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ARTICLE

Association between MeCP2 and Smad7 in the pathogenesis and development of pathological scars

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ABSTRACT

To explore the relationship between methylated binding protein 2 (MeCP2) and mothers against decapentaplegic homolog 7 (Smad7) in the pathogenesis and development of pathological scars. Immunohistochemistry, Western blot and real-time polymerase chain reaction (RT-PCR) were used to detect the expression of MeCP2 in different types of human scars and hypertrophic scars at different growth times. The methylation status of Smad7 gene promoter in different scar tissues was determined by methylation-specific PCR. After transfection with MeCP2-siRNA (small interfering RNA) in human keloid fibroblasts, MTT assay was used to assess the proliferation activity of keloid fibroblasts, while RT-PCR and Western blot assays were used to detect the expression levels of MeCP2, transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), phospho-Smad2 (p-Smad2) and Smad7. MeCP2 was mainly expressed in the nucleus of fibroblasts. The mRNA and protein levels of MeCP2 were significantly higher in keloids than in hypertrophic scars, normal scars and normal skin ($p<.05$). The expression level of MeCP2 in hypertrophic scars during the growth period of <6 months was markedly higher than that of >6 months ($p<.05$). The methylation level of Smad7 was significantly higher in keloids compared to normal skin. After MeCP2 silencing, the proliferation rate of human keloid fibroblasts was decreased, the mRNA and protein levels of Smad7 were increased, and the expression levels of TGF-β1, α-SMA and p-Smad2 were decreased ($p<.05$). MeCP2 and Smad7 play an important role in formation of pathological scars. During keloid formation, MeCP2 weakens the inhibitory effect of Smad7 on p-Smad2/3 by downregulating the expression of Smad7, which in turn promotes fibrosis and scar hyperplasia.

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KEYWORDS
MECP2; Smad7 methylation; keloid; hypertrophic scar

1. Introduction

Pathological scars are one of the most common complications of plastic surgery. It is characterized by local hyperplasia, itching and limited activity, which not only affects the appearance but also affects the organ's function. Previous research has shown that the pathological features of keloids are uncontrolled proliferation of fibroblasts and excessive deposition of extracellular matrix [1]. Transforming growth factor-β1 (TGF-β1) is most closely related to scar formation, and TGF-β/Smad signaling pathway plays an indispensable role in promoting injury repair and pathological scar formation [2]. Studies have shown that there is a negative regulator in TGF-β/Smad signaling pathway, namely, mothers against decapentaplegic homolog 7 (Smad7). In pathological scars, its expression is reduced [3,4]. In the process of renal fibrosis, Smad7 activator can enhance the effect of Smad7 in inhibiting fibrosis [5]. However, why the expression of Smad7 is decreased in keloids still needs to be further explored. Bian et al. [6] reported that the decreased expression of Smad7 in liver fibrosis could be related to the hypermethylation status of Smad7 gene promoter. This provides a new entry point for the association between Smad protein and scar formation.

Methyl-CpG binding protein family inhibits gene expression by combining with hypermethylated promoters, and is involved in the subsequent effects of DNA methylation [7]. As an important member of the methyl-CpG binding protein family, methylated binding protein 2 (MeCP2) is widely considered to be a transcription repressor that silences methylated genes. Under the synergy of histone deacetylase (HDAC), MeCP2 inhibits gene transcription by combining with a hypermethylated gene promoter [8]. Relevant studies have shown that the expression of MeCP2 is increased in the fibroblasts derived from fibrotic tissues [9]. After inhibiting MeCP2 expression, the expression of fibrosis-related inflammatory cytokines is decreased, and the degree of tissue fibrosis is also significantly reduced [10]. In addition, the effect of TGF-β on fibrosis induction is weakened after MeCP2 gene silencing [11].

At present, there are few reports on the expression of MeCP2 in pathological scars. To further explore the role of MeCP2 and Smad7 in keloid formation, we analyzed the expression levels of MeCP2 in different types of human scar tissues and the methylation status of Smad7 gene promoter. The mRNA and protein levels of fibrosis-related cytokines in keloid fibroblasts were also examined after MeCP2 gene silencing.

2. Methods and materials

2.1. Reagents

MeCP2 rabbit monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA). Smad7, TGF-β1, α-smooth muscle actin (α-SMA), phosphorylated Smad2 (p-Smad2) and β-actin rabbit monoclonal antibodies were obtained from Bimake (Houston, TX). Dulbecco's modified eagle medium (DMEM),

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Opti-minimal essential medium (Opti-MEM) and fetal bovine serum were supplied by Gibco (Carlsbad, CA). Lipofectamine 2000 was purchased from Invitrogen (Waltham, MA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody, SP immunohistochemistry kit, DAB color development kit and RNA extraction kit Trizol were purchased from TAKARA (Kyoto, Japan). Reverse transcription reaction system and SYBR Green Real Master Mix were obtained from the Bimake (Houston, TX). The microplate reader was obtained from TECAN (Männedorf, Switzerland), spectrophotometer, reverse transcription and real-time PCR instruments were supplied by Thermo (Waltham, MA), high-speed centrifuge was purchased from Sigma (Munich, Germany) and gel imager was supplied by Vilber Lourmat (Paris, France) were also used in this study.

2.2. Specimen collection

The experimental specimens were all collected from the Department of Plastic and Burn Surgery, the First Affiliated Hospital of Chongqing Medical University. There were 33 normal skin tissue samples and 118 scar tissue samples, which were confirmed by pathological examination. Based on the pathological examination results, the scar tissues were divided into three groups: (i) normal scar group ($n = 32$), (ii) hypertrophic scar group ($n = 51$) and (iii) keloid group ($n = 35$). The morphological characteristics of normal scars were flat, slightly rough, slight pigmentation and no adverse effects on body function, and the scar growth time was typically 1–20 years. Hypertrophic scars were obviously higher than the surrounding normal skin, accompanied by abnormal thickness, irregular shape, a color of red or purple, hard texture and obvious itching symptoms, and the scar growth time was 3 months to 8 years. The clinical manifestations of keloids are that the scar was more obvious than the surface of the normal skin, along with uneven, pink or purplish red, hard texture, obvious pain, burning and itching, and the scar growth time was 1–20 years.

According to the growth time, the hypertrophic scars were divided into four groups: (i) 0–3 months ($n = 10$), where the scar tissue was characterized by swelling, a color of red or purple, and itching; (ii) 3–7 months ($n = 11$), where the scar tissue protruded from the surface of the surrounding skin, and was purplish red, hyperemic and swollen, along with obvious itching and tenderness; (iii) 7–12 months ($n = 13$), where the scar tissue became less swollen, softer in texture and more shallow, but some tissues were still higher than the surface of the surrounding normal skin; and (iv) >12 months ($n = 17$), where the scar was soft, flat, pale in color, and the symptoms such as itching and pain were alleviated.

