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ARTICLE

A comparison of proliferation levels in normal skin, physiological scar and keloid tissue

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ABSTRACT

Proliferation is an important characteristic of life, and many signaling pathways participate in this complicated process. The MAPK/Erk pathway is a classic pathway in cell proliferation. In this study, expression levels of key factors in the MAPK/Erk pathway were measured to assess the proliferation level among normal skin, physiological scar, and keloid tissue. Thirty patients were selected randomly from the Department of Plastic Surgery at Peking Union Medical College Hospital from January 2019 to December 2020. Histological appearance and fiber tissue content were observed by Hematoxylin and eosin staining and Masson staining. Expression levels of key factors in the MAPK/Erk pathway (ATF2, c-Jun, c-Myc, p38 and STAT1) and relative proteins (HIF-1α and PCNA) in tissues were detected by immunohistochemistry and analyzed as the percentage of positively stained cells in both the tissue epidermis and dermis. Western blot was used for quantitative analysis of the above factors. In results, keloid tissue showed a significantly higher fiber and less cell content. In the immunohistochemical result, higher expression of key factors was observed in the epidermis than in the dermal layer, and the expression of all factors was increased remarkably in keloid tissue. In western blot analysis, all factors (except STAT1) showed higher expression in keloid tissue. In our former research, keloid showed similar apoptosis level as physiological scar and normal skin. On combining our former conclusion and results in this study, an imbalance condition between the high proliferation level and normal apoptosis level may lead to the growth characteristics of keloid.

Abbreviations: MAPK: mitogen-activated protein kinase; Erk: extracellular regulated protein kinases; ATF2: activating transcription factor 2; STAT1: signal transducer and activator of transcription 1; HIF-1α: hypoxia-inducible factor 1-alpha; PCNA: proliferating cell nuclear antigen

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Introduction

Scars, whose main content is fibrous tissue, are a natural part of the physiological wound healing process. Keloid is usually considered as the result of abnormal wound healing, and it presents with excessive scar tissue beyond the area of original skin injury and is accompanied by discomfort due to severe itchiness, pain, and functional limitations [1,2].

Proliferation is an essential part of cell life, and many signaling pathways participate in this process. The MAPK/Erk signaling pathway is a classic pathway that plays an important role in cell proliferation and differentiation management, in which factors, such as ATF2, c-Jun, c-Myc, p38, and STAT1, are the key factors in regulating the process [3,4].

Keloid scars are benign with continuous growth characteristics, and a high proliferation level may be the main cause for the growth. In previous research, keloid showed a higher proliferation level, but few studies had focused on the MAPK/Erk pathway in keloid tissue and compared the expression of key factors with those in normal skin and physiological scar. In addition, our former research evaluated the apoptosis level among keloid tissue, physiological scar, and normal skin, and it concluded that they have a similar apoptosis level. Higher proliferation was also found in our previous study, but there was a lack of systematic assessment of the classic pathway of proliferation. In this study, we focused on the key factors of the MAPK/Erk pathway (ATF2, c-Jun, c-Myc, p38 and STAT1) and relative key proteins (HIF-1α and PCNA) in the proliferation process in the above three different types of skin tissue. Combined with our previous conclusion, the growth characteristics of a keloid can be further explained.

Materials and methods

Patients, grouping and sample management

This study protocol was reviewed and approved by the Bioethical Committee of Peking Union Medical College Hospital. An informed consent form was signed by all patients. Thirty patients

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(10 patients with no obvious scars, 10 patients with physiological scars, and 10 patients with keloid) participated in this research and were selected randomly from the Department of Plastic Surgery in Peking Union Medical College Hospital from January 2019 to December 2020. The gender ratio was 1:1 (5 females and 5 males in each patient group). Samples were divided into normal skin (S), physiological scar (C), and keloid tissue (K). Patients' age ranged from 20 to 56 years (S: 35.30 ± 8.08 years; C: 37.90 ± 8.52 years; K: 38.70 ± 10.51 years). Keloid and physiological scar were caused by trauma and diagnosed by pathological examination. None of the patients had any systemic diseases or were receiving drug treatment that might affect the results. No significant differences were found between each group in patients' age, gender, and scar site.

