



# Exploring a Novel Method to Inhibit Scar Formation

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## Abstract

Scars result in aesthetic sequelae and functional disorder and increase social and personal economic burdens. However, this problem still has not been resolved. Clinical studies have demonstrated that scars often form when the deep dermis is injured, and in these situations, fat tissues are also involved, which suggests that fat tissues might play important roles in scar formation. The key cause of scar formation is the excess number of fibroblasts and myofibroblasts that occur during wound repair. Therefore, to repair the fat tissue and decrease the number of fibroblasts in the wound, fibroblast trans-differentiation into fat cells, which would decrease the number of fibroblasts and myofibroblasts in the wound, was explored. Scar fibroblasts and normal fibroblasts were chosen as experimental samples, and the cells were cultured in adipocyte differentiation medium that included both a Wnt inhibitor and Peroxisome Proliferator-Activated Receptor  $\gamma$  (PPAR $\gamma$ ) agonist. After treatment, RT-PCR showed that myofibroblast differentiation was inhibited in the scar fibroblast groups, while myofibroblast differentiation was not inhibited in the normal fibroblast groups. These preliminary results suggested that fibrosis was inhibited after the treatment and showed that the biological behavior of the scar fibroblasts changed, which could be beneficial for the inhibition of scar formation.

**Keywords:** Scar formation; Adipogenesis; Fibroblast; Wnt signal; Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )

## Introduction

Skin is the largest organ and is on the outside of the human body [1]. Thus, it is susceptible to injury, and a scar often forms after wound healing. Scar tissue results in aesthetic sequelae and functional disorder, which increases social burdens and can have detrimental effects on the patient's mental and physical health. The mechanism of scar formation is still not clear, and clinicians have not found ideal ways to treat this condition. Therefore, it is imperative to conduct research and find the best method to treat this condition.

The skin is divided into three layers [2]: The epidermis, dermis and hypodermis. The epidermis, the outermost layers of the skin, is a stratified squamous epithelium composed of proliferating basal and differentiated suprabasal keratinocytes, which cluster together and have a "characteristic tight pavement-like appearance". The dermal tissue is mostly composed of collagen fibers as scaffolds that weave throughout the tissue. The hypodermis is mostly composed of adipocytes and other cells. Anatomically, the three layers are not parallel, and the two interfaces among the three layers appear wavy or as a zigzag. Clinically, superficial dermal injury cannot produce scar formation, whereas deep dermal injury can [3]. After deep dermal tissue injury, the adipocytes and fat tissues are also involved; thus, adipocytes and fat tissues may participate in wound repair and scar formation. Hajime et al. [4] also demonstrated that the involved fat tissue affected scar formation. Pathologically, the fat tissue is under stress, inflammatory cells are recruited, and various cytokines and chemokines are released into the wound area. Mechanically, the neighboring fat tissues contract and are distorted when the scar is formed. Therefore, it is possible to inhibit scar formation if the stress state was reverted to a non-stress state.

Currently, fibroblasts have been demonstrated to play an important role during wound healing [5]. During this process, fibroblasts are recruited from many sources, migrate to the wound, and differentiate into myofibroblasts; thus, abundant Extracellular Matrix (ECM) is synthesized, and the wounds are contracted, leading to hypertrophic scar formation [5-8]. Therefore, one strategy to inhibit scar formation is to decrease the number of myofibroblasts. Furthermore, to decrease the number of myofibroblasts, the number of fibroblasts should be decreased. To decrease the number of fibroblasts, proliferation should be decreased, and apoptosis should be increased [9-11].

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In addition, in view of the fat tissue repair after injury, another different hypothesis was formed to eliminate the excess effect caused by adipocytes involved in decreasing the number of fibroblasts during wound repair. To reverse the stress state, the cells were cultured in an adipogenic microenvironment; to decrease excessive fibroblasts during wound repair, the fibroblasts were trans-differentiated into adipocytes. Therefore, to test our hypothesis, fibroblasts were chosen and examined. The preliminary results suggested that the trans-differentiation of fibroblasts might be a good strategy to inhibit scar formation.

## Materials and Methods

### Ethics statement

All of the experimental procedures were conducted under a protocol reviewed and approved by the Institutional Ethical Committee of the Ruijin Hospital, School of Medicine, Shanghai Jiaotong University.