All specimens have not received any treatment before collection. The protocol for specimen collection was approved by the Ethics Review Committee of the First Affiliated Hospital of Chongqing Medical University. Written informed consent was obtained from all patients who provided the scar tissue specimen.

2.3. Immunohistochemistry

All samples were fixed in formalin for 24 h, paraffin-embedded and sliced, followed by antigen retrieval and antibody incubation according to the immunohistochemistry kit process. In this experiment, sodium citrate was used for antigen retrieval. The acquired immunohistochemical images were scored according to the number of MeCP2-positive cells and the staining intensity of fibroblasts. The number of positive cells was graded in: none, 1–25%, 26–50%, 51–75% and 76–100% and translated to 0, 1, 2, 3 and 4 points, respectively. The intensity of the staining was graded as: negative, weakly positive, positive and strongly positive and translated to 0, 1, 2 and 3 points, respectively. A final score was achieved by multiplying the two separate scores [12]. Each section was scored by two experienced pathologists and performed under double-blind conditions.

2.4. Western blotting

After protein extraction, the concentrations of total protein in the collected tissue samples were quantified. Electrophoresis, primary and secondary antibody incubation, and enhanced chemiluminescence (ECL) development were carried out in order. Grayscale image analysis and statistical analysis were conducted using Image-Pro Plus after ECL development.

2.5. Real-time polymerase chain reaction (PCR)

Total RNA from fibroblasts was isolated by Trizol, cDNA was prepared using All-in-One cDNA Synthesis Super Mix. SYBR Green PCR Master Mix was used for the quantitative detection of mRNA levels. PCR was performed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The cycle threshold (CT value) of the target genes was normalized to that of β-actin, in order to obtain the delta CT ($^{\triangle}$CT) values, $^{\triangle}CT = CT(target) - CT(\beta\text{-actin})$. The ratio of the target gene expression to β-actin expression was calculated using the $2^{(-\triangle CT)}$ formula, i.e. $2^{(-[CT(target) - CT(\beta\text{-actin})])}$. The sequences of primers are listed in Table 1.

2.6. Human keloid fibroblast culture and MeCP2-siRNA intervention experiment

Primary keloid fibroblasts were obtained by culturing fresh human scar tissue. Single cell method was used for culturing keloid fibroblasts. The 2nd or 3rd generation cells were selected, when they were 60% confluent of bottom for the next experiments. Keloid fibroblasts were randomly divided into three groups: (i) blank control group, only add Opti-MEM; (ii) reagent control group, Lipofectamine 2000 working fluid and Opti-MEM were added; and (iii) experimental group, the diluents of FITC-siRNA (small interfering RNA) and Lipofectamine 2000 were added.

Table 1. Primer sequences and SiRNA nucleotide sequences.

Gene name	Primer	Sequence
MECP2	Forward primer	5′-TTCGCTCTAAAGTGGAGTTGATTGC-3′
	Reverse primer	5′-GGAGATTTGGGCTTCTTAGGTGGTT-3′
TGF-β1	Forward primer	5′-CTAATGGTGGAAACCCACAACG-3′
	Reverse primer	5′-TATCGCCAGGAATTGTTGCTG-3′
α-SMA	Forward primer	5′-TGGCCACTGCTGCTTCCTCTTCTT-3′
	Reverse primer	5′-GGGGCCAGCTTCGTCATACTCCT-3′
Smad7	Forward primer	5′-CAGCTCAATTCGGACAACAAGA-3′
	Reverse primer	5′-GTACACCCACACACCATCCAC-3′
P-Smad2	Forward primer	5′-TCCACCAGGCTGTAATCTGAAGA-3′
	Reverse primer	5′-GACATGCTTGAGCAACTGACT-3′
β-actin	Forward primer	5′-TGGAATCCTGTGGCATCCATGAAAC-3′
	Reverse primer	5′-ACGCAGCTCCAGTAACAGTCCG-3′
MeCP2-SiRNA	Sense	5′-GCUUAAGCAAAGGAAAUCUTT-3′
	Antisense	5′-AGAUUUCCUUUGCUUAAGCTT-3′
Control SiRNA	Sense	5′-UUCUUCGAACGUGUCACGUTT-3′
	Antisense	5′-ACGUGACACGUUCGGAGAATT-3′
Smad7 methylated	Forward	5′-GTAGTCGGAGCGTAGGTTTTC-3′
	Reverse	5′-GGTAGTTGGAGTGTAGGTTTTT-3′
Smad7 unmethylated	Forward	5′-TTGTGAGAGTTTTTTTATTTTGTTAGAT-3′
	Reverse	5′-CTCTATATCCTTAGTAACCAAATCTCCT-3′

2.7. MTT assay

The pretreated cells were selected and divided into experimental group, control group and blank control group. The siRNA concentrations in the experimental group were 20, 40 and 60 μM, and the cells in the 60 μM group were collected at 12, 24, 48 and 72 h for determination. MTT solution and dimethyl sulphoxide were added, and the absorbance value of each well was measured at OD 490 nm. Each experiment was repeated at least three times. The measured OD value was plotted, with time as the abscissa and cell proliferation as the ordinate. Proliferation rate=(OD value of the experimental group – zero setting group)/(OD value of the control group – zero setting group)×100%, inhibition rate = 1 – proliferation rate.

2.8. Methylation-specific polymerase chain reaction (MSP)

The methylation levels of Smad7 gene promoters in both scar and skin tissues were determined by MSP assay. PCR with bisulfite-modified DNA was carried out under the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s, with a final extension at 60 °C for 30 min. The methylated (M) and unmethylated (U) PCR products were detected by gel electrophoresis.

2.9. Statistical analysis

According to the grouping conditions, the data obtained from the experiment were statistically analyzed by SPSS software version 22.0 (SPSS Inc., Chicago, IL). The experimental results were normally distributed or approximately normally distributed, and all data were expressed as mean ± standard deviation (SD). Comparison between two independent samples was performed using *T* test. One-way ANOVA was used to test the differences among multiple groups. *p* Values of <.05 indicated that the differences were statistically significant.

