Inhibition of keloid by ^{32}P isotope radiotherapy through suppressing TGF-β/Smad signaling pathway

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ABSTRACT

Background: Keloid seriously affects the appearance, and is accompanied by some symptoms including pain, burning, itching. Radioactive nuclides such as ^{32}P have been proved to be effective in inhibiting the formation of keloid, but the mechanism remains unclear.

Methods: The keloid animal model was established through keloid tissues implantation. Hematoxylin-Eosin (HE) and Masson staining were performed to investigate histological changes and collagen deposition. The mRNA and protein expression were assessed using RT-PCR and western blotting, respectively. Cell apoptosis and cycle were evaluated through flow cytometry.

Results: Both ^{32}P isotope injection and skin path significantly reduced the size of keloid, and inhibited TGF-β/Smad signaling pathway. SRI-011381, the agonist of TGF-β/Smad signaling pathway, markedly reversed the influence of ^{32}P isotope on cell proliferation, cell apoptosis, cell cycle of LNCaP cells and TGF-β/Smad signaling pathway.

Conclusions: ^{32}P isotope injection and skin path greatly reduced the size of keloid, and the TGF-β/Smad signaling pathway was remarkably inhibited by ^{32}P isotope treatment. The regulation of dermal fibroblast by ^{32}P isotope was reversed by SRI-011381. ^{32}P isotope might inhibit keloid through suppressing TGF-β/Smad signaling pathway. Our study provides a novel therapeutic strategy for the treatment of keloid.

INTRODUCTION

Keloid is a benign skin disease, but it often seriously affects the appearance, and can be accompanied by pain, burning, itching and other symptoms, and even affect the function of the limbs, which has a great negative impact on life [1]. In the process of skin wound healing, keloid, due to the interaction of molecules, cells, physiology, biochemistry, physics and other factors, leads to the imbalance of body repair mechanism, abnormal keloid tissue grows excessively [2], gradually bulges from the skin surface, exceeds the original damage range, and invades the surrounding normal tissue without natural regression [3]. At present,

the treatment methods are roughly divided into surgical treatment, laser therapy, physical therapy, and drug therapy [4]. Each of the above treatment methods has its own advantages and has a certain inhibitory effect on scars, but they all have shortcomings, some of which have little effect, some are expensive, and the clinical effect is not ideal [5, 6].

Using radioactive nuclides such as ^{32}P to release β-radiation can significantly inhibit scar hyperplasia and is one of the best treatment methods [7]. Purely released β-rays can treat keloid by influencing the activity of fibroblasts, inhibiting the synthesis of collagen fibers, blocking microvessels, and reducing the blood supply

to keloid tissue through electric radiation [8]. Among them, the use cost of ^{32}P is the lowest, making it suitable for clinical treatment. The total treatment course is relatively cumbersome, and other relevant clinical departments still have little understanding and application [9]. Therefore, this study aims to study a more effective ^{32}P treatment plan within a safe range, in order to further promote its clinical application and understand possible treatment mechanisms.

Keloid is a benign skin tumor beyond the original damage range formed by excessive hyperplasia and hyaline degeneration of skin connective tissue [10]. It has gene susceptibility, epigenetic regulation, and is of great significance to gene expression, regulation, and inheritance, including block non coding RNA, histone modification, etc. [11]. Activating or inhibiting TGF-β/Smad can induce or inhibit fibroblast proliferation and collagen synthesis, and promote or inhibit the formation of keloid [12].

The TGF-β/Smad signaling pathway plays a crucial role in keloid formation, contributing to the excessive and abnormal deposition of extracellular matrix (ECM) components, which characterizes this fibrotic skin disorder [13]. Keloids are an aberrant response to wound healing, where an overproduction of collagen and other ECM proteins leads to the formation of raised, thickened, and fibrous scars that extend beyond the original wound site [14]. Whether ^{32}P isotope radiotherapy could regulate keloid has not been reported.

