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ORIGINAL ARTICLE

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The deubiquitinating enzyme USP37 promotes keloid fibroblasts proliferation and collagen production by regulating the c-Myc expression

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Abstract

Previous research testifies that c-Myc may promote keloid fibroblast proliferation and collagen accumulation. Ubiquitin-specific peptidase 37 (USP37)-mediated deubiquitination and stabilisation of c-Myc are vital for lung cancer proliferation, while the potential role of USP37 in keloid fibroblasts is not investigated. Elevated USP37, c-Myc, and Collagen I content were detected in keloid tissue with RT-PCR or ELISA assay. USP37 over-expression plasmids or USP37 short hairpin RNAs (shRNAs) were transfected into keloid fibroblasts with Lipofectamine 3000 to decipher the role of USP37 in keloid fibroblasts. USP37 overexpression could promote the proliferation of keloid fibroblasts with increased c-Myc and Collagen I expression. On the other hand, USP37 shRNAs inhibited the proliferation of keloid fibroblasts with diminished c-Myc and Collagen I expression. It was worth noting that C-Myc overexpression promoted the proliferation of keloid fibroblasts inhibited by USP37 shRNAs with increasing Collagen I expression. All of these results demonstrate that USP37 could regulate c-Myc to promote the proliferation and collagen deposit of keloid fibroblasts, and USP37 could be targeted in future keloid therapy.

KEYWORDS

c-Myc, collagen I, fibroblasts, keloid, USP37

Key Messages

- USP37 shRNAs inhibited the proliferation of keloid fibroblasts with diminished c-Myc and collagen I expression
- USP37 overexpression could promote the proliferation of keloid fibroblasts with increased c-Myc and collagen I expression
- USP37 could regulate c-Myc to promote the proliferation and collagen deposit of keloid fibroblasts

1 | INTRODUCTION

Keloid is a group of pathological fibroproliferative skin disorders with unremitting extracellular matrix (ECM) accumulation, remodelling, and stiffness.[1,2] Although the pathogenesis of keloid is complex, strong genetic predisposition, growth factors' profile alteration, collagen turnover, and higher stretching tension contribute to keloid formation and development.[3-5] Surgery, steroid, and radiation treatment are recommended in clinics, while the high recurrence and accompanying psychological distress still pose a challenge for affected patients.[6,7]

Myofibroblasts derived from resident skin fibroblasts are the principal mediators responsible for ECM accumulation, demonstrating apoptosis resistance and hyperproliferation characteristics.[8] It is shown that c-Myc can function as an intracellular integrator to coordinate growth regulatory signalling in proliferating fibroblasts.[9-11] At the post-translational level, ubiquitin/proteasome-dependent degradation is vital to regulate the abundance of c-Myc. Ubiquitin-specific peptidase 37 (USP37) demonstrates cysteine-type endopeptidase activity, protein kinase binding activity, and thiol-dependent deubiquitinase function. It is well-known for its full catalytic activity without ubiquitin chain substrate specificity.[12] In lung cancer, USP37 is testified to have the ability to modulate the relative expression of c-Myc with direct deubiquitination and stabilisation.[13] On the other hand, USP36 is testified to be c-Myc target gene. All of these suggest that USP36 and c-Myc form a positive feedback regulatory loop. In comparison, the potential regulation between USP37 and c-Myc in keloid is not investigated.

In this study, gene silencing or overexpression analysis of USP37 is performed on keloid fibroblasts, and our results demonstrate that USP37 could promote the proliferation and collagen accumulation effect. C-Myc overexpression analysis further testifies that the role of USP37 on keloid fibroblast proliferation and Collagen I production is dependent on the expression of c-Myc. These data indicate that USP37 could regulate c-Myc -mediated fibroblast proliferation and ECM accumulation, and USP37 is an attractive therapeutic target in keloid.

2 | METHODS & MATERIALS

2.1 | Participants

Normal scars (three women, mean 35.7 years; n = 8) and keloid tissues (11 women, mean 37.4 years; n = 20) were obtained during reconstructive surgery as indicated in our previous research, and the clinical information could be referred.[14] Informed and written consents were signed by all participants enrolled, and the whole study was approved by the Ethics Committee of the Wuxi Second People's Hospital. This study followed the Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects.