### Human hypertrophic scar tissue and normal skin tissue

The patients were hospitalized in our department. Paired normal skin and hypertrophic scar tissue were collected from eight patients who received no treatment before surgery. These patients were recruited without micro- and macro-vascular complications, and other chronic diseases, such as hypertension, endocrine diseases, and diabetes. These patients were diagnosed as hypertrophic scars and considered as a representative of a larger population by our senior clinicians before sample collection. The recruited date was ranged from Sep. 2014 to Oct. 2016. The age of the eight patients ranged from 10 to 40 years old. The relevant demographic details were showed in Table 1. Written consent was obtained from patients or their legal guardians. After excision, the tissue samples were immediately placed in ice-cold Dulbecco's Modified Eagle's Medium (DMEM) and was subsequently processed for cell culture.

### Cell culture and treatment

The researches were made in our lab. Dermal fibroblasts were isolated and cultured as described previously [12]. Briefly, tissues were trimmed to remove excessive adipose tissue and then rinsed with Phosphate Buffer Solution (PBS) three times. Then, tissues were minced into small pieces and incubated in DMEM (Gibco, USA) containing 0.1% collagenase type I (Sigma, St. Louise, Missouri, USA) at 37°C for 3 h. The isolated fibroblasts were then cultured in DMEM containing 10% fetal calf serum (Gibco), 1% penicillin and 1% streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Fibroblasts from the 3<sup>rd</sup> to the 5<sup>th</sup> passages were used in all experiments. Before any treatment, fibroblasts reaching 70% to 80% confluence were incubated in serum-depleted medium for another 12 h.

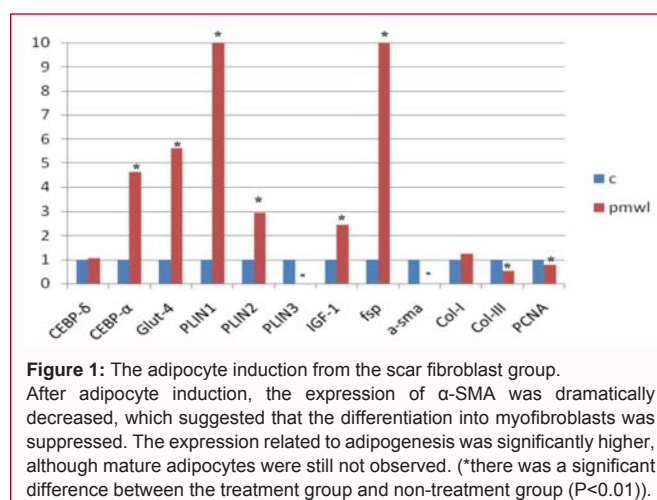
Normal fibroblasts and hypertrophic fibroblasts, which were seeded into 6-well plates, were randomly arranged into different groups (n=6).

### Experimental design

Cells (normal fibroblasts and hypertrophic fibroblasts) were separately seeded into 6-well plates and expanded with preadipocyte medium for 3 days and then changed into preadipocyte differentiation medium. The medium was changed every 3 days, and the induction period was 9 days. After induction, the cells were incubated in adipocyte medium and maintained for 6 days. The cells were treated with a Wnt antagonist (IWP-2, Santa Cruz, USA) and PPAR $\gamma$  agonist

**Table 1:** The demographic of the participants.

	Sex	Age	Race	Region	Etiology	Cause of the disease
1	M	10	Han. Asia	Hand	Burns	1.5 yrs
2	F	35	Han. Asia	Abdomen	Cesarean section	3 yrs
3	F	30	Han. Asia	Upper back	Skin abscess	2 yrs
4	M	12	Han. Asia	Front chest	Burns	3 yrs
5	F	29	Han. Asia	The auricle	Mosquito bites	1.8 yrs
6	M	40	Han. Asia	Shoulders	Burns	5 yrs
7	F	30	Han. Asia	Thigh	Burns	4 yrs
8	M	22	Han. Asia	Frontal	Trauma	2 yrs



**Figure 1:** The adipocyte induction from the scar fibroblast group.

After adipocyte induction, the expression of  $\alpha$ -SMA was dramatically decreased, which suggested that the differentiation into myofibroblasts was suppressed. The expression related to adipogenesis was significantly higher, although mature adipocytes were still not observed. (\*there was a significant difference between the treatment group and non-treatment group ( $P < 0.01$ )).