RESULTS

Both ^{32}P isotope injection and skin path significantly reduced the size of keloid

Two types of treatments, ^{32}P isotope injection and ^{32}P isotope skin path, were applied to treat keloid. Both ^{32}P isotope injection and skin path significantly reduced the size of keloid (Figure 1A). HE staining was performed to investigate the histological changes. The disorder tissues were improved after ^{32}P isotope injection and ^{32}P isotope skin path treatment (Figure 1B). Masson's trichrome staining (Figure 1C) revealed that ^{32}P isotope injection and ^{32}P isotope skin path treatment remarkably suppressed the collagen deposition.

Both ^{32}P isotope injection and skin path significantly inhibited TGF-β/Smad signaling pathway

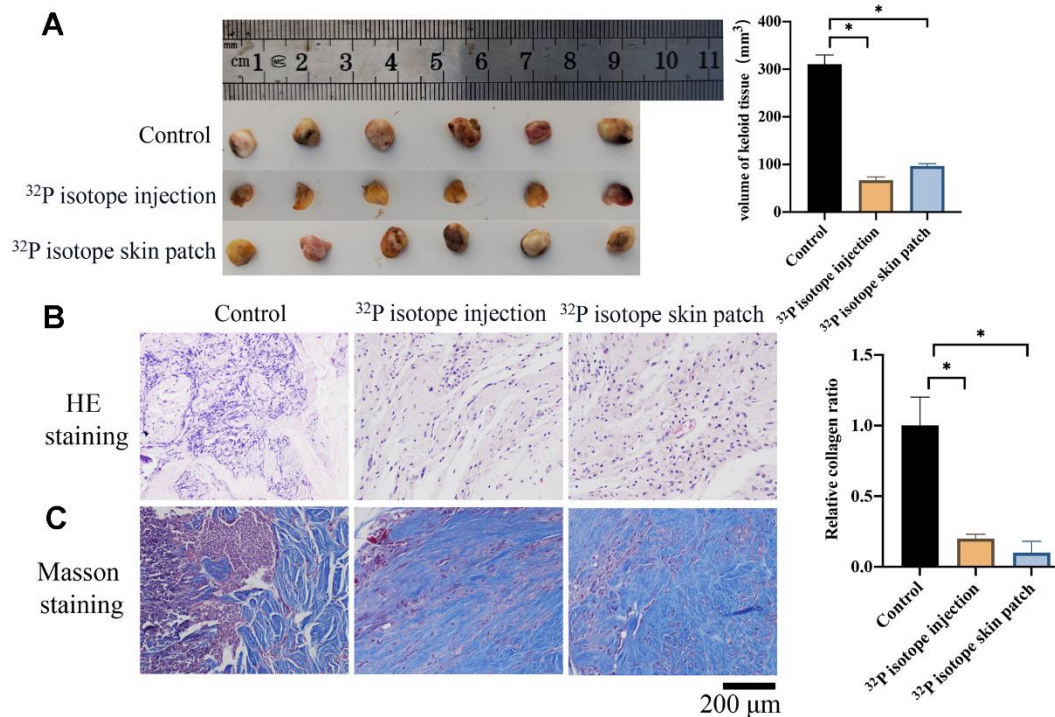HIF-1α and TGF-β/Smad signaling pathway are closely linked with the formation of keloid. Coll1α1 and

Figure 1. Both ^{32}P isotope injection and skin path significantly reduce the size of keloid. (**A**) ^{32}P isotope injection and skin path significantly reduced the size of keloid (n=6); (**B**) HE staining was performed to investigate the histological changes (n=3); (**C**) Masson's trichrome staining was performed to investigate the collagen deposition (n=3). * indicates p <0.05.

Coll3α1 are representative markers of collagen deposition. We found that Both ^{32}P isotope injection and skin path treatments could markedly inhibit the protein and mRNA expression of HIF-1α, TGF-β, p-Smad2/3/Smad2/3 (Figure 2A, 2B), Coll1α1, and Coll3α1 (Figure 2A, 2C). Therefore, ^{32}P isotope treatment might inhibit keloid through targeting TGF-β/Smad signaling pathway.