3. Results

3.1. Expression of MeCP2 in scar and skin fibroblasts

3.1.1. The expression of MeCP2 protein in different scars and skin fibroblasts

Immunohistochemical results showed that MeCP2 was mainly expressed in the nucleus of fibroblasts. Its expression levels in normal skin, normal scar, hypertrophic scar and keloid were gradually increased (Figure 1). Specifically, the expression of MeCP2 was negative or weakly positive in normal skin (Figure 1(A)) ($n = 33$), weakly positive in normal scar with a lesser number of positive cells (Figure 1(B)) ($n = 32$), positive in hypertrophic scar (Figure 1(C)) ($n = 34$) and strongly positive in keloid with a greater number of positive cells (Figure 1(D)) ($n = 35$). The differences between the four groups were analyzed by one-way ANOVA, and a statistically significant finding was observed ($F = 38.52$, $p < .001$).

3.1.2. Quantitative analysis of MeCP2 protein and mRNA in different scars and skin fibroblasts

Western blot analysis and real-time PCR analysis indicated that the protein and mRNA expression of MeCP2 was gradually increased in normal skin, normal scar, hypertrophic scar and keloid (Figure 2, Table 2). This shows that the expression of MeCP2 is closely related to the degree of scar hyperplasia.

3.2. Mecp2 affects the development of hypertrophic scars

3.2.1. Expression of MeCP2 protein in hypertrophic scars at different growth stages

Immunohistochemistry showed that the expression levels of MeCP2 decreased with the prolongation of hypertrophic scar growth time (Figure 3). At 0–3 months ($n = 10$), MeCP2 was positively expressed in fibroblasts, and the number of positive cells was the highest (Figure 3(A)). At 4–7 months ($n = 11$), the expression of MeCP2 in fibroblasts remained strongly positive, and the number of positive cells was not significantly different from that at 0–3 months (Figure 3(B)). At 7–12 months ($n = 13$), MeCP2 was still positively expressed in fibroblasts, but the number of positive cells was significantly reduced (Figure 3(C)). When the growth time exceeded 12 months ($n = 17$), the expression of MeCP2 in fibroblasts was weakly positive, and the number of positive cells was the lowest (Figure 3(D)). The difference among the four groups was statistically significant ($F = 26.19$, $p < .001$).

3.2.2. Quantitative detection of MeCP2 protein in hypertrophic scars fibroblasts

Quantitative analysis of MeCP2 protein in scar tissue showed that there was no significant difference in the expression of MeCP2 between 0–3 months and 4–7 months ($p = .98$, $> .05$; Figure 2(B)). When the growth time of hypertrophic scars exceeded 12 months, the expression level of MeCP2 was the lowest ($F = 25.8$, $p < .001$).

3.3. The methylation level of Smad7 is highest in the keloid group

To clarify the methylation status of Smad7 gene promoter in scar tissue and skin fibroblasts, MSP method was used for the detection. The results showed that the methylation level of Smad7 was significantly higher in the keloid group ($n = 10$) compared to the normal skin group ($n = 10$) (Figure 4).

3.4. Mecp2-siRNA transfection inhibits the proliferative activity of human keloid fibroblasts

After transfection with MeCP2-siRNA in human keloid fibroblasts, the expression levels of MeCP2 were significantly altered. The mRNA expression of MeCP2 was significantly lower ($F = 15.28$, $p < .001$) in the experimental group compared to the control (2.19 ± 0.47) and blank (2.19 ± 0.31) groups (Figure 5(A)). The protein level of MeCP2 was also markedly lower ($F = 49.458$, $p < .001$) in the experimental group (622.42 ± 180.34) compared with the control (3110.76 ± 311.45) and blank (2991.43 ± 478.98) groups (Figure 5(B)).

MTT assay results showed that the proliferation activity of keloid fibroblasts was decreased by the inhibition of MeCP2 expression. After MeCP2-siRNA interference for 24 h, the proliferation rates of fibroblast cells were $70.63 \pm 1.91\%$, $40.49 \pm 2.14\%$ and $27.66 \pm 1.81\%$ ($p < .001$) in 20, 40 and 60 μM MeCP2-siRNA groups, respectively (Figure 6(A)). When MeCP2-siRNA concentration was constant, the inhibition rates of fibroblast cells were significantly increased with increasing transfection times. At a high concentration of MeCP2-siRNA (60 μM), the inhibition rates of fibroblast cells were $22.9 \pm 3.82\%$, $55.7 \pm 1.71\%$, $70.29 \pm 3.05\%$ and $83.2 \pm 0.59\%$ ($p < .001$) after 12, 24, 48 and 72 h of transfection (Figure 6(B)).

