent with previous findings, we also pointed out the positively regulation of USP37/SALL4 on PI3K/AKT pathway activity. Wang et al. suggested that inactivation of PI3K/AKT pathway repressed the proliferation, migration and invasion of keloid fibroblasts[37]. Here, we discovered that PI3K pathway inhibitor LY294002 reversed the promotional effects of SALL4 on keloid fibroblast functions, indicating that SALL4 might contribute to keloid formation by activating PI3K/AKT pathway.

Based on the above, the proposed USP37/SALL4/PI3K/AKT axis provides a new idea for the development of clinical keloid treatment targets. However, there are still some limitations to this study. So far, the study is informative at the cellular level. In vivo experiments are essential to confirm the therapeutic potential of targeting the USP37/SALL4/PI3K/AKT axis in keloid formation. This is crucial for translating the findings into clinical applications. In the future, in vivo studies are still needed to further confirm the important role of USP37/SALL4/PI3K/AKT axis in keloid formation. Also, the universality of USP37 and SALL4 expression still needs to be confirmed in larger cohorts to provide more evidence for them to be potential targets for keloid treatment. Besides, we demonstrate that USP37 stabilizes SALL4 expression through deubiquitination, but the molecular mechanism behind this interaction needs to be further investigated. In the future, understanding how USP37 specifically recognizes and deubiquitinates SALL4 would provide important mechanistic insights

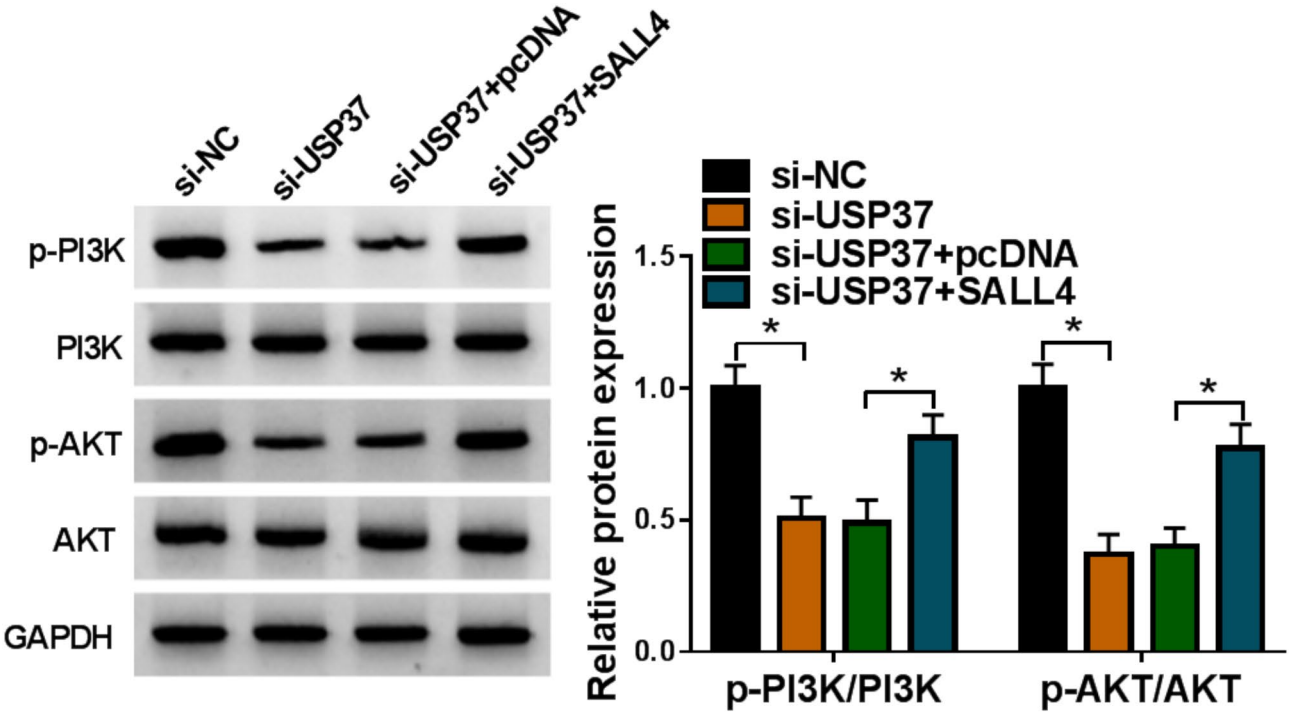

Fig. 6. Effect of USP37/SALL4 axis on PI3K/AKT pathway. Keloid fibroblasts were co-transfected with si-USP37 and SALL4 overexpression vector ($n = 3$). The protein levels of p-PI3K/PI3K and p-AKT/AKT were measured by WB analysis. One-way ANOVA. $^{*}P < 0.05$.

that enhance the robustness of this study. In addition, our study only reveals that PI3K/AKT is a downstream pathway of USP37/SALL4 axis so far, and whether the USP37/SALL4 axis regulates other pathways to affect keloid formation is still unclear. Previous studies have shown that SALL4 may mediate cell proliferation, invasion and apoptosis by promoting the Wnt/β-catenin pathway[29,40]. Therefore, we speculate that the USP37/SALL4 axis may also mediate keloid fibroblast growth, invasion, migration, ECM accumulation and glycolysis by regulating the Wnt/β-catenin pathway, which requires further investigation in the future.