According to our previous methods of managing samples, the central part of the keloid, physiological scar, and normal skin tissue was obtained for study. Fat tissue and hair were removed before samples were washed in phosphate-buffered saline (PBS) and blood was washed off. Samples were divided into two parts for further experiment. Samples for staining were placed in 10% formalin solution for 48 h and embedded in paraffin. Other fresh samples were frozen in liquid nitrogen for protein extraction and quantitative analysis of expression.

H&E and Masson staining

The histological appearance of the three groups was observed by H&E and Masson staining. All protocols were basically designed according to our previous study [5]. All specimens were sectioned and mounted on slides after paraffin embedding. A total of 90 slides were stained for observation. In H&E staining, slides were dewaxed at 70 °C for 30 min and washed in xylene twice for 15 min. Then, slides were rehydrated in ethanol gradient (100%, 95%, 85%, 75%, and deionized water, 3 min each). After 5 min of hematoxylin staining and 10 s of 1% hydrochloric acid, running water was used for washing slides for 15 min. Eosin staining lasted for 1 min of counterstain. The backward concentration of the alcohol gradient was used for dehydration. Then slides were washed by xylene and mounted. In H&E staining, red color referred to the cytoplasm and other components, while blue color referred to the nuclei.

Fiber content was observed by Masson staining. A staining kit (Heart Biological Technology, Xian, China) was used, and all the staining procedures were based on the kit instructions. In Masson staining, red color indicated cells, and blue color indicated fibrils.

Immunohistochemical observation

The immunohistochemical staining procedures were conducted according to our previous study [5]. In immunohistochemical analysis, 3 slides of each factor from each sample were stained. All slides were dewaxed and rehydrated routinely and incubated in 3% H_2O_2 for 10 min. After 95 °C and 15 min of antigen retrieval in citrate buffer, slides were incubated with normal goat serum at 37 °C for 30 min. Antibody incubation were performed at 4 °C overnight (12–16 h) with anti-ATF2 (1:100, Abcam, Cambridge, United Kingdom), anti-c-Jun (1:100, Abcam), anti-c-Myc (1:100, Abcam), anti-p38 (1:100, Abcam), anti-STAT1 (1:100, Abcam), anti-HIF-1α (1:100, Abcam), and anti-PCNA (1:100, Abcam). After PBS washing 3 times (3 min each), a horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO, Beijing, China) was used to mark the primary antibody under 37 °C. Then, slides were stained with 3,3′-diaminobenzidine (DAB, 1×), hematoxylin (5 min), and 1% hydrochloric acid for 10 s. In immunohistochemical staining, brown staining implied positive expression of the target factor. The percentage of positive cells was also calculated.

Western blot assessment

The protein extraction and western blot protocols were conducted according to our previous study [5]. Tissue or Cell Total Protein Extraction Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used for protein extraction from 50 mg samples. Samples were incubated on ice in cell lysis buffer (246 μl of lysis buffer, 1.25 μl of phosphatase inhibitor, 0.25 μl of a protease inhibitor, and 2.5 μl of PMSF) for 10 min and centrifuged under 4 °C, 14,000 rpm for 15 min. The supernatant protein of 60 μg was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes for about 40–150 min. After 2 h of blocking with 5% blocking buffer (Li-cor, Lincoln, NE, USA), the membranes were incubated with anti-ATF2 (1:500, Abcam, Cambridge, United Kingdom), anti-c-Jun (1:500, Abcam), anti-c-Myc (1:500, Abcam), anti-p38 (1:500, Abcam), anti-STAT1 (1:500, Abcam), anti-HIF-1α (1:500, Abcam), and anti-PCNA (1:500, Abcam) at 4 °C overnight (12–16 h) on a shaker. The membranes were then incubated with the secondary antibody (1:1000, Li-cor) for 1 h after being washed by TBST (TBS buffer + Tween 20, three times, 5 min each), and then they were flushed with TBST (three times, 5 min each) and TBS (once, 5 min). Double-color infrared laser imaging system (Odyssey, Li-cor) was used for imaging scanning and analysis of membranes.