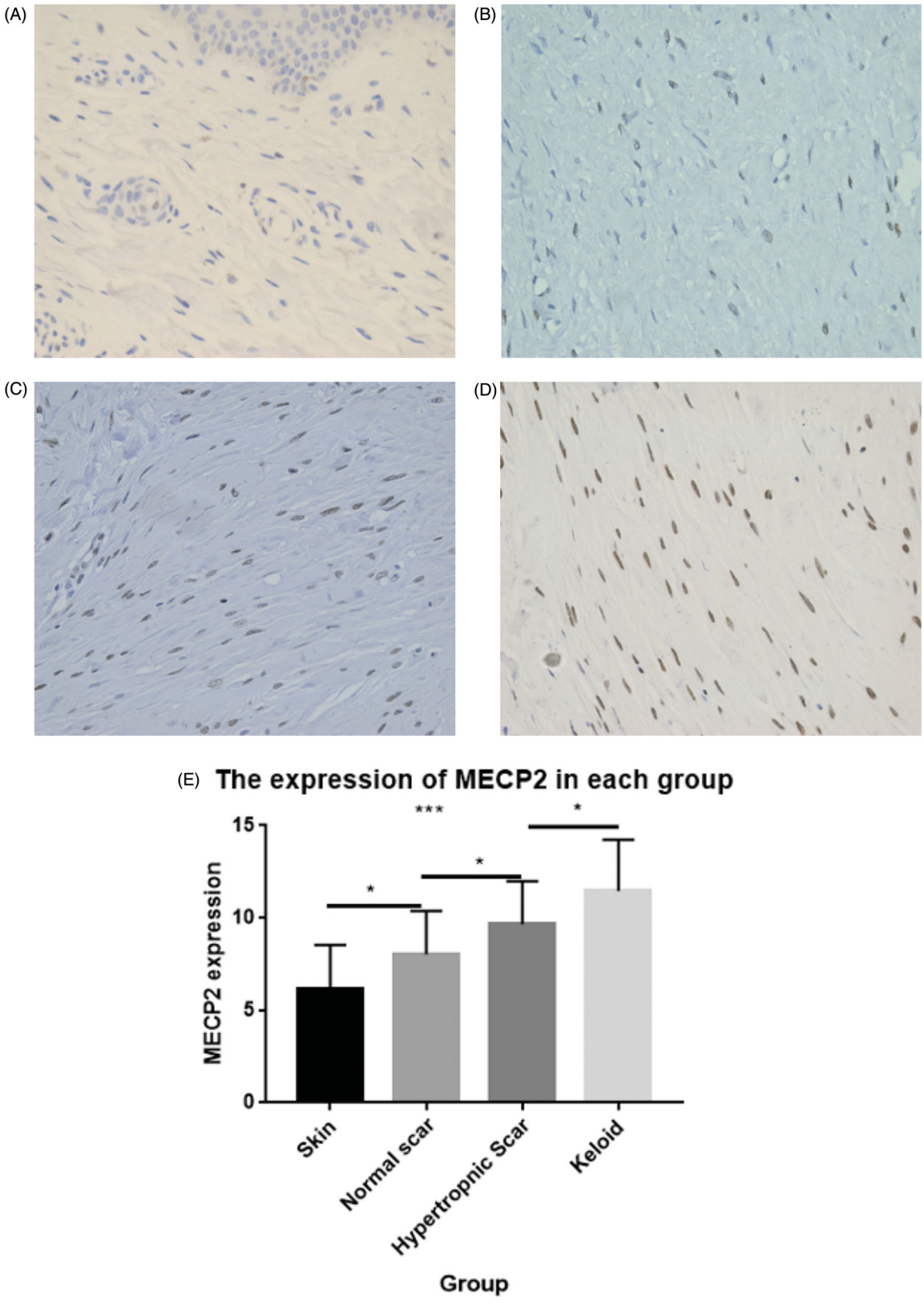


Figure 1. MECP2 protein expression in normal skin and scar tissues (×400); (A) normal skin: MECP2 expression is negative or weakly positive. (B) Normal scar: MECP2 expression is weakly positive and the number of positive cells is less. (C) Hypertrophic scar: MECP2 expression is positive; (D) keloid: MECP2 expression is strongly positive and had a large number of positive cells (arrow points to fibroblasts). (E) The result of statistical analysis.

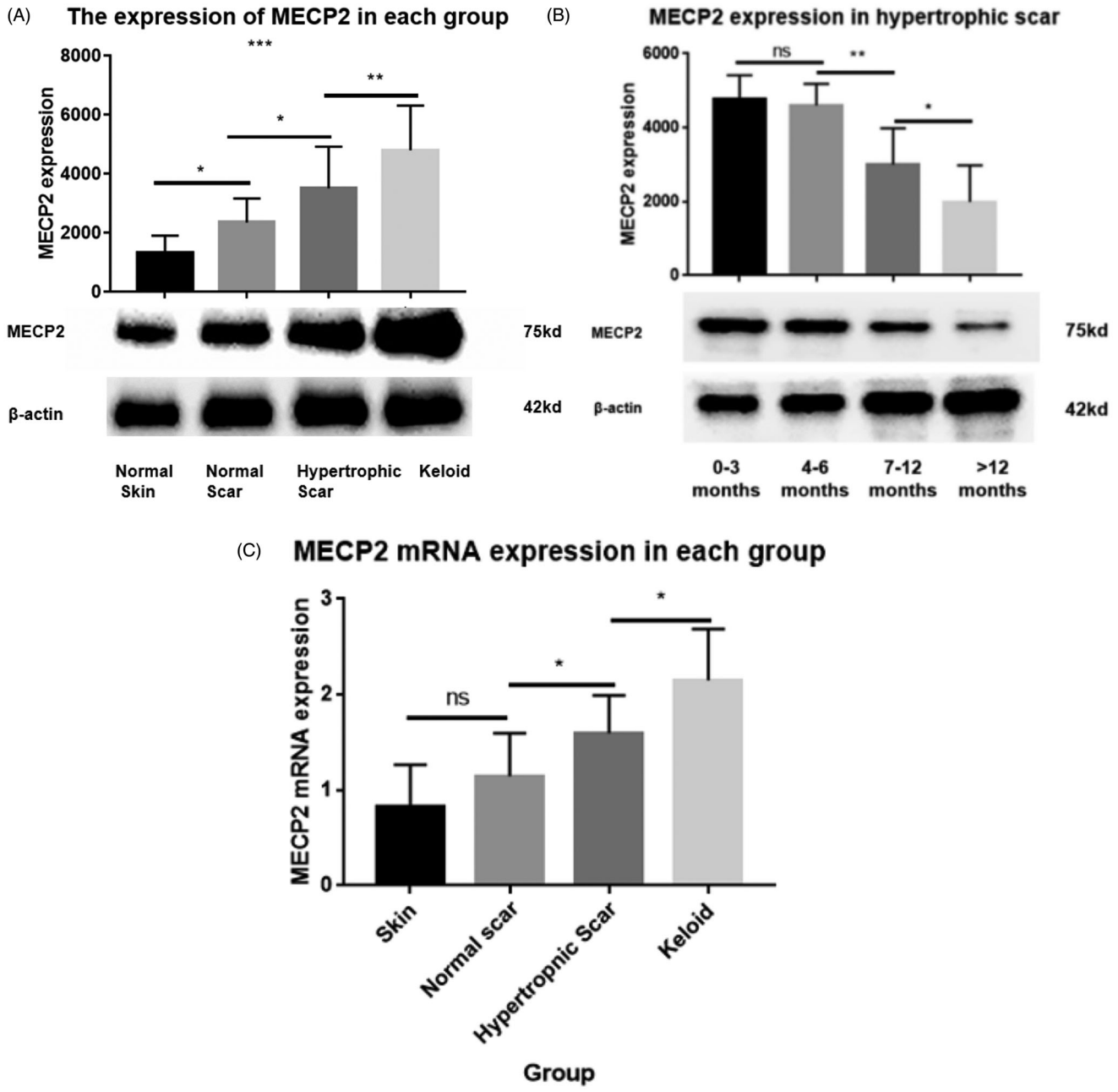


Figure 2. (A) The expression of MECP2 protein in various scars and normal skin; (B) the expression of MECP2 protein in hypertrophic scar; (C) the expression of MECP2 mRNA in various scars and normal skin.

Table 2. The expression of protein and mRNA MECP2 in various scars and normal skin (mean ± SD).