(LG100754, Tocris Bioscience, R&D Systems, USA) from the first day of induction and continued to the end of induction. To examine the effectiveness of the treatment, both stimuli were divided into three concentrations: High concentration (h), middle concentration (m) and low concentration (l). To observe the optimum combination, nine combinations of the two chemical agents (WhPh, WhPm, WhPl, WmPh, WmPm, WmPl, WlPh, WlPm, and WlPl) were applied in this research. The three concentrations of each of them were as follows: the high concentration of the Wnt antagonist (Wh) was 67.5 nm/ml, the middle concentration of the Wnt antagonist (Wm) was 13.5 nm/ml, and the low concentration of the Wnt antagonist (Wl) was 2.75 nm/ml. The high concentration of the PPAR $\gamma$  agonist (Ph) was 2.5  $\mu$ m/ml, the middle concentration of the PPAR $\gamma$  agonist (Pm) was 0.5  $\mu$ m/ml, and the low concentration of the PPAR $\gamma$  agonist (Pl) was 0.1  $\mu$ m/ml. The cells were collected for RT-PCR during the maintenance period.

### RT-PCR

After the cells were cultured to 80% confluence, total RNA was extracted by TRIzol reagent (Gibco, Grand Island, New York, USA). RT-PCR was performed using reagents from Promega (Promega, Shanghai, China) according to the manufacturer's instructions. Briefly, 2  $\mu$ g of RNA sample was reverse transcribed using antisense primers (Invitrogen, Shanghai, China). The PCR cycles consisted of 94°C for 30 s, Tm for 30 s, and 72°C for 30 s for 30 cycles. GAPDH was used as an internal control, with the PCR protocol being 94°C for 30 s, 58°C for 30 s and 72°C for 30 s for 30 cycles (primer sequences in Table 2).

The gene expression related to adipocytes (CCAAT/enhancer-

**Table 2:** Optimal primer pairs for RT-PCR.

Gene	Sense	Antisense	TM (°C)	Cycles	Size (bp)
FSP-1	5' GGA CAA CGA GGT GGA CTT C	5' CTT CCT GGG CTG CTT ATC TG	83.2	40	103
$\alpha$ -SMA	5' GGA CAT CAA GGA GAA ACT GT	5' CCA TCA GGC AAC TCG TAA CT	82.5	40	105
COL1A1	5'CGAAGACAT CCCACCAATC	5'CAAGAAACCACCGGCGTCGAGG	91	40	227
COL3A1	5'TGAAGGGCAGGGAACAACCTT	5'CTTCTCGCTCTGCTTCATCCC	81.9	40	143
PCNA	5' TTA GCT CCA GCG GTG TAA AC	5' CAG CGG TAG GTG TCG AA	86.7	40	97
GLUT4 (SLC2A4)	5' GAA TAC CTT CTT CGC TGC TA	5' GCT CTC TGC TCT GGT TTG T	82.7	40	85
IGF1	5' TCC TCG CAT CTC TTC TAC CT	5' AAA AGC CCC TGT CTC CAC AC	88.7	40	130
PLIN1	5' CTC ACA GCC ACA TTT CCA TT	5' GTT CCC CAG CAT CAA AAG AG	79.4	40	71
PLIN2	5' GCC AAC AGA CCA TTT CTC A	5' CTC CTT TTC CAC TCT ACC CA	81.8	40	136
PLIN3	5' GAA GAA GTA GGG GGA GAG GA	5' CCA AGT GGA CAG CAC AGA AG	86.3	40	138
CEBP- $\alpha$	5' GAC CCT CAG CCT TGT TTG TA	5' ATC GTG CTT CGT GTT CCT A	79.5	40	60
CEBP- $\delta$	5' GCA GTT TCT TGG GAC ATA GG	5' GCT TCT CTC GCA GTT TAG TG	81.5	40	72
GAPDH	5' GGG AAG GTG AAG GTC GGA GT	5' GGG GTC ATT GAT GGC AAC A	84.1	40	105

binding protein  $\alpha$  (CEBP- $\alpha$ ), CCAAT/Enhancer-Binding Protein  $\delta$  (CEBP- $\delta$ ), Glucose transporter type-4 (Glut-4), Perilipin-1 (PLIN1), Perilipin-2 (PLIN2), Perilipin-3 (PLIN3), Insulin Growth Factor-1 (IGF-1)), Fibroblasts, (FSP), and scar formation,  $\alpha$ -SMA, PCNA, Col-I, and Col-III were assessed.