Statistical analysis

In this study, one-way analysis of variance (ANOVA) followed by least significant difference (LSD) *t*-test for statistical data analysis through SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Data are shown as means ± standard deviation (SD). Statistical significance was set at $p < 0.05$.

Results

Histological observations

H&E and Masson staining are shown in Figure 1. Compared with the physiological scar and normal skin, keloid tissue showed a relatively thicker epidermis and an increased number of disordered arrayed collagen fibrils with less cell content. By contrast, normal skin dermis presented more cellular content and fewer fibrils, which was a loose dermal structure. The physiological scar showed moderate condition among the three different skin tissues.

Immunohistochemical studies

Immunohistochemical staining images are presented in Figure 2. Compared with the C group and S group, factors, such as ATF2, c-Jun, c-Myc, STAT1, p38, and HIF-1α, showed the strongest protein expression intensity in both the epidermis layer and dermis layer of keloid tissue. However, PCNA showed similar expression intensity among all groups in the epidermis layer, but it still showed the highest expression in the keloid dermis.

Furthermore, the percentage of positive cells was calculated and statistically evaluated according to immunohistochemical staining (Figure 3, Table 1). In both the epidermis layer and dermis layer, keloid tissue showed significantly the highest positive

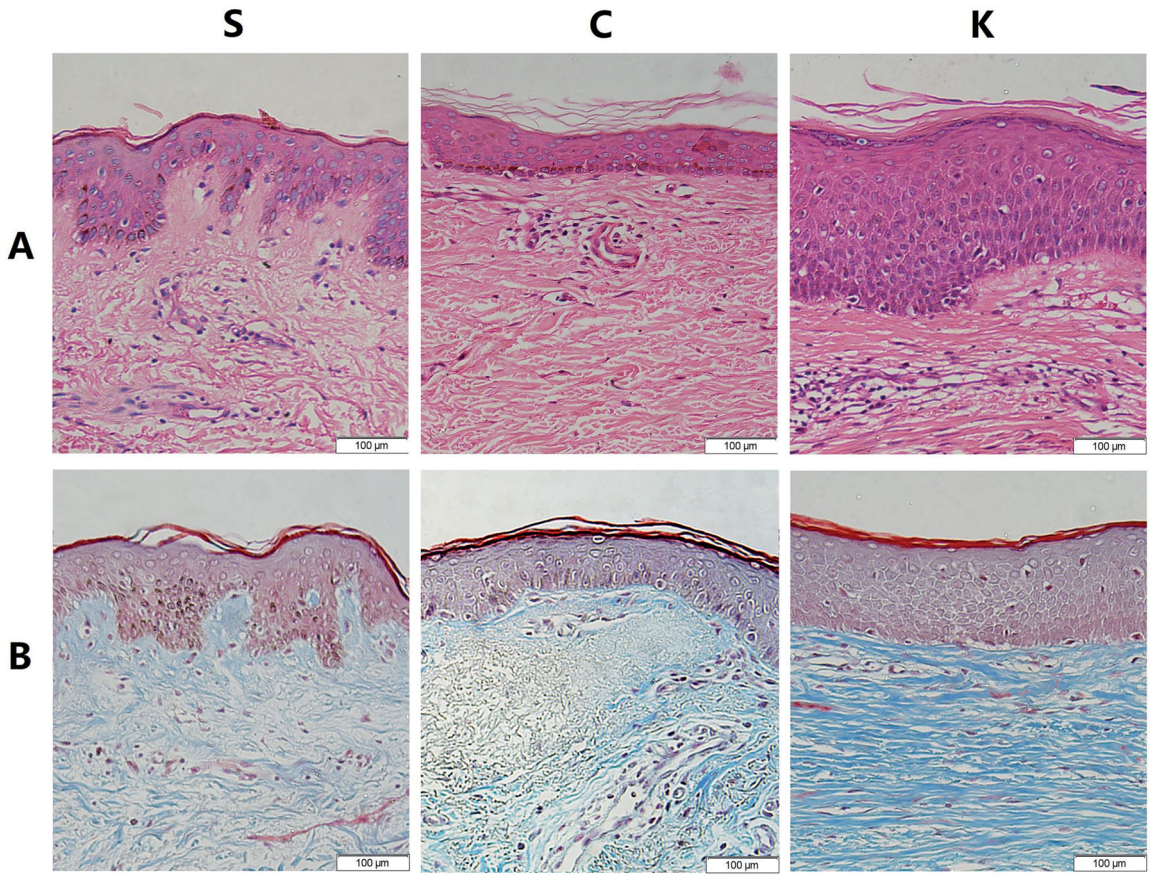

Figure 1. The histological image of H&E staining (A) and of Masson staining (B) in all groups (200×). The epidermal layer was thicker in the K group, while the dermal layer presented disordered arrayed fibrils with less cell content. By contrast, less fibrils and more cell content was observed in the S group. The C group showed the moderate condition compared with the K group and S group.

cell percentage of all factors; however, no significant difference was found between normal skin and physiological scar tissue.

Protein expression of proliferation factors

Western blotting was used for quantitative analysis of the protein expression levels of MAPK/Erk pathway factors and related proliferation proteins HIF-1α and PCNA. In these results, normal skin and physiological scars showed similar expression of all factors. Compared with the non-keloid group, the K group still had higher expression of ATF2, c-Jun, c-Myc, p38, HIF-1α, and PCNA. Interestingly, no significant difference was found among all groups for STAT1 expression (Figure 4, Table 2).

Discussion