	Number (case, *n*)	MeCP2	MeCP2 mRNA
Normal skin	33	1326.45 ± 572.34	0.82 ± 0.43
Normal scar	32	2341.4 ± 816.22	1.14 ± 0.45
Hypertrophic scar	34	3500.7 ± 1407.66	1.59 ± 0.39
Keloid	35	4787.95 ± 1514.33	2.14 ± 0.53
F Value		33.82	23.45
p Value		<.001	<.001

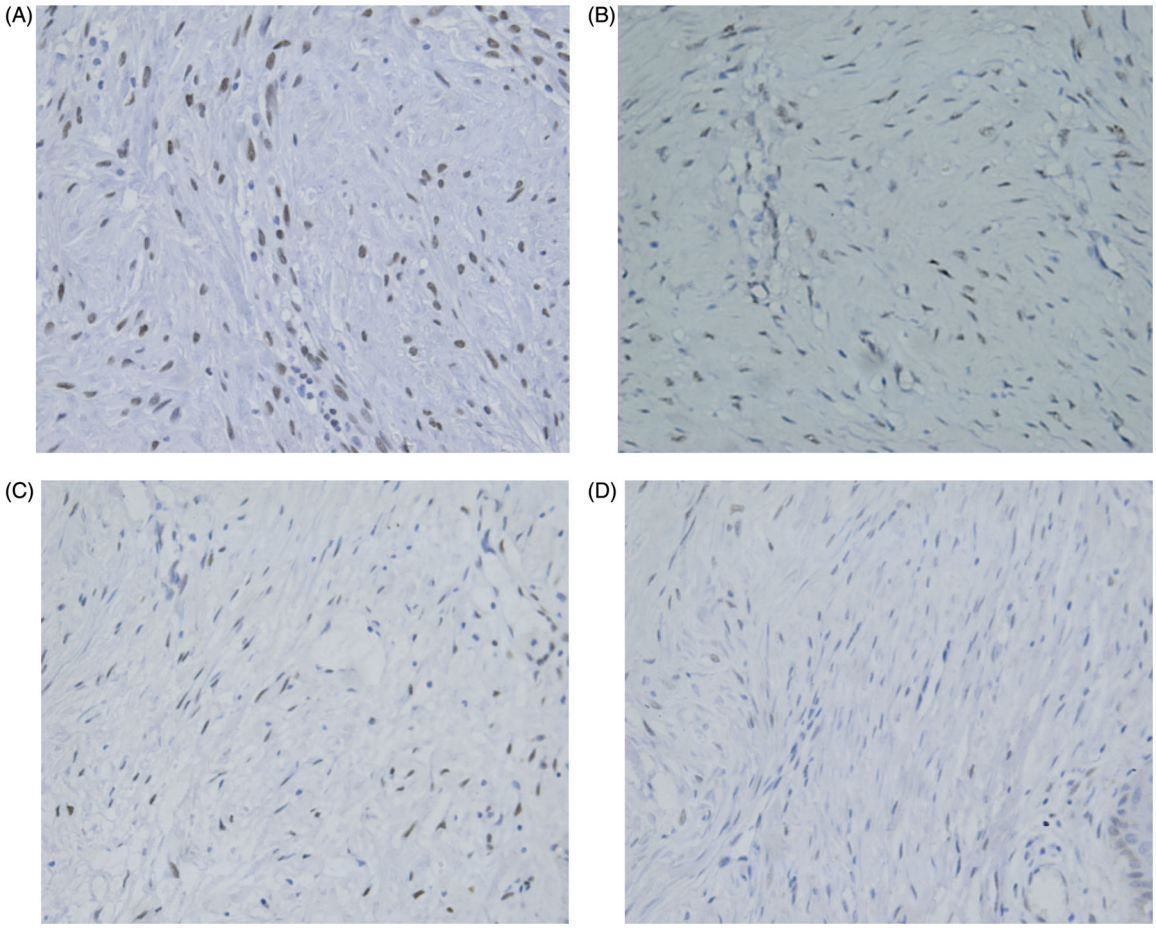
3.5. MeCP2 silencing affects the expression levels of keloid fibrosis-associated factors

3.5.1. The mRNA expression of TGF-β1, p-Smad2, α-SMA and Smad7 in keloid fibroblasts after MeCP2-siRNA transfection

After transfection with MeCP2-siRNA in keloid fibroblasts, the mRNA levels of MeCP2 and Smad7 in the experimental group were significantly decreased and increased, respectively, compared to the control and blank groups. Besides, the mRNA expression levels of TGF-β1, p-Smad2, α-SMA were significantly decreased ($p<.05$) in the experimental group (Table 3, Figure 7).

3.5.2. The protein expression of TGF-β1, α-SMA, p-Smad2 and Smad7 in keloid fibroblasts after MeCP2-siRNA transfection

When MeCP2-siRNA was transfected into keloid fibroblasts, the protein level of MeCP2 was significantly decreased, while that of Smad7 was remarkably increased. At the same time, the protein expression levels of fibrosis-associated cytokines TGF-β1, p-Smad2 and α-SMA were markedly lower in the experimental group ($p<.05$) than in the control and blank groups (Table 4, Figure 8).



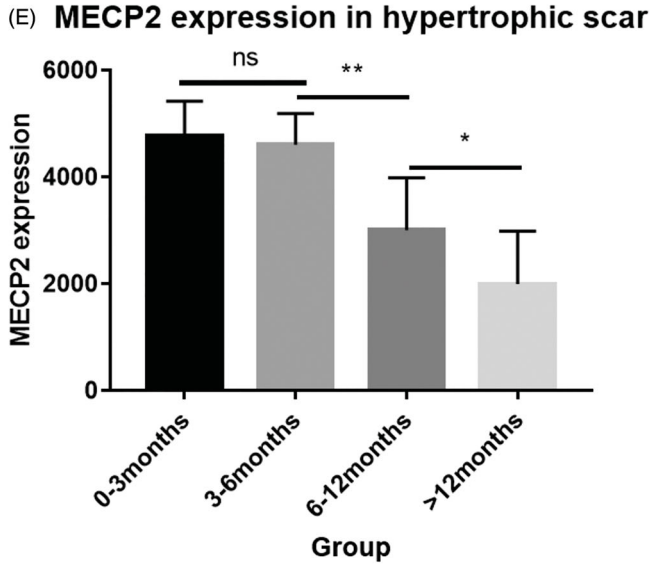


Figure 3. The expression of MECP2 in hypertrophic scar fibroblasts (×400); (A) 0–3 months ($n = 10$): MECP2 is positively expressed and much number; (B) 4–7 months ($n = 11$): MECP2 is strongly positive in fibroblast; (C) 7–12 months ($n = 13$): MECP2 is positively expressed but small number; (D) MECP2 expression in fibroblasts at >12 M is low and weak ($n = 17$) (arrow points to fibroblasts). Methylation-specific polymerase chain reaction (MSP) analysis of Smad7 gene promoter in human keloids and skin. M and U, PCR products of methylated and unmethylated alleles (K: keloid; S: skin).