Both ^{32}P isotope injection and skin path significantly promoted cell apoptosis and regulate cell cycle

To investigate if ^{32}P isotope treatment could affect keloid through targeting TGF-β/Smad signaling pathway. The agonist of TGF-β/Smad signaling pathway, SRI-011381, was used. We found that the ^{32}P isotope greatly increased cell apoptosis, inhibited cell
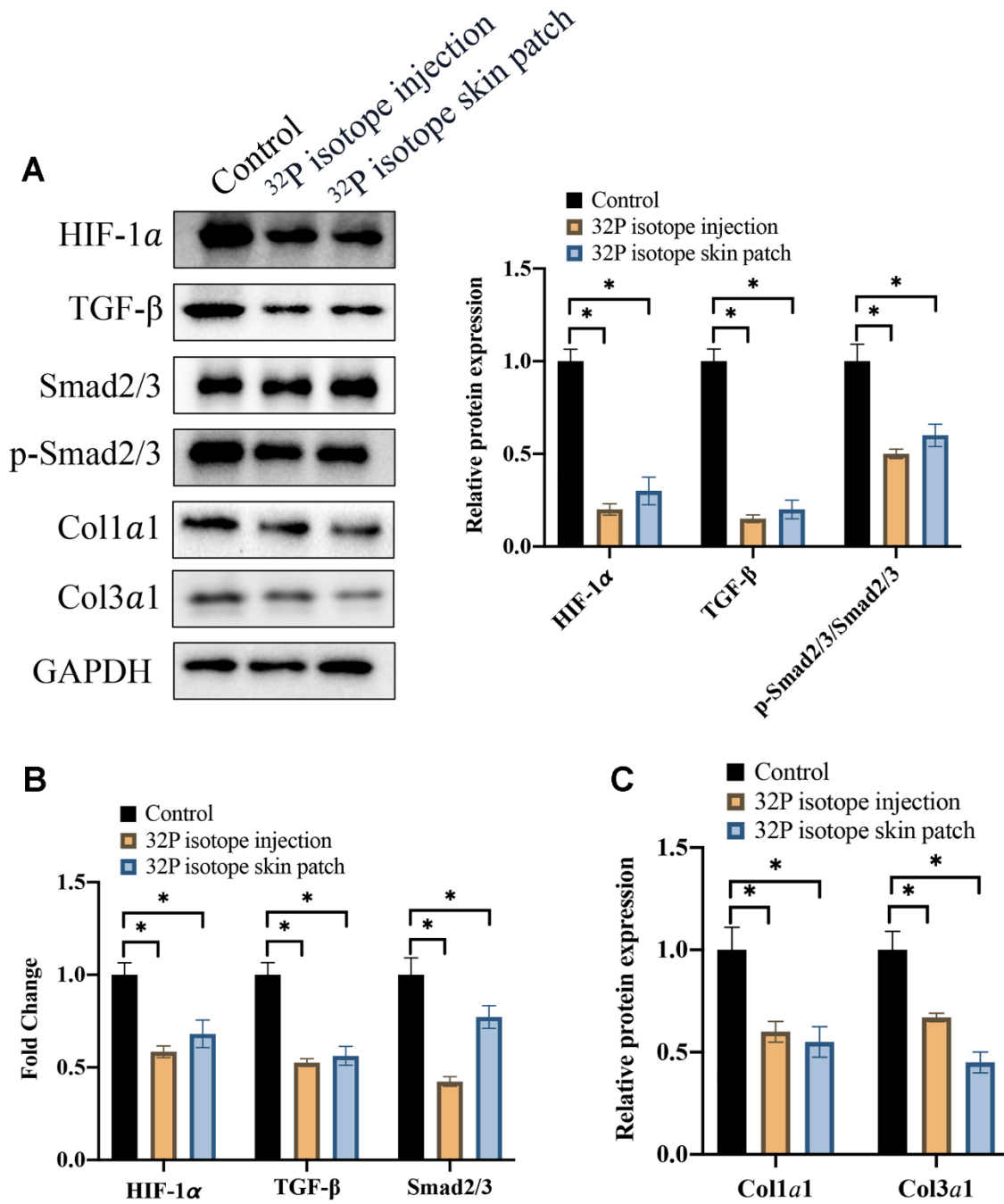