Keloids, usually considered to be a result of irregular wound healing, are characterized by red, tickling, and hard tissues, and they tend to outgrow their origin. Although it is benign, its growth characteristics and accompanying symptoms, such as itchiness, pain, functional limitations, and psychological morbidities, can seriously affect keloid patients' quality of life [6–8].

Proliferation and apoptosis are essential processes during cell and organism life, and they are controlled by many signaling pathways. The balance between cell proliferation and apoptosis leads to the life outcome of cells [9,10]. The growth characteristics of keloids imply that keloid tissue may present a higher proliferation level. This conclusion had been presented in many studies [11–13]. However, histological studies usually focused on one or two proliferation factors, while relatively systematic studies about keloid proliferation usually remain at the cytological level. In this case, a systematic evaluation of the proliferation level in keloid tissue may be necessary, especially compared with the other skin tissue types of normal skin and physiological scar. Among many pathways that play roles in the proliferation process, the MAPK/Erk signaling pathway is the most classic pathway [3,14]. In the MAPK/Erk signaling pathway, ATF2, c-Jun, c-Myc, p38, and STAT1 are key factors for cell proliferation [15–17]. Among these key factors, p38 plays an essential role in regulating cellular processes, including inflammation, cell differentiation, cell growth, and death [18]. High expression of p38 indicates the activation of the MAPK/Erk pathway [18]. As the downstream effect factor of p38, increased expression of c-Jun, c-Myc, ATF2, and STAT1 would induce cell proliferation [15,19]. Besides, other relative proteins, such as HIF-1α and PCNA, may also take part in this process. According to Liang's study [20], HIF-1α may play an indirect role in proliferation, which indicates the hypoxia microenvironment of tissue and can be activated by the MAPK/Erk pathway. PCNA expression is significantly upregulated in proliferating tissue, especially tumors [21,22], which has been considered an effective indicator for evaluating proliferation in tissues.

In this study, three different kinds of skin tissue, including normal skin, physiological scar, and keloid, were studied to assess the level of proliferation. H&E and Masson staining were used to observe histological differences among these tissues. In the results, keloid tissue had a thicker epidermis. In addition, more disordered arrayed fibrils and less cell content were observed in keloid dermal layers. In former studies, Marneros also reported

