

# Effect of Optimized Concentrations of Basic Fibroblast Growth Factor and Epidermal Growth Factor on Proliferation of Fibroblasts and Expression of Collagen: Related to Pelvic Floor Tissue Regeneration

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## Abstract

**Background:** Fibroblasts were the main seed cells in the studies of tissue engineering of the pelvic floor ligament. Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were widely studied but at various concentrations. This study aimed to optimize the concentrations of combined bFGF and EGF by evaluating their effects on proliferation and collagen secretion of fibroblasts.

**Methods:** Fibroblasts were differentiated from rat adipose mesenchymal stem cells (ADSCs). Flow cytometry and immunohistochemistry were used for cell identification. The growth factors were applied at concentrations of 0, 1, 10, and 100 ng/ml as three groups: (1) bFGF alone, (2) EGF alone, and (3) bFGF mixed with EGF. Cell proliferation was evaluated by Cell Counting Kit-8 assays. Expression of Type I and III collagen (*Col-I* and *Col-III*) mRNAs was evaluated by real-time quantitative reverse transcription-polymerase chain reaction. Statistical analysis was performed with SPSS software and GraphPad Prism using one-way analysis of variance and multiple *t*-test.

**Results:** ADSCs were successfully isolated from rat adipose tissue as identified by expression of typical surface markers CD29, CD44, CD90, and CD45 in flow cytometry. Fibroblasts induced from ADSC, compared with ADSCs, were with higher mRNA expression levels of *Col I* and *Col III* ( $F = 1.29$ ,  $P = 0.0390$ ). bFGF, EGF, and the mixture of bFGF with EGF can enhanced fibroblasts proliferation, and the concentration of 10 ng/ml of the mixture of bFGF with EGF displayed most effectively (all  $P < 0.05$ ). The expression levels of *Col-I* and *Col-III* mRNAs in fibroblasts displayed significant increases in the 10 ng/ml bFGF combined with EGF group (all  $P < 0.05$ ).

**Conclusions:** The optimal concentration of both bFGF and EGF to promote cell proliferation and collagen expression in fibroblasts was 10 ng/ml at which fibroblasts grew faster and secreted more Type I and III collagens into the extracellular matrix, which might contribute to the stability of the pelvic floor microenvironment.

**Key words:** Adipose Mesenchymal Stem Cells; Basic Fibroblast Growth Factor; Epidermal Growth Factor; Fibroblasts; Tissue Engineering

## INTRODUCTION

With the development of life science, biomicroenvironments had attracted increasing attention. Research on the microenvironments of tissue engineering in the field of regenerative medicine and various growth factors in cell functions was also gaining more attention.<sup>[1]</sup> Tissue engineering was expected to mimic microenvironments *in vivo*.<sup>[2]</sup> In this study to construct sacral ligament tissue *in vitro* by tissue engineering, we focused on the role of growth factors in fibroblasts as seed cells. The fibroblasts

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in this study were differentiated from adipose-derived mesenchymal stem cells (ADSCs). The effects of growth factors were evaluated by measuring the proliferation and collagen secretion of fibroblasts to determine optimal growth factor concentrations.

Fibroblasts are one of the main components of pelvic floor tissue. They are important for the healing process of pelvic floor tissue defects.<sup>[3]</sup> In a previous study on the reconstruction of pelvic floor tissue defects, Ruiz-Zapata *et al.*<sup>[4,5]</sup> found that basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and vascular endothelial growth factor are expressed in the damaged area to promote angiogenesis and granulation tissue regeneration. In advanced defect repair, a large number of fibroblasts mature to secrete collagen, which facilitates wound healing and repair functions.<sup>[6]</sup> Therefore, the collagen secretion and proliferation of fibroblasts are important for defect healing. In this study, fibroblasts were induced from ADSCs.<sup>[7,8]</sup> In studies on the effects of bFGF to promote fibroblast functions, bFGF concentrations vary from 0.005 to 2000 ng/ml,<sup>[2,9-12]</sup> with no defined optimal concentration. EGF is a widely used growth factor to promote the repair of skin fibroblasts.<sup>[13]</sup> In this study, we found that EGF plays a synergistic role with bFGF in promoting the proliferation of fibroblasts. It is difficult to detect the physiological effects of various growth factor concentrations around the pelvic floor ligament. Therefore, this study was designed to optimize the concentrations of combined bFGF and EGF to promote pelvic floor microenvironment stability.

This study was only a part of microenvironments' research of tissue engineering reconstruction of pelvic sacral ligament. ADSCs performed as original seed cells for the feature of low immunogenicity, widely source, high survival rate, and conducive to subsequent experiments *in vivo*; in addition, ADSCs could be differentiated to specific cells according to the demand of tissue engineering. In the whole tissue engineering reconstruction experiment, ADSCs were induced to differentiate into fibroblasts and smooth muscle cells, and the homology of the two kinds of cells was conducive to the development of cell culture *in vitro* and the transplantation of reconstruction of the sacral ligament of tissue engineering in the future. This article only introduced the experimental results of fibroblast growth microenvironment study.

## METHODS

### Primary culture of ADSCs

Adipocytes were isolated from groin adipose tissue of Sprague-Dawley rats (8–10 weeks old). All procedures were approved by the Ethics Committee of our hospital. The adipose tissue was rinsed with phosphate-buffered saline (PBS) three times, visible blood vessels and fascial fibers were removed,<sup>[14]</sup> and the tissue was cut into 1 mm<sup>3</sup> pieces. Digestion was performed with 0.01% Type I collagenase at 37°C in a water bath for 30 min. The digestion was terminated by adding PBS (HyClone, Logan, Utah, USA) containing 10% fetal bovine serum (Gibco, Grand

Island, New York, USA) and 1% antibiotics (100 U/ml penicillin/streptomycin) (Gibco). The cells were cultured at 37°C with 5% CO<sub>2</sub>. The medium was changed after 3 days and then every 3 days. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hospital (No. 2015-35).