Figure 2. Both ^{32}P isotope injection and skin path significantly inhibited TGF-β/Smad signaling pathway. (**A**) The protein expression of TGF-β/Smad signaling pathway was measured with western blotting (n=3); (**B**) The mRNA levels of HIF-1α and TGF-β/Smad signaling pathway were analyzed (n=3); (**C**) The protein levels of Coll1α1 and Coll3α1 were analyzed (n=3). * indicates p <0.05.

proliferation, arrested cells in the S stage while reduced the cell percentage in the G2 stage (Figure 3A–3C). However, simultaneous treatment with SRI-011381 significantly reversed the effects of ^{32}P isotope, leading to a decreased cell apoptosis, increased cell proliferation and cell percentage in the G2 stage (Figure 3A–3C). We demonstrated that activation of TGF-β/Smad signaling pathway *in vitro* could remarkably reverse the influence of ^{32}P isotope.

C16PAF markedly reversed the influence of ^{32}P isotope on cell apoptosis of LNCaP cells and TGF-β/Smad signaling pathway

In addition, the influence of ^{32}P isotope treatment and SRI-011381 on the protein and mRNA expression of HIF-1α, TGF-β/Smad2/3, Coll1α1, and Coll3α1 were also measured. We demonstrated that the inhibitions of HIF-1α, TGF-β, p-Smad2/3/Smad2/3 by ^{32}P isotope were greatly reversed after SRI-011381 treatment (Figure 4A–4C).

DISCUSSION

The prevalence of keloid in the population is about 4.5-16%, often accompanied by uncomfortable symptoms

[15]. ^{32}P radionuclide brachytherapy has a good aesthetic treatment effect on scars, with low recurrence rate, low complications, high patient satisfaction and comfort, which can greatly alleviate the physical and mental injuries caused by the disease [16]. It is an effective treatment method that can be widely used in clinical practice, and has a good market prospect.