2.2 | Primary keloid fibroblasts culture

Collected keloid tissues were disassociated with 0.5 mg/mL type-I collagenase (Gibco, Grand Island, NY) and 0.2 mg/mL trypsin (Gibco) supplemented in DMEM medium for six hours at 37°C to obtain primary keloid fibroblasts. The fibroblasts were further cultured in a low-glucose DMEM medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Gibco), and three–four generations of primary fibroblasts were used.

2.3 | Transient transfection

Human USP37 cDNA was subcloned into pcDNA3.1-Flag vector to construct USP37-overexpressing plasmid as indicated by the previous report,[13] which was transfected into primary fibroblasts with Lipofectamine® 3000 (Invitrogen, Waltham, MA) for 48 h. The design of shRNAs against USP37 was referred to a previous report,[15] and USP37 shRNAs (shRNA#1, 5′-CCGAAGAACTGGAGTATTC-3′; shRNA#2, 5′-CCTAGTAGTTCACTACAAT-3′) were synthesised (GenePharma Company, Suzhou, China) and transfected into primary keloid fibroblasts with Lipofectamine® 3000 (Invitrogen) for 48 h, which was further transfected with a c-Myc-PT3EF1a plasmid (Addgene, #92046, Watertown, MA).

2.4 | Cell viability

Untransfected or transfected keloid fibroblasts (5×10^5 cells) were incubated in 96-well plates for 24 h. Then, 10 μL cell-counting kit-8 (CCK-8) solution (Dojindo Laboratories, Kumamoto, Japan) was further incubated for two hours at 37°C. The absorbance was detected with the SpectraMax® Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

2.5 | Real-time PCR

TRIzol (Invitrogen) was used to extract total RNA from keloid fibroblasts, and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) was used to reverse-transcribe total RNA into cDNA.

SYBR Green (TaKaRa, Dalian, China) was applied to show the amplification with the following reaction: 96°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for one minute on Roche Cycler 480. *GAPDH* was used as an internal control. Differential expression was calculated with the $2^{-\Delta\Delta Ct}$ method. Primers were listed: *GAPDH*, forward, 5′-GGTATCGTGGAAGGACTCATGAC-3′, reverse, 5'-ATGCCAGTGAGCTTCCCGTTCAG-3′; *USP37*, forward, 5′- TCTCTATTGACCTTCCTCGTAGG-3′, reverse, 5′- TGC CTGACAAGAGCACACTTCC-3′.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

Skin tissue was homogenised with T 10 basic ULTRA-TURRAX® (IKA) in RIPA Buffer (Abcam, Cambridge, MA, USA) to obtain keloid tissue lysate. Keloid tissue lysate and the supernatant of keloid fibroblasts were collected and used to detect the contents of USP37, c-Myc, and Collagen I with relevant ELISA kits (Human USP37 ELISA Kit, Cat. No. HUF108315, AssayGenie, Ireland; Human c-Myc ELISA Kit, Cat. No. ab287834, Abcam, Cambridge, MA; Human collagen type I ELISA Kit, Cat. No. ab285250, Abcam) according to the manufacturer's instructions. All standards and samples were measured with SpectraMax® Plus 384 microplate reader at a wavelength of 450 nm.

2.7 | Western blotting

The relevant fibroblasts lysates obtained with Cell Extraction Buffer (Invitrogen) were separated with 10% SDS-PAGE and transferred to NC membrane, which were sequentially incubated with 5% non-fat milk (2 h, at room temperature), primary antibodies (overnight, at 4°C), and secondary antibody (1 h, at room temperature). ECL system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was applied to develop the signal, and the intensity of the bands was calculated with NIH-Image J1.51p 22. Primary antibodies used were indicated: anti-USP37 (1:1000 dilution, PA5-31266, Invitrogen), anti-c-MyC (1:1000 dilution, MA1-980, Invitrogen), and anti-GAPDH (1:2000 dilution, MA1-16757, Invitrogen).

2.8 | Statistical analysis

All data were presented as mean ± standard deviation (SD). Differences between groups were assessed with the Student *t*-test, one-way or two-way ANOVA analysis with a post hoc test. All statistical analyses were performed with GraphPad Prism.