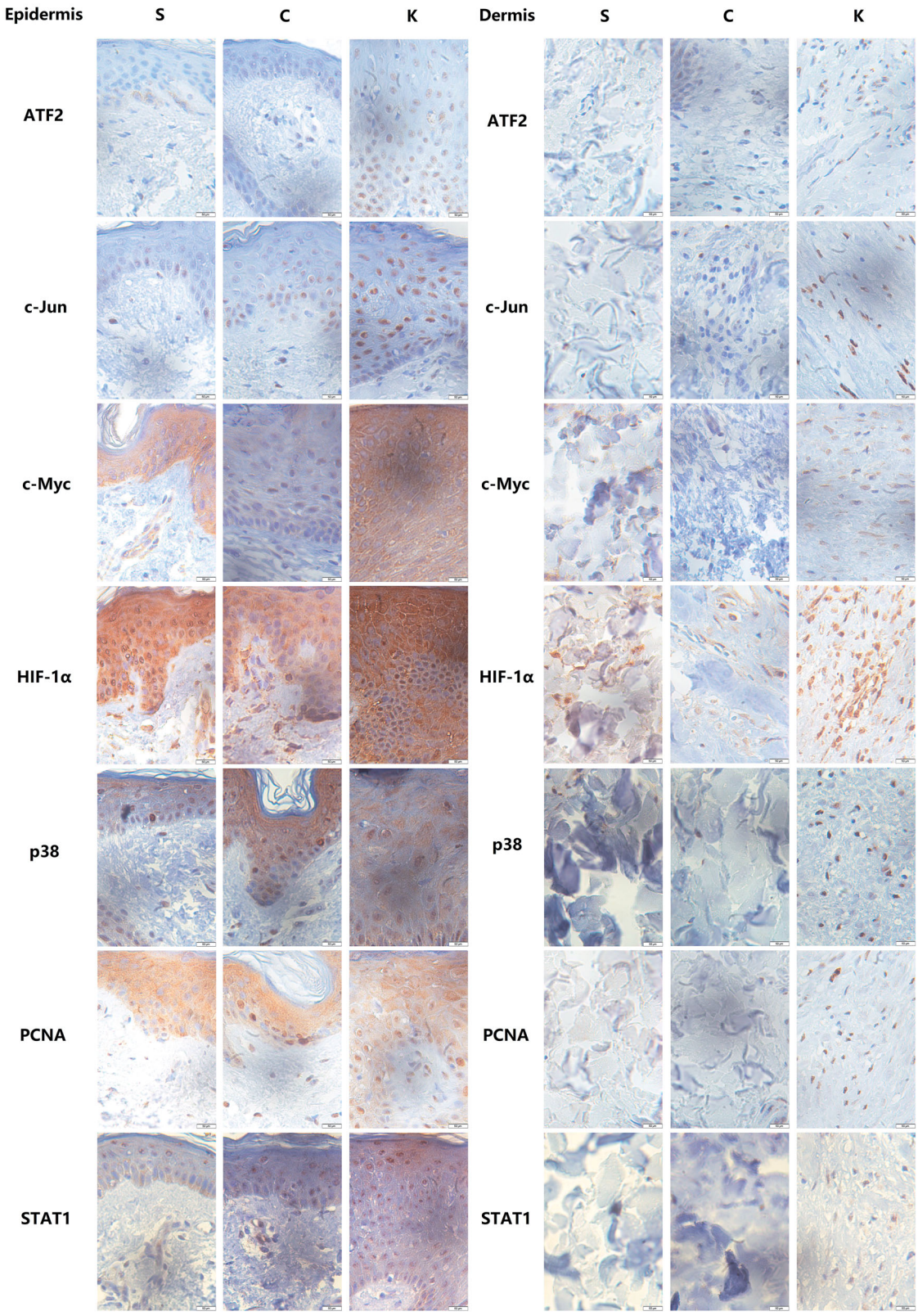

Figure 2. Immunohistochemical micrographs (400×) of epidermal and dermal skin tissue for ATF2, c-Jun, c-Myc, HIF-1α, p38, PCNA, and STAT1 in all groups. Brown staining indicates the positive expression areas of the target protein. In both the epidermal and dermal layers, all factors (except PCNA in the epidermal layer, which showed similar expression level among all groups) showed highest expression in the K group.