4. Discussion

Scars are the product of human healing after trauma. The occurrence and progression of scars are determined by environment, gene expression and other factors [13]. According to the histomorphological characteristics of scars, they can be divided into normal scars, hypertrophic scars and keloids. Different types of scars have different clinical and pathological manifestations. Pathological scars include hypertrophic scars and keloid, which not only seriously affect the appearance and bodily function, but also have the characteristics of identification and treatment difficulties. Therefore, it is necessary to elucidate the molecular mechanisms involved in the occurrence and progression of pathological scars.

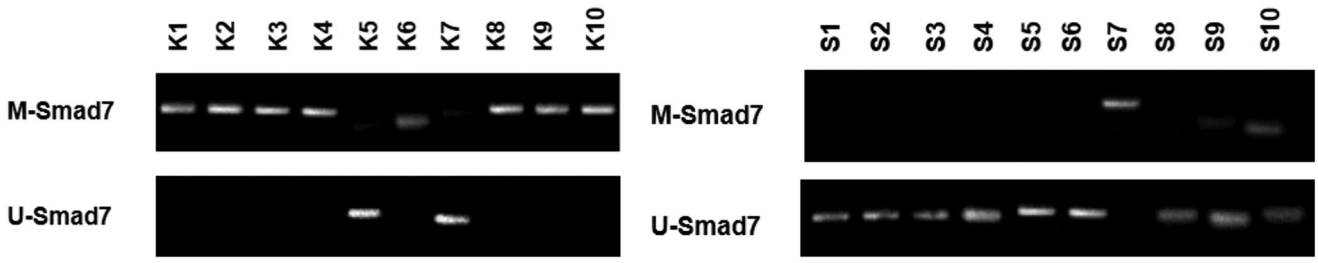


Figure 4. Methylation-specific polymerase chain reaction (MSP) analysis of Smad7 gene promoter in human keloids and skin. M and U, PCR products of methylated and unmethylated alleles (K: Keloid; S: Skin).

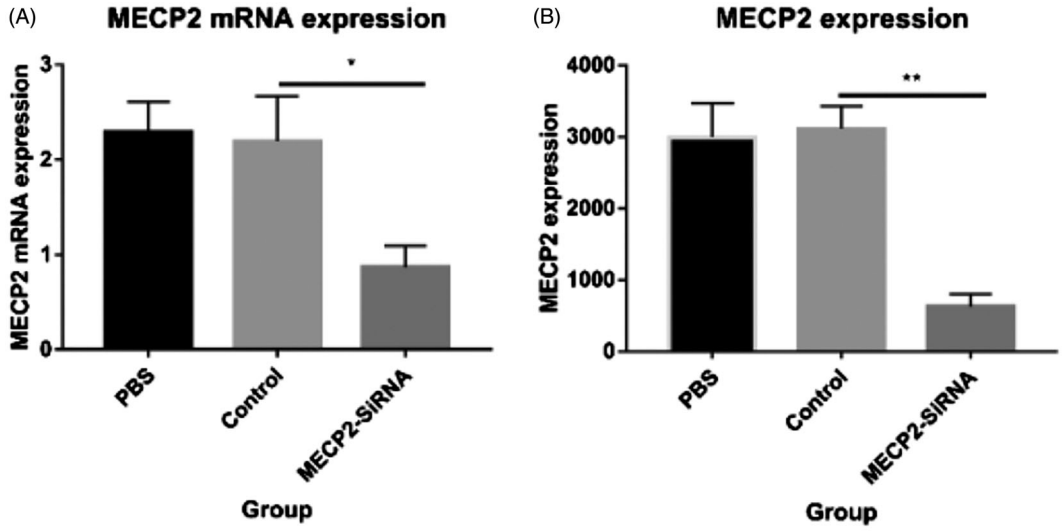


Figure 5. MECP2-SiRNA transfected fibroblasts with MECP2 changes.

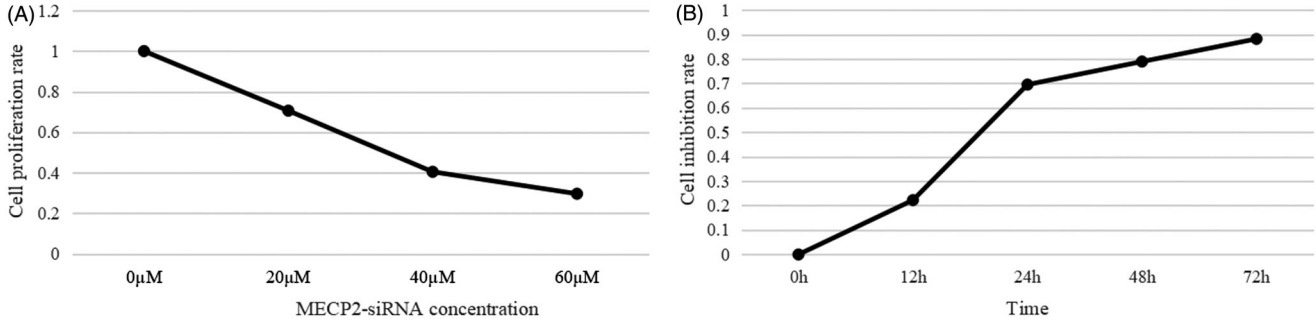


Figure 6. (A) Cell proliferation rate after MECP2-siRNA interference for 24 h. (B) Cell inhibition rate at MECP2-siRNA concentration of 60 μM.

Table 3. mRNA expression of related proteins after MECP2 transfection (mean ± SD).

	Blank control	Reagent control	MECP2-SiRNA	*F* Value	*p* Value
TGF-β	2.41 ± 0.31	2.19 ± 0.47	0.86 ± 0.22	10.35	$p=.01$
α-SMA	1.74 ± 0.24	1.59 ± 0.37	0.6 ± 0.27	12.57	$p<.05$
Smad7	0.97 ± 0.15	0.91 ± 0.37	2.41 ± 0.28	26.31	$p<.05$
P-smad2	1.89 ± 0.12	2 ± 0.41	0.88 ± 0.21	15.14	$p=.05$

Fibroblasts are the main cellular component in scar formation. TGF-β1 is a cytokine known to be most closely related to wound repair and scar formation, and its signaling is transmitted through TGF-β/Smad pathway [14]. Appropriate amount of TGF-β is beneficial for wound healing, but excessive expression of TGF-β can lead to scar hyperplasia [15]. The expression level of TGF-β is directly proportional to fibrosis, and it was used as an important evaluation standard for fibrosis in this experiment. Similarly, the expression level of α-SMA is positively correlated with the deposition of extracellular matrix, thus, it was used as another evaluation index of fibrosis in keloid fibroblasts.