3 | RESULTS

3.1 | Up-regulated USP37 and c-Myc in keloid tissues

Up-regulated USP37 (about two folds) was observed in keloid tissue lysates testified by both RT-PCR (Figure 1A, mean value: 2.5 folds) and ELISA (Figure 1B, USP37 mean value: 137.8 pg/mg protein) analysis. On the other hand, up-regulated c-Myc (Figure 1C, c-Myc mean value: 48.7 ng/mg protein) and Collagen I (Figure 1D, Collagen I mean value: 9.3 ng/mg protein) were also detected in keloid tissue lysates compared with normal skin lysates. All of these results indicated the potential role of USP37 in keloid.

3.2 | Knockdown of USP37 inhibits keloid fibroblast proliferation and collagen production

USP37 shRNAs were transfected into keloid fibroblasts to knock down USP37. As expected, downregulated USP37 expression was detected in both mRNA (Figure 2A) and protein (Figure 2B) levels. Functionally, knockdown of USP37 could significantly inhibit keloid fibroblast proliferation (Figure 2C, $P < .001$ versus shNC on Day 5) and Collagen I production (Figure 2D, $P < .001$ versus shNC).

3.3 | Overexpression of USP37 promotes collagen production and cell proliferation in keloid fibroblasts

On the other hand, USP37-overexpressing plasmids were transfected into keloid fibroblasts, and up-regulated USP37 expression was detected (Figure 3A), which showed the success of USP37 transfection. USP37 overexpression could significantly promote cell proliferation (Figure 3B, $P < .001$ versus EV on Day 5) and Collagen I production in keloid fibroblasts (Figure 3C, $P < .001$ versus EV). Both the knockdown and overexpression experiments demonstrated the effect of USP37 on promoting Collagen I production and cell proliferation in keloid fibroblasts.

3.4 | USP37 regulates c-Myc expression in keloid fibroblasts

The relative expression of c-Myc after USP37 silencing was showed by Western blot analysis (Figure 4A), and