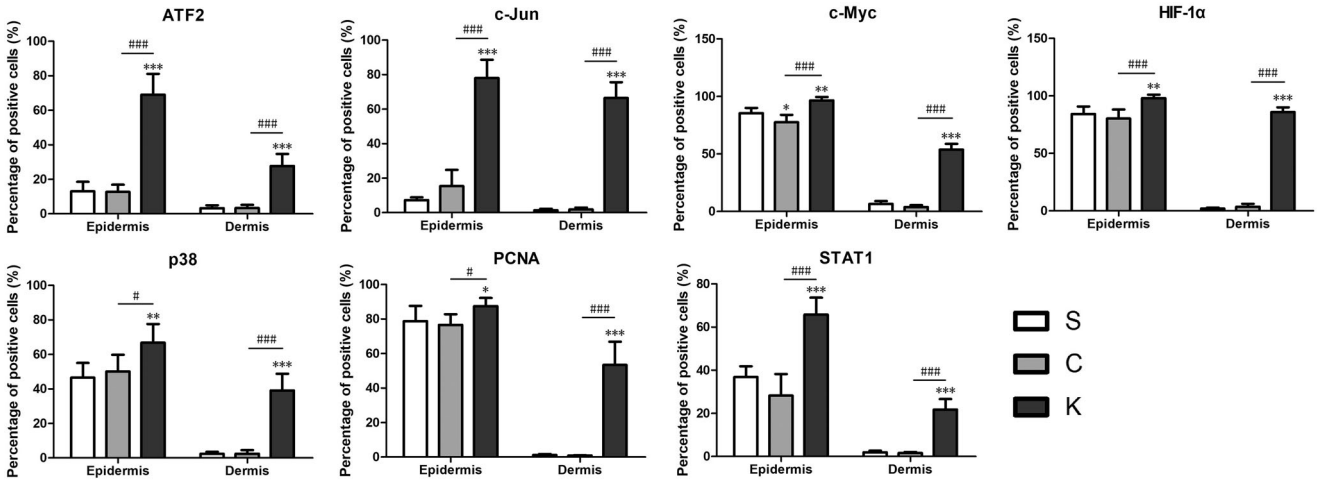

Figure 3. Results of the percentage of protein-positive cells. Either in the epidermal layer or in the dermal layers, all factors showed a significantly higher percentage of protein-positive cells in the keloid tissue. Values are shown as means ± SD; ($n = 10$ in each group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the S group; $\#p < 0.05$, $\#\#\#p < 0.001$ vs. the C group).

Table 1. Percentage of immunohistochemical staining positive cells in each group. Values are means ± SD.

	Percentage of positive cells			*p*-Value		
Factor	S	C	K	K vs. S	K vs. C	S vs. C
ATF2						
Epidermis	13.0433 ± 5.4934	12.6750 ± 4.2217	69.0633 ± 12.0782	<0.001	<0.001	0.938
Dermis	3.3333 ± 1.6275	3.3467 ± 1.8728	27.7383 ± 6.9617	<0.001	<0.001	0.996
c-Jun						
Epidermis	7.2667 ± 1.6433	15.3750 ± 9.4165	78.0750 ± 10.5280	<0.001	<0.001	0.108
Dermis	1.3733 ± 0.9500	1.8517 ± 1.0751	66.4550 ± 9.1643	<0.001	<0.001	0.879
c-Myc						
Epidermis	85.4800 ± 4.5301	77.5383 ± 6.4618	96.5067 ± 3.0995	<0.01	<0.001	0.013
Dermis	6.5533 ± 2.4608	3.7800 ± 1.8270	53.8017 ± 5.0648	<0.001	<0.001	0.180
HIF-1α						
Epidermis	84.2567 ± 6.6110	80.3367 ± 7.8260	98.0233 ± 3.0755	<0.01	<0.001	0.289
Dermis	2.0133 ± 0.9242	3.6717 ± 2.4895	85.8900 ± 4.1402	<0.001	<0.001	0.325
P38						
Epidermis	46.5433 ± 8.4747	50.0850 ± 9.7026	66.7717 ± 10.8670	<0.01	<0.05	0.538
Dermis	2.3383 ± 1.1897	2.4517 ± 2.0773	39.0867 ± 9.6835	<0.001	<0.001	0.973
PCNA						
Epidermis	78.7800 ± 8.8409	76.6517 ± 6.1564	87.4317 ± 4.7478	<0.05	<0.05	0.596
Dermis	1.1783 ± 0.6409	0.9933 ± 0.1226	53.3483 ± 13.5408	<0.001	<0.001	0.968
STAT1						
Epidermis	36.8333 ± 4.9804	28.2600 ± 9.9916	65.7450 ± 7.8784	<0.001	<0.001	0.079
Dermis	1.9517 ± 0.7705	1.6067 ± 0.4987	21.7683 ± 4.8570	<0.001	<0.001	0.837