DNA methylation plays an essential role in the evolution of mammals. The hypermethylated state of gene promoter inhibits gene transcription, while the hypomethylated state can induce gene activation and expression [16]. Methylated binding proteins, such as MeCP2, MBD1, MBD2, MBD3 and MBD4, are a class of proteins that bind to methylated-CpG dinucleotides. Previous studies demonstrated that MeCP2, MBD1 and MBD2 could bind to the hypermethylated promoter to inhibit gene transcription [12], and the mRNA expression of MeCP2 was upregulated in chronic hepatitis B [17], pulmonary fibrosis [18] and scleroderma [19]. During the process of organ fibrosis, MeCP2 acts as a transcriptional

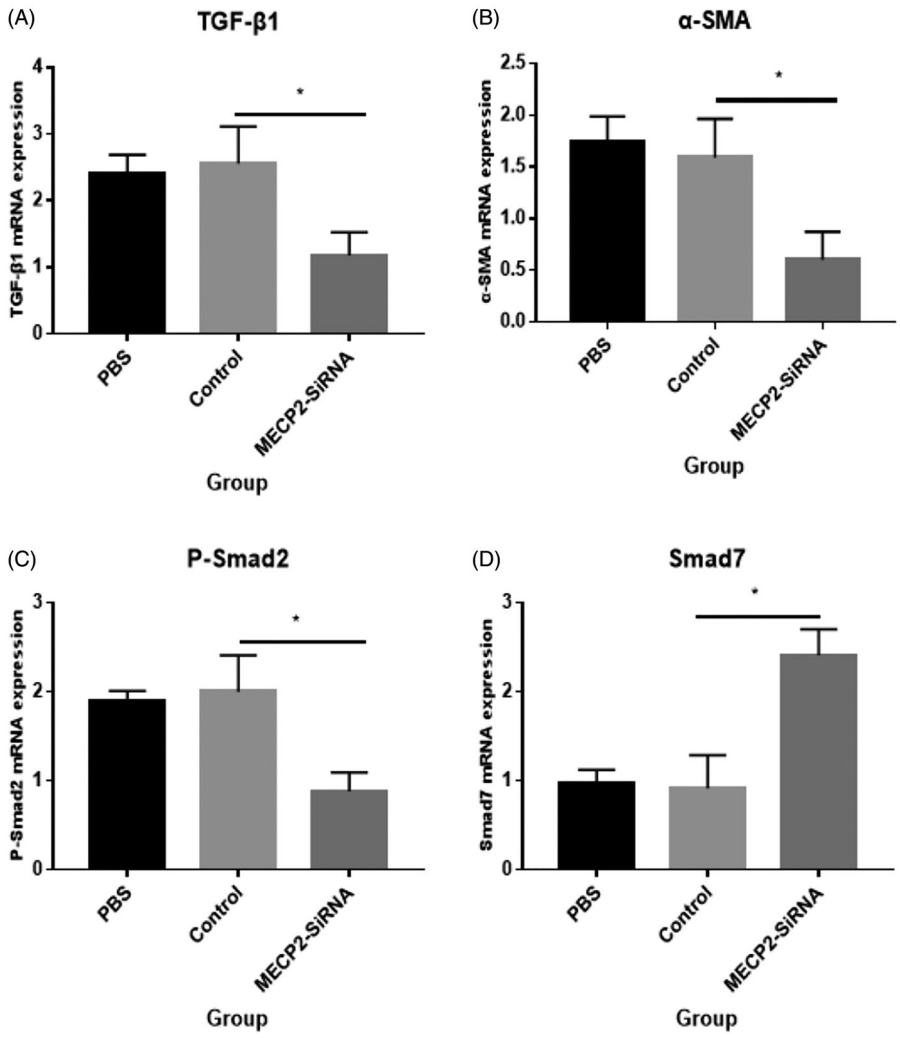


Figure 7. Expression of related cytokine mRNA after MECP2 transfection with fibroblasts.

Table 4. Expression of related proteins in keloid fibroblasts after MECP2 transfection (mean ± SD).

	Blank control	Reagent control	MECP2-SiRNA	*F* Value	*p* Value
TGF-β	2557 ± 535	2246 ± 486.5	1101.3 ± 190.5	9.448	$p=.014$
α-SMA	2491 ± 148.6	2403 ± 433.6	1145 ± 354.1	13.218	$p<.05$
Smad7	975 ± 143.6	1207 ± 515	2737 ± 525.1	14.683	$p<.05$
P-smad2	2511 ± 504.91	2316 ± 697.8	879.3 ± 276.3	8.732	$p=.017$

repressor to inhibit the function of negative regulators of fibrogenesis, thereby promoting tissue fibrosis in various organs. In the lung fibrosis model, when the expression of MeCP2 was inhibited, the expression levels of fibrosis-related cytokines were significantly decreased, and the severity of fibrosis was also reduced [20]. This indicates that DNA methylation acts as an inhibitory regulator in the TGF-β/Smad signaling pathway, and is involved in scar formation after wound healing. Based on the commonality of epigenetic modifications of cancer and fibrosis [21], DNA hypermethylation of specific genes may contribute to the occurrence of fibrosis. In this study, Western blot and real-time polymerase chain reaction (RT-PCR) experiments showed that MeCP2 expression was significantly increased in keloid and hypertrophic scars, which is consistent with the increased expression of MeCP2 in cirrhotic fibroblasts. A significant difference in MeCP2 levels was also observed in hypertrophic scars at different growth stages. Compared with the regression and maturity stages, MeCP2 expression was significantly upregulated during the early growth and hyperplasia stages. Overall, these results suggest that MeCP2 may serve as a new target for inhibiting scar formation.