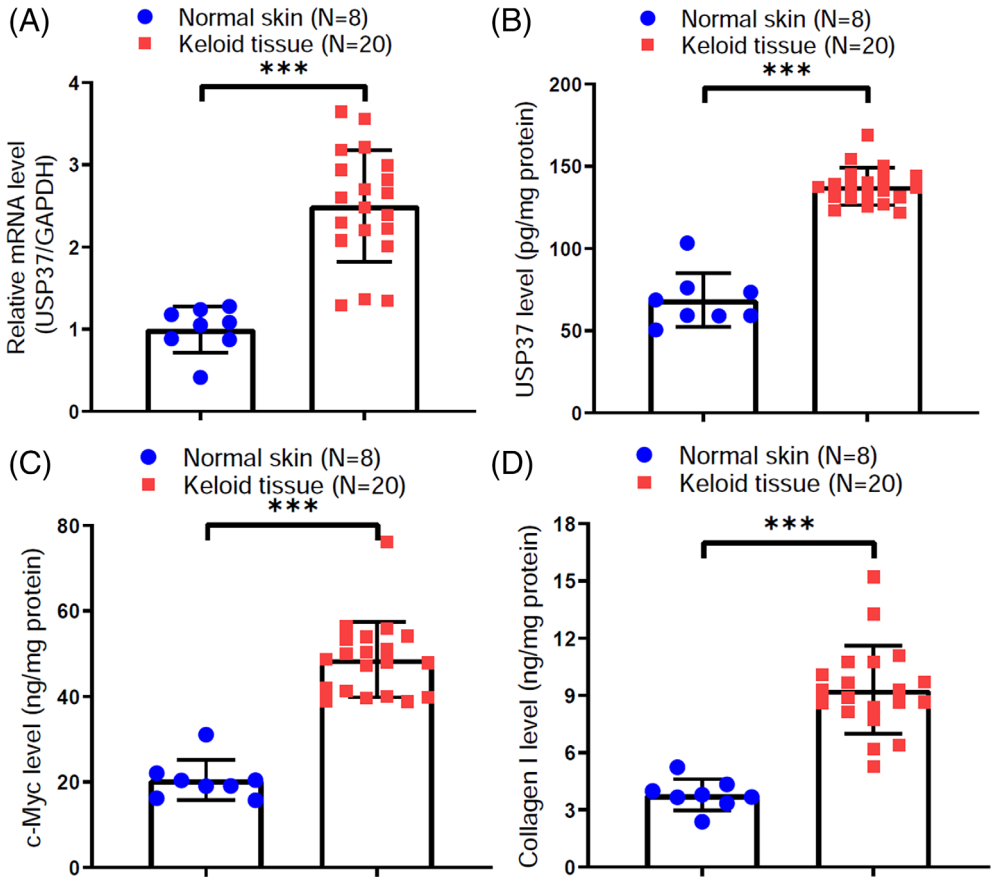

FIGURE 1 USP37 is up-regulated in keloid tissues. (A) Keloid tissues and normal skin were prepared for qRT-PCR against USP37. GAPDH was used as an internal control. (B–D) Keloid tissues and normal skin were also prepared for ELISA analysis of USP37 (B), c-Myc (C), and Collagen I (D). ***$P < .001$

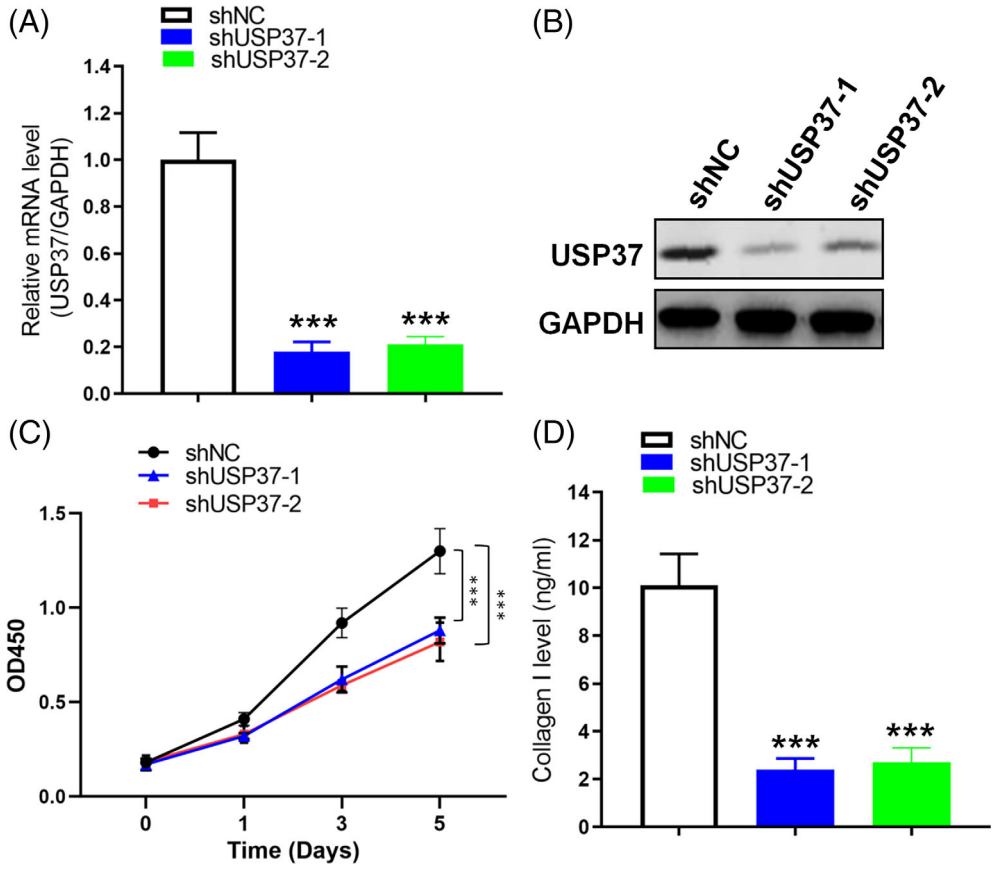

FIGURE 2 Knockdown of USP37 inhibits the proliferation of keloid fibroblasts and relevant Collagen I production. (A,B) Keloid fibroblasts were transfected with indicated shRNAs for two days, and the relative expression of USP37 was detected with qRT-PCR (A) and Western blot (B). (C) The proliferation of shRNAs transfected keloid fibroblasts for 0, 1, 3, or 5 days of culture was showed by CCK-8 assay. (D) The secretion of Collagen I in the supernatant of keloid fibroblasts after two days of culture was assayed with ELISA. ***$P < .001$