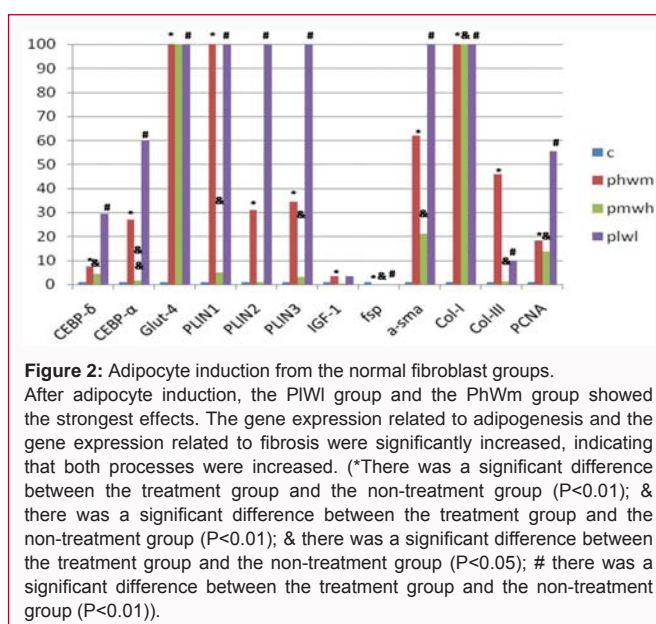
### Statistical analysis

All data are expressed as the mean  $\pm$  SD of at least three independent experiments. One-way analysis of variance was used for statistical differences. Statistical significance was set at  $P < 0.05$ .

## Results

After induction, the number of cells decreased, indicative of reduced proliferation. The phenotype of the cells changed, but the cells did not show translucent oil droplets. The oil red staining was negative, which suggested that the cells did not change into adipocytes for both normal fibroblasts and scar fibroblasts. RT-PCR showed that the results of the induction in the PmW1 group were the best in the scar groups, while the results of induction in the PhWm, PmWh, and PIW1 groups were better than others in the normal groups. In the PmW1 group of scar fibroblasts, the expression levels of CEBP- $\alpha$ , Glut-4, PLIN1, PLIN2 and IGF-1 were significantly increased, while the expression levels of  $\alpha$ -SMA, PCNA, and Col-III were significantly decreased (Figure 1). The expression of FSP was also dramatically higher after induction in the same groups. The results suggested that the profibrotic effects and ability to contract of scar fibroblasts were inhibited after adipo-induction treatment and that cell could change their characteristics from fibroblasts to adipocytes. The higher expression of FSP in these cells was characteristic of fibroblasts.

In the normal fibroblast groups, the expression levels of CEBP- $\alpha$ , CEBP- $\delta$ , Glut-4, PLIN1, PLIN2, PLIN3 and IGF-1 were significantly increased in the PhWm, PIW1 and PmWh groups, while the expression levels of  $\alpha$ -SMA, Col-I, Col-III and PCNA were still dramatically increased in these groups (Figure 2). The expression of FSP was decreased significantly. The other groups showed that the trans-differentiation treatment was not effective. These results indicated that the fibroblasts showed heterogeneity. After adipo-induction treatment, some of the cells showed differentiation to adipocytes, while others showed increased profibrotic characteristics. Considering that the normal cells were quiescent in a normal

**Figure 2:** Adipocyte induction from the normal fibroblast groups.

After adipocyte induction, the PIW1 group and the PhWm group showed the strongest effects. The gene expression related to adipogenesis and the gene expression related to fibrosis were significantly increased, indicating that both processes were increased. (\*There was a significant difference between the treatment group and the non-treatment group ( $P < 0.01$ ); & there was a significant difference between the treatment group and the non-treatment group ( $P < 0.01$ ); # there was a significant difference between the treatment group and the non-treatment group ( $P < 0.05$ ); # there was a significant difference between the treatment group and the non-treatment group ( $P < 0.01$ )).

microenvironment, the induction experiment activated these cells. After activation, some of the cells produced high levels of ECM, and thus, they might behave like scar fibroblasts.

## Discussion

Skin covers the outside of the human body and is easily injured [1]. After healing, a scar often occurs; its unattractive appearance and functional disorder may increase the patient's social burden and is detrimental for the patient's mental and physical health. Currently, the mechanism of scar formation is not clear, and clinicians have not found ideal ways to treat this condition [1]. Therefore, it is imperative to conduct research on this issue and develop optimal treatments.

Generally, scar formation occurs after deep dermal injury, which is often accompanied by hypodermal injury. In dermal tissues [3], fibroblasts are an important type of effect or that is activated after injury. During wound repair, fibroblasts are recruited from many sources to the wound and differentiate into myofibroblasts, which can abundantly synthesize Extracellular Matrix (ECM),

leading to hypertrophic scar formation [5-8]. Therefore, decreasing myofibroblasts by decreasing the proliferation and increasing the apoptosis of fibroblasts is one possible strategy [9-11].