similar findings [1]. The physiological scar is regarded as a mature scar condition, which presents a moderate state between keloid and normal skin tissue. An analysis of proliferation factors, keloid showed higher expression of all mentioned factors in immunohistochemical staining and percentage of positive cells. Even in protein quantitative evaluation of western blot, expression of all proliferation factors (except STAT1) was increased significantly in keloid tissue. Daian's study also showed that activating p38 and ATF2 may cause a positive effect towards keloid formation [23]. In other studies, c-Jun and c-Myc also played a pivotal role in the proliferation process in cancer and other normal organs [24–26]. Interestingly, STAT1 expression showed no significant difference among the three groups in this study. STAT1 was the first found member of the STATs family. To date, no research has explored STAT1 expression in keloid tissue. According to former studies, loss of STAT1 activation and/or expression was found in malignant cells derived from various histological types of tumors [27], indicating that STAT1 expression may cause a different effect on different tumors. Based on our results, keloids showed similar expression of STAT1 as physiological scar and normal skin; in this case, STAT1 may not play an essential role in the keloid proliferation process. As a result of activation of the MAPK/Erk pathway, high expression of HIF-1α and PCNA could be natural. In addition, despite the increased fiber content and reduced cell content in keloid tissue, the expression of the above proliferation key factors was still higher, indicating that the keloid tissue cells may have more active function than that of other tissues.

In our previous study [5], we investigated the expression levels of key factors in the mitochondrial pathway and compared the apoptosis levels among normal skin, physiological scar, and keloid tissue. Our findings suggested that the apoptosis level in keloid and non-keloid skin tissue was statistically similar, but PCNA expression was remarkably higher in keloid. But PCNA was quite one-sided to evaluate the tissue proliferation level systematically and comprehensively. To compensate for the defect of the former study, we conducted this work. Combining the results of this study and previous study, we could primarily draw a conclusion that keloid may be in a state of imbalance between a high proliferation level and a