In conclusion, our study revealed that USP37 promoted keloid fibroblast growth, invasion, migration, ECM accumulation and glycolysis through activating PI3K/AKT pathway by deubiquitinating SALL4. Our results suggest that targeted inhibition of USP37/SALL4/PI3K/AKT axis may inhibit keloid formation by inhibiting keloid fibroblast functions, which may become a new strategy for keloid treatment and provide a new direction for clinical drug development of keloid.

Ethics approval

The research was carried out following the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital of the Naval Medical University (No.112012098).

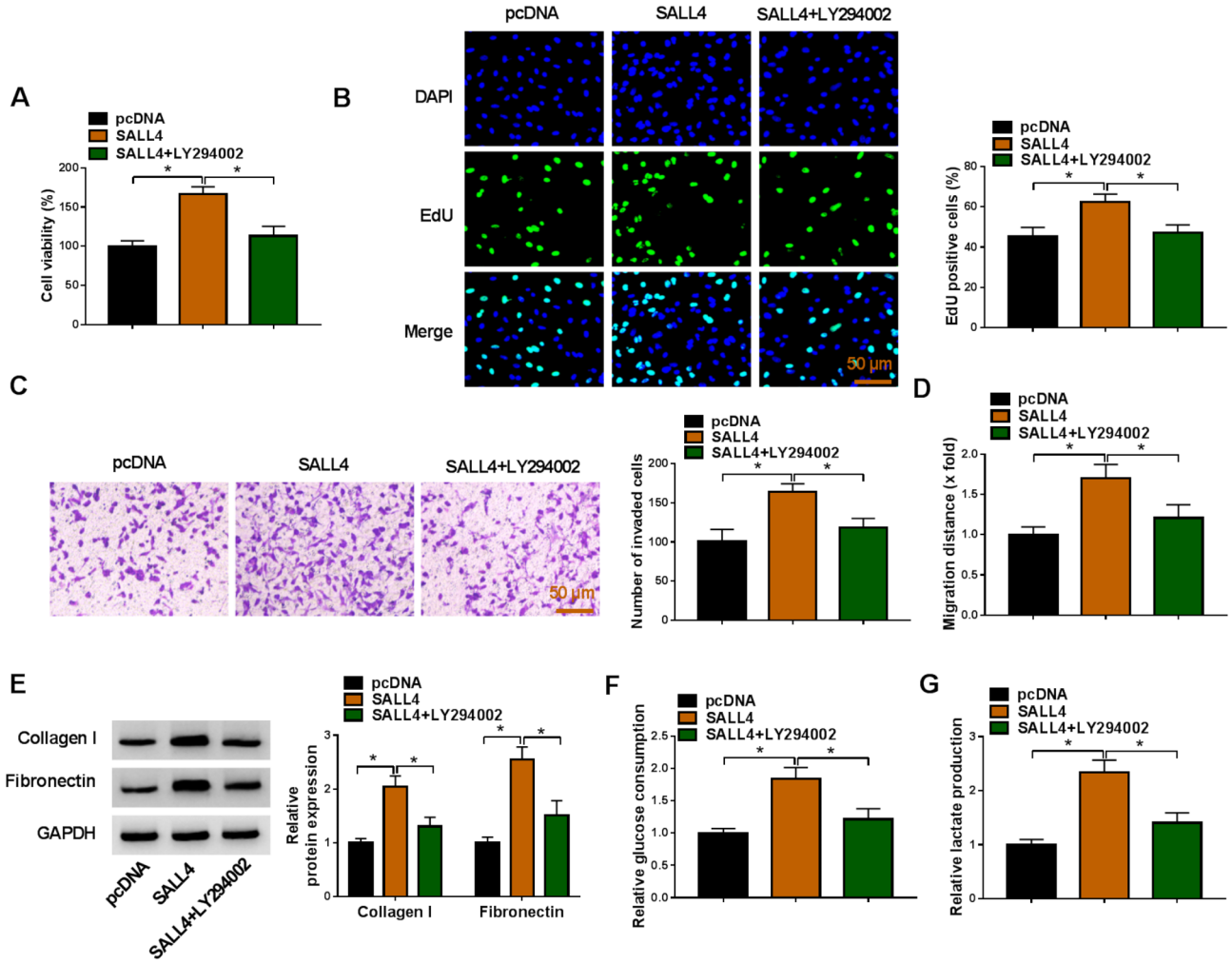

Fig. 7. Effects of SALL4 and PI3K pathway on keloid fibroblast functions. Keloid fibroblasts were transfected with SALL4 overexpression vector and treated with LY294002 ($n = 3$). Cell viability, EdU positive rate, invasion and migration were evaluated by CCK8 assay (A), EdU assay (magnification 200×) (B), transwell assay (magnification 200×) (C) and wound healing assay (D). (E) Collagen I and fibronectin levels were measured using WB analysis. (F-G) Glucose consumption and lactate production were detected with the corresponding kits. One-way ANOVA. $^*P < 0.05$.

Data availability

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

Received: 3 December 2024; Accepted: 24 February 2025
Published online: 04 March 2025

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Author contributions

S. F. performed all the experiments and wrote the first draft of the manuscript. Z. W. and J. X. provided with the statistical assistance. M.X .and J. Z. revised the manuscript for important intellectual content. Y. Z. and C. X. contributed to the conception and design of the study. All authors contributed to the manuscript revision, and read and approved the submitted version.

Funding

This research was sponsored by National Natural Science Foundation of China (Grant Nos. 81701923).

Declarations

Competing interests

The authors declare no competing interests.

Disclosure of interest

The authors declare that they have no conflicts of interest.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/1

0.1038/s41598-025-91776-5.

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