In the process of skin healing, fibroblasts proliferate excessively and secrete a large amount of extracellular matrix, and cell apoptosis is delayed and degradation is insufficient, and local microvascular formation is increased, many factors promote its gradual formation of keloid [17, 18]. ^{32}P released β-rays can directly or indirectly ionize and destroy DNA molecular structure, inhibit cell division and proliferation, inhibit fibroblast migration, proliferation and collagen synthesis and secretion, weaken immune cell function and reduce dysfunctional blood vessels, strongly inhibit inflammatory reaction, and can also effectively induce cell apoptosis and reduce TGF-β [19], Reduce the synthesis of extracellular matrix and collagen fiber, thus inhibiting the formation of keloid. Other kinds of radiotherapies treating keloid have been reported. X-ray, cobalt-60, and linear accelerator have been reported to treat keloid [20]. Meanwhile, brachytherapy may
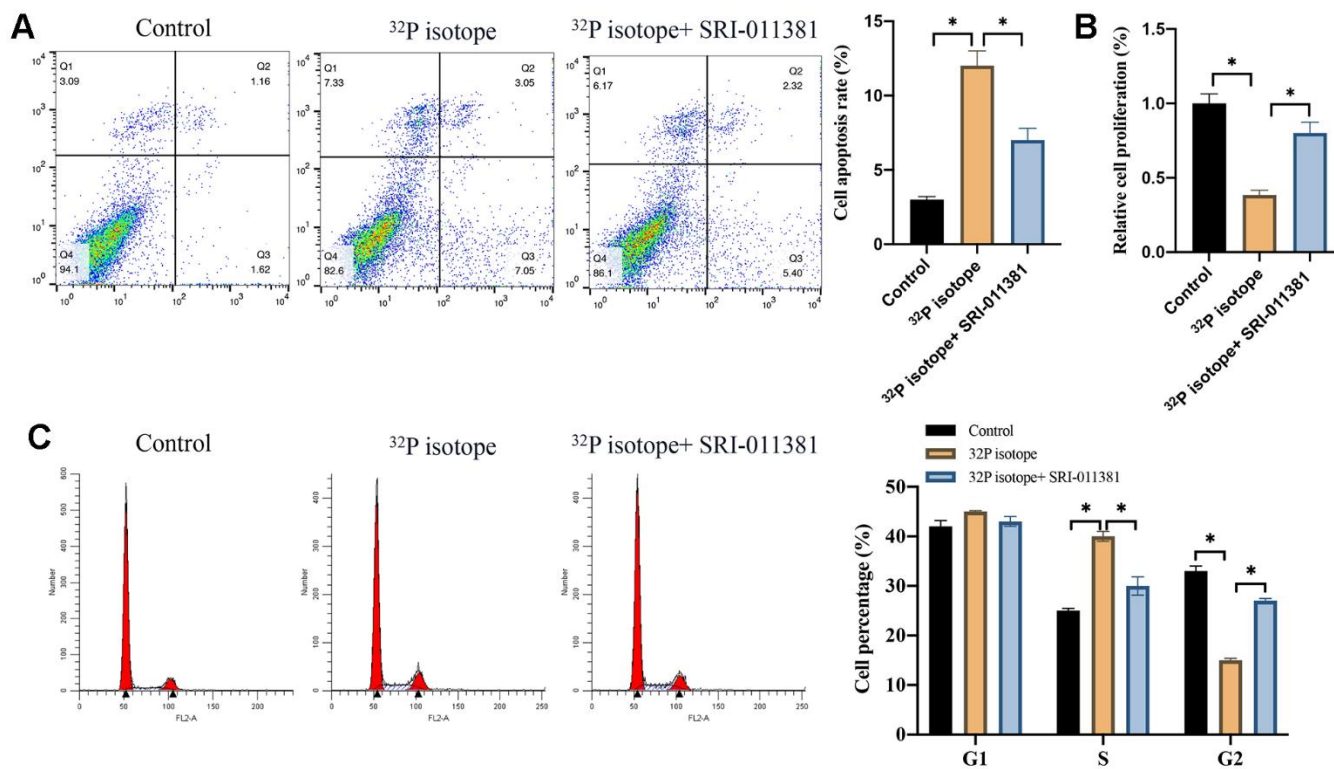

Figure 3. Both ^{32}P isotope injection and skin path significantly promoted cell apoptosis and regulated cell cycle. (**A**) The cell apoptosis was measured with flow cytometry (n=3); (**B**) The cell proliferation ability was detected with CCK8 assay (n=3); (**C**) The cell cycle change was measured with flow cytometry (n=3). * indicates p <0.05.

significantly reduce the recurrence of keloid compared with X-ray or electron beam [21]. The overall complication rate of radiotherapy for keloids was 19% including acute complications (erythema, wound dehiscence, infection, and peeling) and chronic complications (skin color changes, telangiectasia, and fibrosis) [22]. In this research, the complications described above were not observed.