Because the hypodermis is always injured with deep dermal injury [4] and the involved fat tissue plays an important role in scar formation, it is necessary to repair fat tissues and diminish the excessive inflammatory reaction [13]. Therefore, to repair the fat cells, we hypothesized that the fat cells could be increased by trans-differentiation from fibroblasts, which could also decrease the redundant fibroblasts in the wound and might inhibit scar formation.

To examine this hypothesis, the Peroxisome Proliferator-Activated Receptor  $\gamma$  (PPAR $\gamma$ ) agonist [14,15] (LG100754) and Wnt signal inhibitor-IWP-2 (a small-molecule antagonist of the Wnt/b-catenin pathway) were chosen as critical stimulating reagents. The nuclear hormone receptor PPAR $\gamma$  is a master regulator of adipogenesis, while Wnt inhibits adipogenesis by maintaining preadipocytes in an undifferentiated state through inhibition of the adipogenic transcription factors CCAAT/enhancer binding protein alpha (C/EBP  $\alpha$ , adipocyte transcription factor) and PPAR $\gamma$  [16]. C/EBP- $\alpha$  is the upstream signal of PPAR $\gamma$ . When Wnt signaling was prevented by over expression of Axin or dominant-negative TCF4, the cells could differentiate into adipocytes [17]. Thus, Wnt plays a role in the switch in adipogenesis [18]. When it occurs, adipogenesis would be inhibited; in contrast, adipogenesis would be initiated. Generally, the two factors were used together because they could act synergistically in adipogenesis [18].

In our experiment, the cells were cultured first in an adipogenic environment. Then, the two factors were added to the cell culture medium, and adipogenesis was tested by RT-PCR and oil red staining. Scar fibroblasts and the normal fibroblasts were assessed in this research. The gene expression levels of Glut4, IGF-1, PLIN1, PLIN2, PLIN3, CEBP- $\alpha$ , and CEBP- $\delta$  [19-22], were measured because they are involved in adipogenesis. Moreover, FSP-1,  $\alpha$ -SMA, COL-I, Col-III and PCNA were selected because they could be used to evaluate fibroblasts and myofibroblasts.

The results showed that the phenotype and biological behavior of the fibroblasts changed dramatically after interference. The number of fibroblasts, both in the normal groups and scar groups, was significantly decreased. In the scar fibroblast groups, the expression of  $\alpha$ -SMA, which was characteristic of myofibroblasts, was decreased to almost zero compared with no interference. The expression of PCNA was also significantly decreased, which was consistent with the microscopic observations. The expression of collagen III was decreased, which was consistent with other studies [23-26]. The PmW1 group showed the strongest effects among the groups. However, in normal groups, the expression levels of  $\alpha$ -SMA, PCNA, collagen I and collagen III were significantly increased compared with no treatment. Another study showed that the production and secretion of type I collagen from normal fibroblasts was inhibited by the Wnt inhibitor IWR-1 [27]. One explanation could be that different inhibitors were used in the experiments. IWP-2 was used in our experiment, which is an inhibitor of WNT processing and secretion, while IWR-1 was used in their experiment, which is a reversible Wnt pathway inhibitor and exerts its effect *via* stabilization of the Axin disruption complex [28].

However, although the expression of all adipogenesis factors was increased significantly compared with no treatment, no lipid droplet cells were observed microscopically, and the oil red staining, which

was used to stain the adipocytes, was negative for all the groups. The expression of adipogenesis parameters was significantly higher in the normal groups than the scar groups. The results suggested that it was difficult to trans-differentiate from fibroblasts to adipocytes despite other successful studies [29,30]. The reason might be that different sources of fibroblasts were used as experimental samples. In our experiment, the fibroblasts were from skin, while in their experiments, the cells were from orbital samples [29] and foreskin [30]. Interestingly, the expression of FSP-1 was higher compared with the no interference in scar groups, which was not reviewed by other studies and requires further analysis.

In conclusion, our research showed that the biological behavior of scar fibroblasts changed, and the characteristics of fibrosis were inhibited after treatment. The results also showed that the biological behavior was different between the normal fibroblasts and the scar fibroblasts after the same stimulus despite the lack of protein expression data. However, if the results of protein expression and the animal tests confirmed the RT-PCR results, it could yet be regarded as a good way to inhibit scar formation.

Finally, our data suggested that the biological behavior was complex, and the trans-differentiation methods require further study. Appropriate methods should be chosen carefully to achieve adipogenesis from fibroblasts.

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