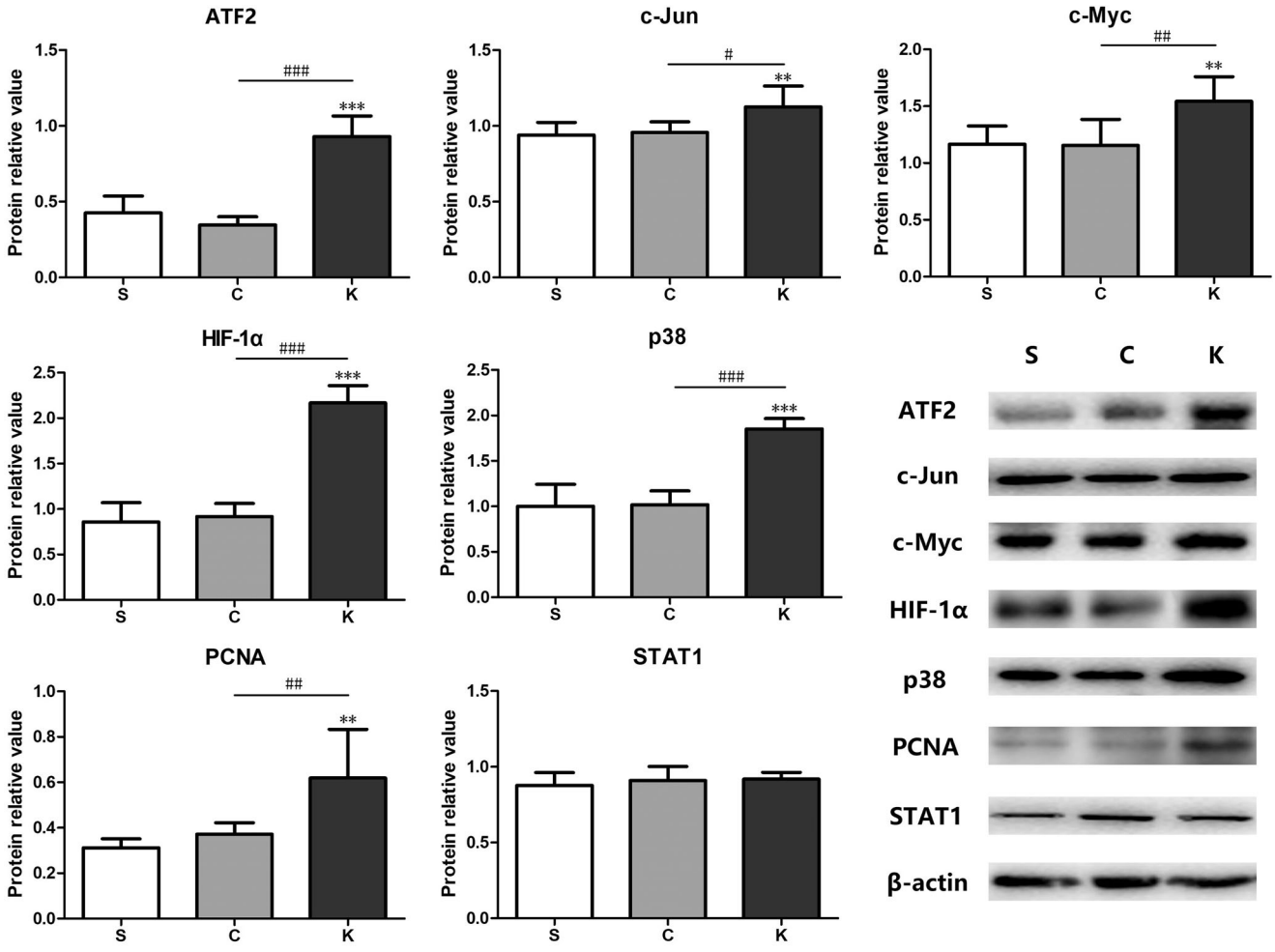

Figure 4. Protein expression amounts for all target factors. Compared with the S group and C group, protein expressions of all target factors (except STAT1) were increased remarkably in the K group. However, no significant difference was found among all groups in the expression of STAT1. Values are shown as means ± SD; ($n = 10$ in each group; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the S group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. the C group).

Table 2. Protein relative value in all groups. Values are means ± SD.

	Protein relative value			*p*-Value		
Factor	S	C	K	K vs. S	K vs. C	S vs. C
ATF2	0.4250 ± 0.1120	0.3461 ± 0.0541	0.9300 ± 0.1358	<0.001	<0.001	0.218
c-Jun	0.9389 ± 0.0847	0.9570 ± 0.0704	1.1258 ± 0.1370	<0.01	<0.05	0.761
c-Myc	1.1662 ± 0.1593	1.1560 ± 0.2279	1.5428 ± 0.2164	<0.01	<0.01	0.932
HIF-1α	0.8597 ± 0.2110	0.9175 ± 0.1439	2.1679 ± 0.1900	<0.001	<0.001	0.594
p38	1.0018 ± 0.2409	1.0181 ± 0.1526	1.8511 ± 0.1136	<0.001	<0.001	0.875
PCNA	0.3122 ± 0.0388	0.3712 ± 0.0508	0.6189 ± 0.2139	<0.01	<0.01	0.441
STAT1	0.8764 ± 0.0856	0.9098 ± 0.0915	0.9183 ± 0.0444	0.359	0.850	0.463

relatively low apoptosis level, which caused continuous growth characteristics. This study was mainly observational; more related pathways could be further investigated to fully evaluate the proliferation and apoptosis levels in keloid tissue.

Disclosure statement

No potential conflict of interest was reported by the authors.

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