Smad7 is the most important negative regulator in the TGF-β/Smad signaling pathway. Overexpression of Smad7 could inhibit the contraction of fibroblasts [22]. Previous studies have indicated that Smad7 plays an important role in suppressing renal [23], heart [24] and liver [25] fibrosis, but the association between Smad7 and fibrosis in keloid is rarely being reported. In addition to histone acetylation and microRNA regulation, Smad7 expression can also be regulated by DNA methylation [26]. The promoter of Smad7 gene was primarily methylated in liver fibrosis and human atherosclerosis tissues [27]. A recent study demonstrated that lncRNA was upregulated in hypertrophic scar tissue and fibroblasts, and it suppressed the proliferation of fibroblasts by promoting Smad7 expression [21]. Moreover, the expression levels of collagen types I and III were reduced, and the proliferation of keloid fibroblast cells was inhibited after transfection with Smad7 overexpression vector [28]. The promoter region of Smad7 gene was strongly hypermethylated in human atherosclerotic plaques and atherosclerosis patients [29], as well as in the liver tissues of CCl_4-treated rats [30]. This indicates that DNA methylation of Smad7 promoter is closely related to fibrosis. From this point of view, the relationship between Smad7 and keloid development was further evaluated. MSP analysis indicated that the methylation level of Smad7 in keloid group was higher than that in

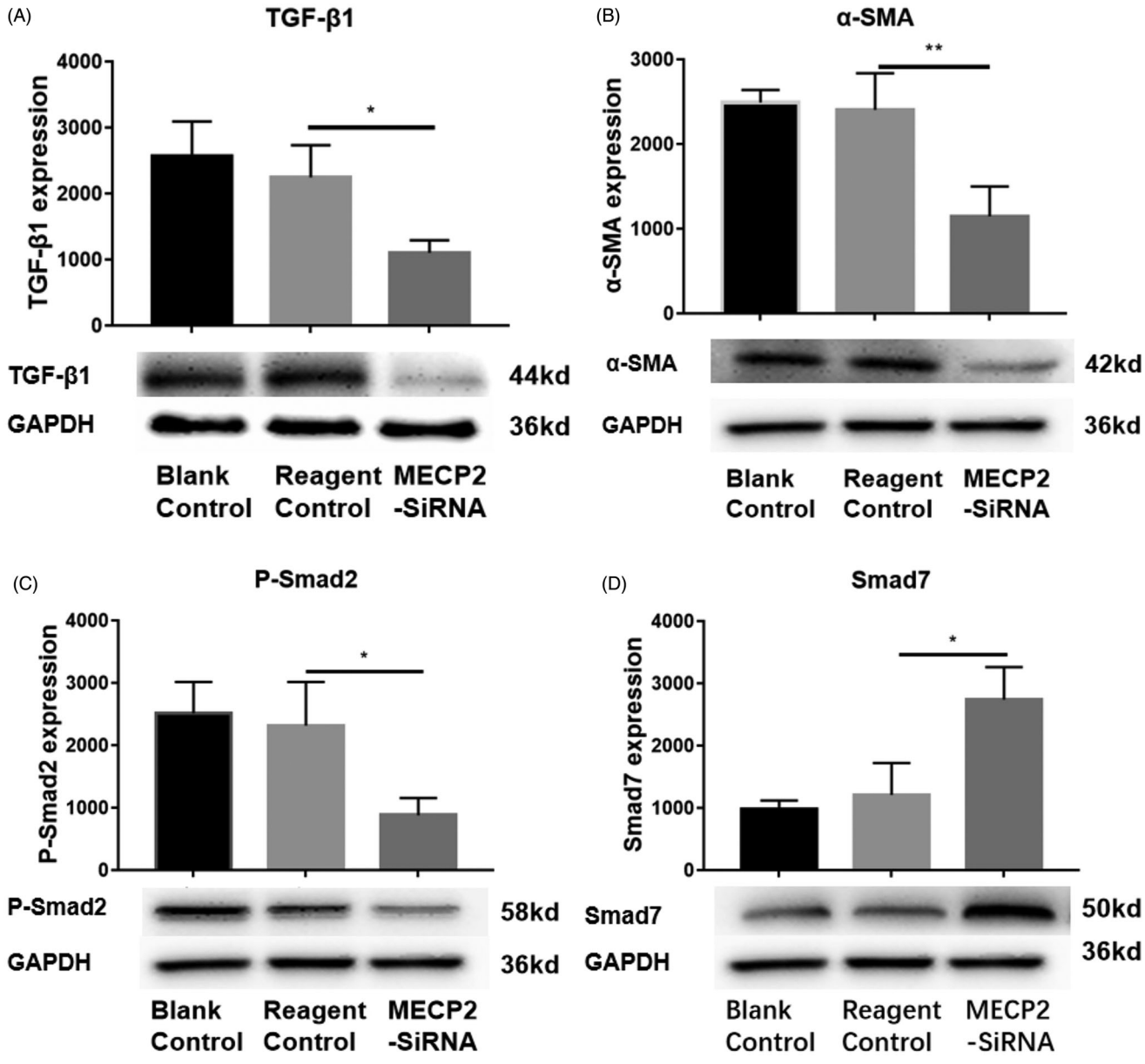


Figure 8. Expression of relevant cytokine proteins in fibroblasts after MECP2 transfection.

hypertrophic scar, normal scar and normal skin groups. This provides a prerequisite for the transcriptional repression of MeCP2.

Previous research showed that, after treatment with methylation transferase inhibitors, TGF-β1 expression decreased, Smad7 expression increased and p-Smad2/3 expression decreased in keloid fibroblasts [31]. In this study, we selected the most representative keloids as the research object, and further explored the relationship between Smad7 methylation and MeCP2 expression in keloid formation. After transfection with MeCP2-siRNA, the proliferation of human keloid fibroblast was inhibited, Smad7 level was increased, and TGF-1, α-SMA and p-Smad2 levels were decreased. Noticeably, Smad7 was overexpressed after downregulation of MeCP2, indicating that MeCP2 inhibits the expression of Smad7 during scar formation, and the negative feedback regulation of Smad7 on the TGF-β/Smad signaling pathway is suppressed. After the inhibitory effect of MeCP2 on Smad7 is abolished, Smad7 can be upregulated and its inhibitory effect on p-Smad2/3 may be restored. MeCP2 was originally identified as a transcriptional repressor, but was found later to also play an important role as a transcriptional activator that binds to the methylated α-SMA gene promoter and results in an increased expression of α-SMA [32], while promoting cell fibrosis and deposition of extracellular matrix. Our results indicated that the expression of α-SMA was decreased after MeCP2 inhibition. However, it can only be inferred that the promoter region of α-SMA in keloid fibroblasts is in a hypermethylated state [6]. Whether α-SMA hypermethylation is associated with MeCP2 and Smad7 still needs further investigation. Nevertheless, it is noteworthy to mention that downregulated MeCP2 expression weakened the transcriptional activation of α-SMA, decreased the expression of α-SMA, and inhibited organ fibrosis. This further confirms that MeCP2 can be used as a transcription repressor to inhibit the formation of keloids.

MeCP2 regulates the occurrence and progression of pathological scars indirectly or directly through Smad7 inhibition and α-SMA activation. It is suggested that MeCP2 plays an indispensable role in regulating scar formation, which provides a new entry point for the treatment of scars. However, the specific methylation sites of Smad7 and α-SMA in keloid fibroblasts are still unknown.

Therefore, more research is needed to better understand the underlying mechanisms for the observed associations.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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