During the normal wound healing process, TGF-β, a multifunctional cytokine, plays a pivotal role in promoting tissue repair and ECM synthesis. However, in keloid formation, the TGF-β signaling pathway becomes dysregulated, leading to an excessive and prolonged activation of downstream Smad proteins [23]. The dysregulation of the TGF-β/Smad signaling pathway in keloid formation leads to the abnormal and
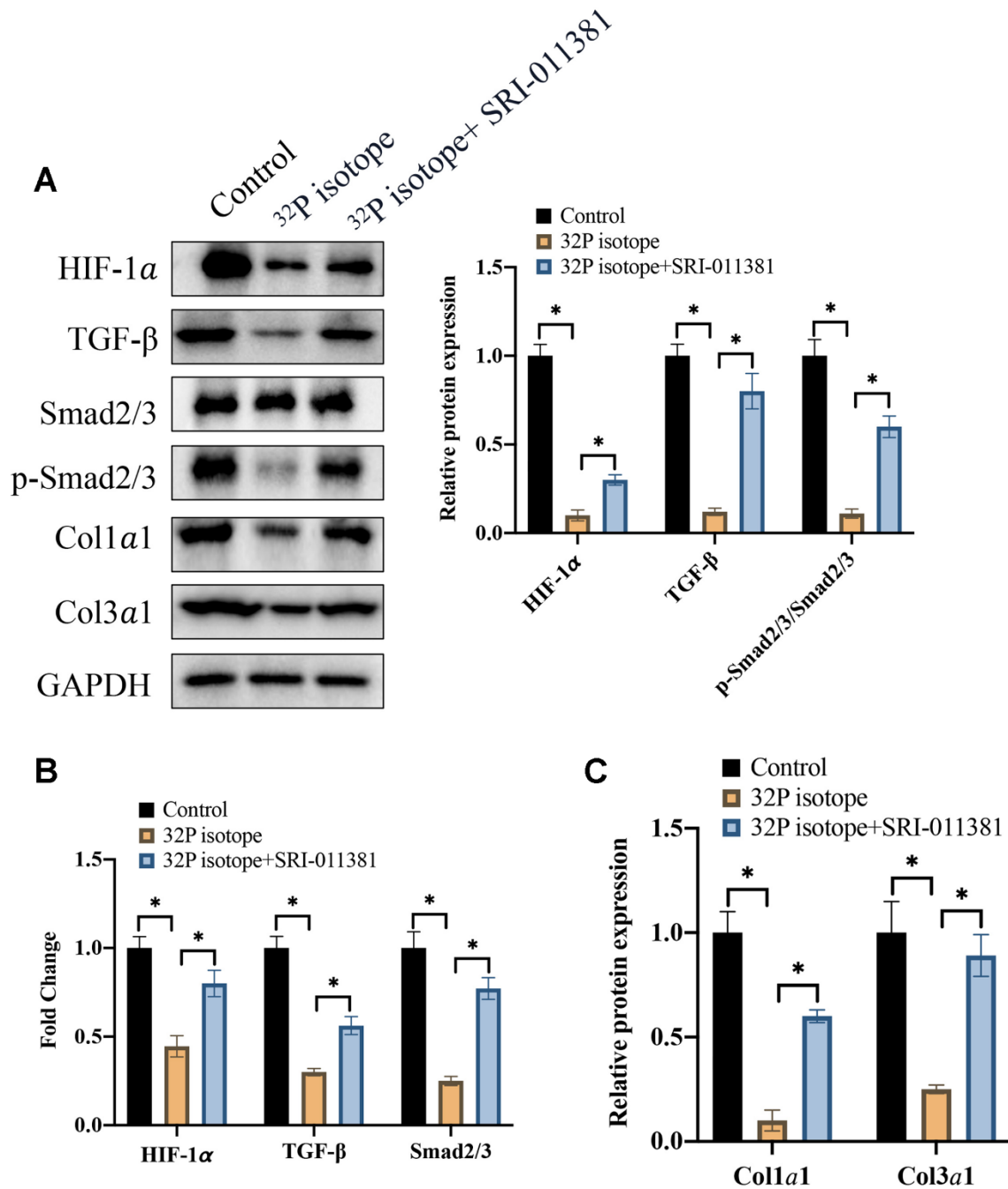

Figure 4. C16PAF markedly reversed the influence of ^{32}P isotope on cell apoptosis of LNCaP cells and EMT process. (A) The protein expression of TGF-β/Smad signaling pathway was measured with western blotting (n=3); (B) The mRNA levels of HIF-1α and TGF-β/Smad signaling pathway were analyzed (n=3); (C) The protein levels of Coll1α1 and Coll3α1 were analyzed (n=3). * indicates p <0.05.

excessive deposition of ECM proteins, resulting in the development of fibrotic scars [24]. Understanding the molecular mechanisms underlying this process may pave the way for the development of targeted therapies aimed at modulating TGF-β/Smad signaling to mitigate keloid formation and improve patient outcomes [25]. In the present study, we found that Both ^{32}P isotope injection and skin path significantly inhibited TGF-β/Smad signaling pathway. Meanwhile, the regulation of dermal fibroblast by ^{32}P isotope was reversed by the agonist of TGF-β/Smad signaling pathway, SRI-011381. These findings indicate that ^{32}P isotope might inhibit keloid through suppressing TGF-β/Smad signaling pathway.