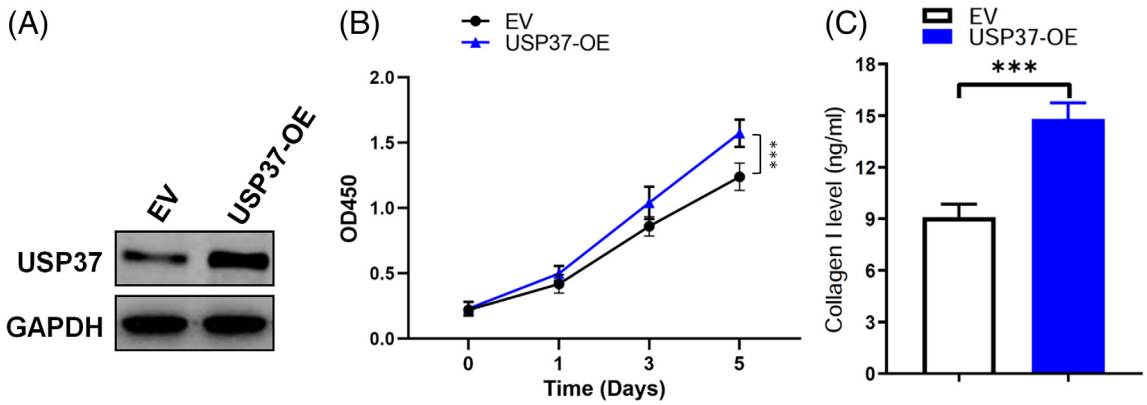

FIGURE 3 Overexpression of USP37 promotes collagen production and cell proliferation in keloid fibroblasts. (A) Keloid fibroblasts transfected with USP37-overexpressing plasmids (USP37-OE) or empty vector (EV) were cultured for two days, and the relative expression of USP37 was detected with Western blot. (B) The proliferation of USP37 transfected keloid fibroblasts was detected with the CCK-8 assay. (C) The level of Collagen I in the supernatant of USP37 overexpressed keloid fibroblasts was detected with ELISA. ***$P < .001$

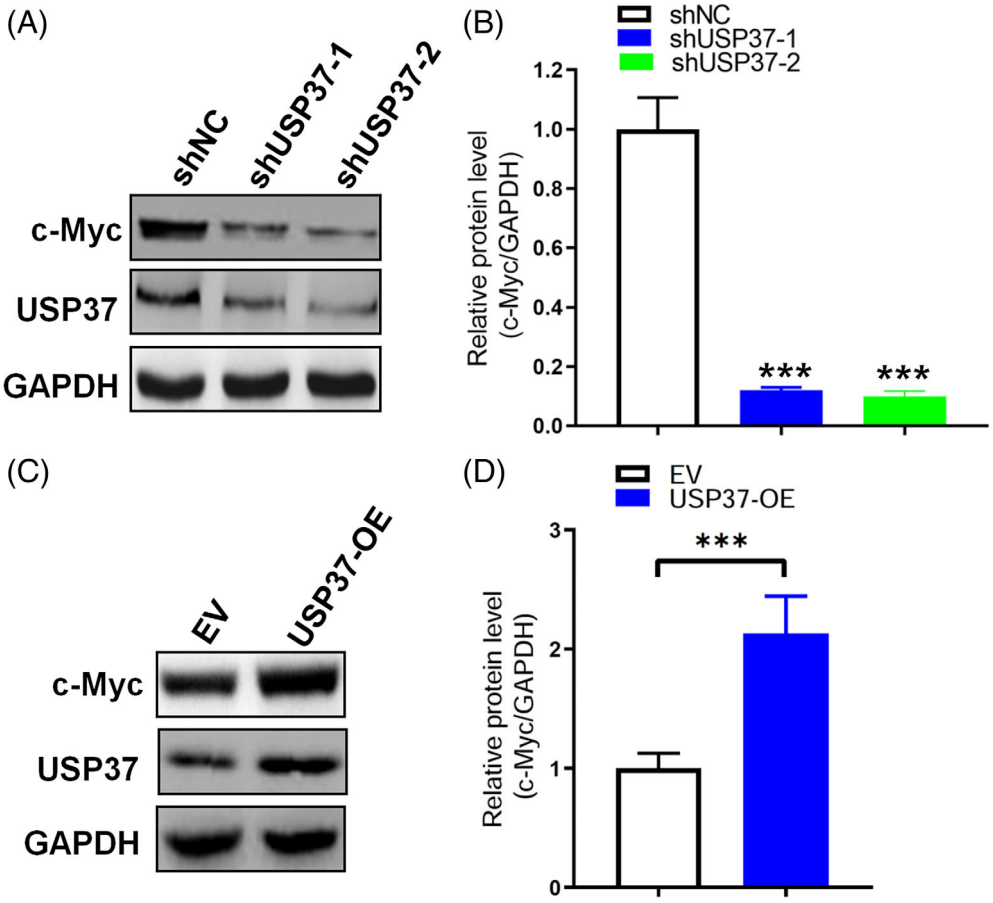

FIGURE 4 USP37 regulates c-Myc expression in keloid fibroblasts. (A,B) The relative expression of c-Myc in shRNAs USP37 transfected keloid fibroblasts was detected with Western blot (A). The optical density for c-Myc was also determined (B). (C,D) The relative expression of c-Myc in USP37 overexpressed keloid fibroblasts was detected with Western blot (C). The optical density for c-Myc was also determined (D). ***$P < .001$

significantly downregulated c-Myc expression was observed (Figure 4B, $P < .001$ versus shNC). On the other hand, USP37 overexpression could significantly up-regulate c-MYC expression in keloid fibroblasts (Figure 4C,D, $P < .001$ versus EV). All of these results demonstrated that USP37 could positively regulate c-Myc expression in keloid fibroblasts.

3.5 | C-Myc overexpression restores the function of shUSP37 in keloid fibroblasts

A large amount of c-Myc expression was detected in EV plasmids transfected keloid fibroblasts, and up-regulated c-Myc expression was detected in c-Myc-overexpressed keloid fibroblasts when compared with EV plasmids

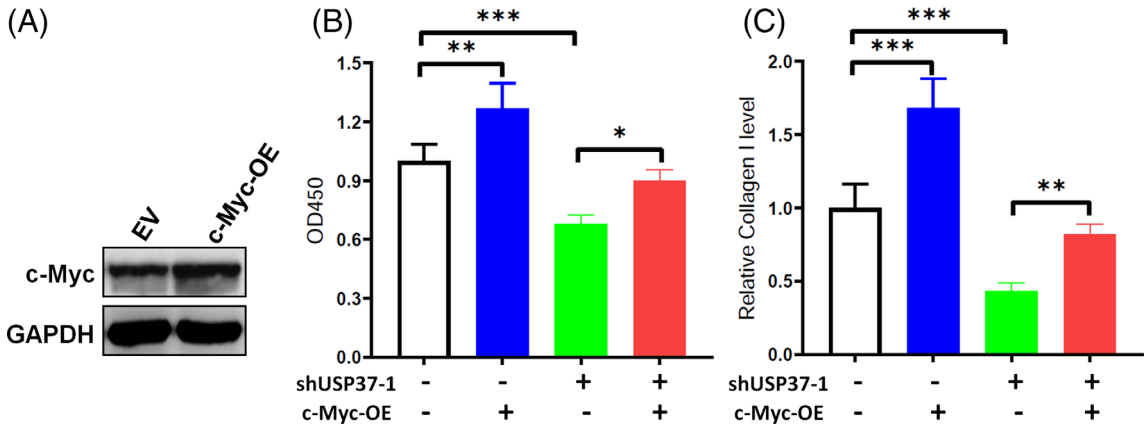

FIGURE 5 Overexpression of c-Myc could block the function of shUSP37 in keloid fibroblasts. (A) Western blot was performed to detect the transfection efficacy of c-Myc-overexpressing (c-Myc-OE) plasmids. (B) Keloid fibroblasts transfected with shUSP37-1 were further transfected with c-Myc-OE plasmids for two days, and the proliferation was showed with the CCK-8 assay. (C) The secretion of Collagen I by keloid fibroblasts was detected with ELISA. *$P < .05$, **$P < .01$, ***$P < .001$