However, our research lacks further investigation about the function mechanism of ^{32}P isotope in inhibiting keloid. In recent years, studies have found that genes related to tumor growth in Keloid lesions may be abnormal. MicroRNAs (miRNAs) expression disorder may promote the development of keloid [26]. MiRNAs and their target genes and corresponding molecular signaling pathways together build a complex and fine network, which jointly regulate the formation of keloid [27]. MiRNA plays an important role in regulating inflammation, cancer, malignant skin diseases, wound healing, fibrotic diseases, and angiogenesis physiology, and also affects TGF- β [28]. However, if ^{32}P isotope radiotherapy could regulate keloid through miRNA needs to be further explored. The abnormal expression of many genes such as epithelial mesenchymal transformation (EMT) also participates in the occurrence and development of keloid [29]. If ^{32}P isotope radiotherapy could affect the EMT process remains unclear.

CONCLUSIONS

Both ^{32}P isotope injection and skin path significantly reduced the size of keloid, and the TGF-β/Smad signaling pathway was remarkably inhibited by ^{32}P isotope treatment. The regulation of dermal fibroblast by ^{32}P isotope was reversed by the agonist of TGF-β/Smad signaling pathway, SRI-011381. [32]P isotope might inhibit keloid through suppressing TGF-β/Smad signaling pathway.

MATERIALS AND METHODS

Keloid animal model establishment

Eighteen healthy male BALB/c nude mice (Charles River, Beijing, China), aged 4–6 weeks, were selected for the experiment. Anesthesia was administered, and an 8-mm incision was made in the scapular area of each nude mouse. Keloid tissues, obtained during surgery, were cut into blocks approximately 4 mm^3 in size and

implanted in the subcutaneous space of the nude mice. The skin was then sutured to secure the implants. After 21 days, the mice were randomly divided into three groups: control, ^{32}P isotope injection, and ^{32}P isotope skin patch. The mice in the group ^{32}P isotope skin patch were treated using the customized ^{32}P isotope therapy patch from China Atomic Energy Research Institute, with an effective treatment area of 1.0 cm × 1.0 cm, with a radiation dose of 1.08 cGy/s·cm^2. 6 layers of tin foil were used to protect the non-irradiated skin, and ^{32}P isotope therapy was applied to the surface of the skin for 150 s each time. The treatment frequency is once every 2 days, with a total of 10 treatments. The gross morphology and histopathology of keloids were observed after 21 days of continuous injection. The dosage of ^{32}P isotope was determined based on our pre-experiment and previous report [9].

Cell culture

Primary dermal fibroblast cells (#PCS-201-012, ATCC, Manassas, VA, USA) were used in this research. The cells were cultured in DMEM medium (Gibco, #12491015, Langley, OK, USA) supplemented with 5% FBS, 40 μg/ml streptomycin, and 40 IU penicillin at 5% CO_2 and 37° C. The concentration for SRI-011381 in the cell incubation was 10 μM.

Hematoxylin-eosin (HE) staining

After sacrificing animals, the keloid was isolated, and the tissues were embedded in paraffin after dehydration. Sections of 5 μm were cut and dewaxed. The tissues were then stained with hematoxylin solution (#G1140, Solarbio, Beijing, China) for 5 minutes, washed with water until no staining solution flowed out, and incubated with PBS for 5 minutes to return to blue. Subsequently, the sections were stained with eosin (#MB9898-3, Meilunbio, Dalian, China) for 15 seconds. After decolorization with 95% ethanol for 10 seconds, the tissues were washed with water. Transparency was achieved by immersing the tissues in xylene for 10 minutes, followed by sealing with neutral gum (#MB9899, Meilunbio).