transfected keloid fibroblasts (Figure 5A). C-Myc-overexpression could promote keloid fibroblast proliferation (Figure 5B, the second column) and Collagen I production (Figure 5C, the second column). In contrast, USP37 silencing could diminish keloid fibroblasts proliferation (Figure 5B, the third column) and Collagen I production (Figure 5C, the third column), which could be significantly reversed by the transfection of the c-Myc-overexpression plasmid (Figure 5B, the fourth column; Figure 5C, the fourth column). All of these data demonstrated USP37-dependent and c-Myc-mediated keloid fibroblast proliferation and Collagen I production.

4 | DISCUSSION

Deubiquitination and ubiquitination, essential post-translational modifications, are getting increasing attention concerning potential phenotype regulation. In this paper, up-regulated USP37 (nearly two-fold in both mRNA and protein levels) is detected in keloid tissues. USP37 overexpression could promote the proliferation of keloid fibroblasts and the relevant Collagen I production with increased c-Myc expression, and USP37 gene silencing could down-regulate such effect, which could be reversed by c-Myc-overexpressing. Thus, this study discovers that USP37 could regulate the relative expression of c-Myc in keloid fibroblasts, which could be attributed to the ubiquitin-specific peptidase function of USP37. USP37-dependent c-Myc mediated proliferation and collagen growth-stimulating effects in keloid fibroblasts could be considered as a potential therapeutic target in keloid.

It is testified that USP37 could directly deubiquitinate c-Myc in a deubiquitinase-dependent manner to promote lung cancer cell proliferation and induce aerobic glycolysis (the Warburg effect).[13,16] As a multifunctional proto-oncogene, c-Myc can regulate cell division, growth, autophagy, and apoptosis.[17-19] In this study, we testify that USP37 could promote fibroblasts proliferation and collagen production in keloid with up-regulated c-Myc accumulation or expression, which indicates the ubiquitin-specific peptidase function of USP37 to post-translational regulate the expression of c-Myc. It is worth noting that post-translational modification could localise c-Myc to the nuclear pore basket to induce histone modification, chromatin remodelling, and paused RNA polymerase release, which can regulate genes related to cellular responses to environmental signals.[20,21] In addition to regulating the relative expression of c-Myc, whether USP37 could re-localise c-Myc in keloid fibroblasts needs further analysis.

Characterised Warburg effect is a unique feature in keloid fibroblasts different from other types of fibroblasts, such as atrophic scar fibroblasts, proliferative stage scar fibroblasts, and hypertrophic scar fibroblasts,[22] which indicates the reprogramming of keloids fibroblasts from oxidative phosphorylation to the Warburg effect. In addition to proliferation and collagen deposit, whether USP37/c-Myc could promote the energy metabolism reprogramming of fibroblasts needs further analysis.

Increased Collagen I expression observed in this study indicates the alteration of collagen composition and cross-linking in keloid tissues, and the relative expression of Collagen I is associated with stable hypoxia-inducible factor-1α (HIF-1α) in keloid tissue.[23,24] It is worth noting that fibroblasts isolated from the growing margin of keloid scars could produce more Collagen I when compared with fibroblasts isolated from the intralesional and

the extralesional regions.[25,26] All of these indicate that USP37-induced Collagen I production may contribute to the development of keloid.

Some limitations should be indicated here. The precise mechanism of USP37 to regulate c-Myc expression is not deciphered in this study. As indicated by previous research, USP37 overexpression could markedly increase the abundance of c-Myc by blocking c-Myc degradation, while USP37 depletion could greatly reduce c-Myc levels by promoting the degradation.[13] It is worth noting that USP37 and c-Myc interact exterior to both pleckstrin and C-19 domain. USP37-C-Myc interactions might play an essential role in keloid pathogenesis, which could be used to design small molecules and peptidyl disruptors.

5 | CONCLUSION

USP37 could promote c-Myc-mediated keloid fibroblast proliferation and collagen accumulation, which could be considered as a post-transcriptional option in c-Myc target research in keloid.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Informed and written consents were signed by all participants enrolled, and the whole study was approved by the Ethics Committee of the Wuxi Second People's Hospital. This study followed the Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects.

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