Masson staining

Masson trichrome staining was performed as follows: The tissues were stained with hematoxylin for 10 minutes, differentiated with ethanol, incubated with Masson blue solution, and then subjected to Ponceau acid fuchsin staining for 10 minutes. After washing with phosphomolybdic acid solution for 2 minutes, aniline blue staining was conducted for 2 minutes. The tissues were then dehydrated with 95% ethanol three times for 50 seconds each, followed by incubation with xylene

three times for 1 minute each. Finally, the tissues were sealed with neutral gum, and an inverted optical microscope was used to observe the stained tissues. The calculation of Masson staining was performed using Image J software.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from tissues using Trizol (#R0016, Beyotime, Shanghai, China), and its purity was measured with the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The Takara PrimeScript RT reagent kit with gDNA eraser kit (#RR047A) was used for reverse transcription. RT-PCR was performed with Bio-Rad (CXF96) (Hercules, CA, USA). The relative expression level of the gene was determined using the $2^{-\Delta\Delta CT}$ method. The primer sequences are provided in the following: HIF-1α (Forward 5′- CAGAAGATACAAGTAGCCTC-3′, Reverse 5′-CTGCTGGAATACTGTAACTG-3′), TGF-β (Forward 5′-GCAACAATTCCTGGCGATACCTC-3′, Reverse 5′-AGTTCTTCTCCGTGGAGCTGAAG-3′), Smad2/3 TGF-β (Forward 5′-ATGAGCACCCAGATCCAGTG-3′, Reverse 5′-CTGTGGGCTTCCTTGTTGTC-3′).

Western blotting

Proteins were lysed using RIPA lysis buffer (Sigma-Aldrich, R0278, St. Louis, MO, USA) containing a protein phosphatase inhibitor. The protein concentration was determined using the BCA method (#A045-4-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and equal amounts of protein from each group were subjected to 10% SDS-PAGE. The proteins were then transferred to a PVDF membrane (Millipore, GVWP02500, Burlington, MA, USA). The membrane was blocked with 5% non-fat milk in TBST (Beyotime, #P0222) for 2 hours. Next, the membrane was incubated with primary antibodies overnight at 4° C and with secondary antibodies for 2 hours at room temperature. Finally, an enhanced chemiluminescence detection kit (Thermo Fisher Scientific) was used to detect the target genes, and ImageJ software was utilized to analyze the bands. The antibodies used in the study are listed below: HIF-1α (ab92498, Abcam, Cambridge, UK), TGF-β (ab215715, Abcam), Smad2/3 (ab232326, Abcam), p-Smad2/3 (phospho T8, ab272332, Abcam), Col1α1 (ab270946, Abcam), Col3α1 (M50852, Zenbio, Durham, NC, USA), GAPDH (ab9485, Abcam).

Flow cytometry

Cells in the logarithmic growth phase were washed three times with PBS (#10010023, Gibco), trypsinized (#108444, Sigma-Aldrich), suspended in serum-free medium, and adjusted to a cell concentration of 1×10^7/ml. A 100 μL cell suspension was added to a 1.5 mL microcentrifuge tube, followed by addition of 100 μL Annexin V (Beyotime, #C1062L) and PI. The cells were then incubated at room temperature in the dark for 20 minutes and analyzed using the Guava® Muse® Cell Analyzer.

Statistical analysis

SPSS 24.0 statistical software was used for analysis, with econometric data expressed as mean ± standard deviation (x ± s). t-tests and analysis of variance were used for inter group differences analysis; count data comparison was done using χ 2 Inspection. Correlation analysis was done using Spearman correlation coefficient test. The results showed a statistically significant difference with $P<0.05$.

Availability of data and material

The data and material used to support the findings of this study are included within the manuscript and supplementary files.

AUTHOR CONTRIBUTIONS

YS conceived and designed the experiments; LX, LH, and GZ performed the experiments; YS and LX wrote the paper.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL STATEMENT

The animal experimental protocol was approved by Fujian Medicine University (2023-377).

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