### Fibroblasts induced from ADSCs

According to the classic report,<sup>[14,15]</sup> the fibroblasts could be induced by ADSCs cultured in the 20 ng/ml bFGF and 20 ng/ml EGF medium. The culture medium was replaced with fibroblast-inducing medium containing 20 ng/ml bFGF (Gibco) and 20 ng/ml EGF (Gibco), and then ADSCs were cultured to 40–50% confluence.<sup>[15,16]</sup> Cells were cultured for another 2 weeks, and the medium was changed every 2–3 days. The cells were then identified by flow cytometry and immunohistochemistry.

### Flow cytometric analysis

The third passage of ADSCs was identified by flow cytometric analysis of CD29, CD44, CD90, and CD45 (Abcam, San Francisco, USA).<sup>[14]</sup> Suspensions of ADSCs were prepared at  $1 \times 10^6$  cells/ml. One hundred microliters of cell suspension were stained with 20  $\mu$ l rat CD29-FITC (1:100, ab93759), CD44-FITC (1:100, ab157107), CD45-FITC (1:100, ab10558), or CD90-FITC (1:20, ab123511) for 30 min at 37°C while protected from light. The samples were centrifuged for 5 min at 200  $\times g$  and then washed with PBS twice. A Becton-Dickinson FACScan flow cytometer (Becton-Dickinson, New York, USA) was used to analyze the samples after resuspension with 0.4 ml PBS.

### Proliferation assay

The effects of the growth factors on fibroblast proliferation were determined by Cell Counting Kit-8 (CCK-8, MedChem Express, New Jersey, USA).<sup>[16]</sup> Fibroblasts were seeded at  $2 \times 10^2$  cells/ml in 96-well plates and treated with various concentrations and combinations of growth factors for 1–7 days. The treatments were as follows: (i) bFGF at 0, 1, 10, and 100 ng/ml, (ii) EGF at 0, 1, 10, and 100 ng/ml, and (iii) both bFGF and EGF at 0, 1, 10, and 100 ng/ml each. The medium was changed every day. A CCK-8 assay was performed each day up to the 7<sup>th</sup> day. All experiments were repeated three times.

### Reverse transcription quantitative real-time polymerase chain reaction of Type I and III collagen mRNAs

ADSCs and fibroblasts were cultured in six-well plates. They were cultured in normal medium and medium of the various groups of growth factors for 7 days. Total RNA of the cells was extracted before and after induction from ADSCs, and total RNA of fibroblasts was extracted at the 7<sup>th</sup> day. RNA samples were reverse transcribed, and cDNA of the following genes was amplified by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR): Type I collagen (*Col-I*), Type III collagen (*Col-III*), and  $\beta$ -actin as the reference gene. Primer sequences [Table 1] were designed using Primer Premier (version 5.0, PREMIER

**Table 1: Primer sequences and molecular size of the product**

Gene	Primer sequences 5'-3'	Product
<i>Col-I</i>	F: ATGGTGGCAGCCAGTTTG R: AGGAATGGCAGGCAGAT	328 bp
<i>Col-III</i>	F: AAGAGCGGAGAATACTGGG R: CAATGTCATAGGGTGCAGATA R: CTCACCAGTCGGGTCTCAGTA	532 bp
$\beta$ -actin	F: GAGGGAAATCGTGCGTGAC R: CTGGAAGGTGGACAGTGAG	445 bp

F: Forward; R: Reverse; *Col-I*: Type I collagen; *Col-III*: Type III collagen.

Biosoft, California, USA).<sup>[17]</sup> PCR samples were subjected to agarose gel electrophoresis and imaging. Expression values were analyzed using Adobe Photoshop. The experiments were repeated three times.

### Immunohistochemistry of ADSCs

ADSCs were cultured on glass coverslips in six-well plates for 7 days. The cells were washed with PBS twice and then fixed in 4% paraformaldehyde. The samples were analyzed by immunohistochemistry of CD29, CD44, CD90, and CD45 and observed by light microscopy.<sup>[14]</sup> Fibroblasts were also cultured on glass coverslips in six-well plates for 7 days and then analyzed by immunohistochemistry of fibroblast-specific protein-1 (FSP-1).

### Statistical analysis

All data are presented as mean  $\pm$  standard deviation. Statistical analysis was performed with SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 6.0, GraphPad, Inc., La Jolla, California, USA) using nonparametric and ordinary one-way analysis of variance and multiple *t*-test. Values of  $P < 0.05$  were accepted as statistically significant.

## RESULTS

### Rat abdominal fat tissue-derived ADSCs isolation and immunophenotyping

ADSCs were derived successfully from rat pelvic tissue [Figure 1a]. After 3 days of primary culture, the ADSCs morphology became stable with an irregular polygonal shape [Figure 1c]. ADSCs grew markedly fast from day 4. Immunocytochemistry revealed expression of specific surface markers of ADSCs [Figure 1b]. ADSCs were positive for CD29, CD44, and CD90 and negative for CD45. Flow cytometry results showed the typical immunophenotype of ADSCs with expression rates of CD29, CD44, CD90, and CD45 at 99.39%, 94.6%, 89.6%, and 0.15%, respectively [Figure 1d].

### Identification of ADSCs-induced fibroblasts

Fibroblasts were induced from the third-passage ADSCs because they had a higher cell proliferation ability compared with the sixth passage cells (day 6:  $1.413 \pm 0.163$  [passage 3, P3] vs.  $0.686 \pm 0.157$  [P6],  $t = 5.547$ ,  $P = 0.0051$ ;

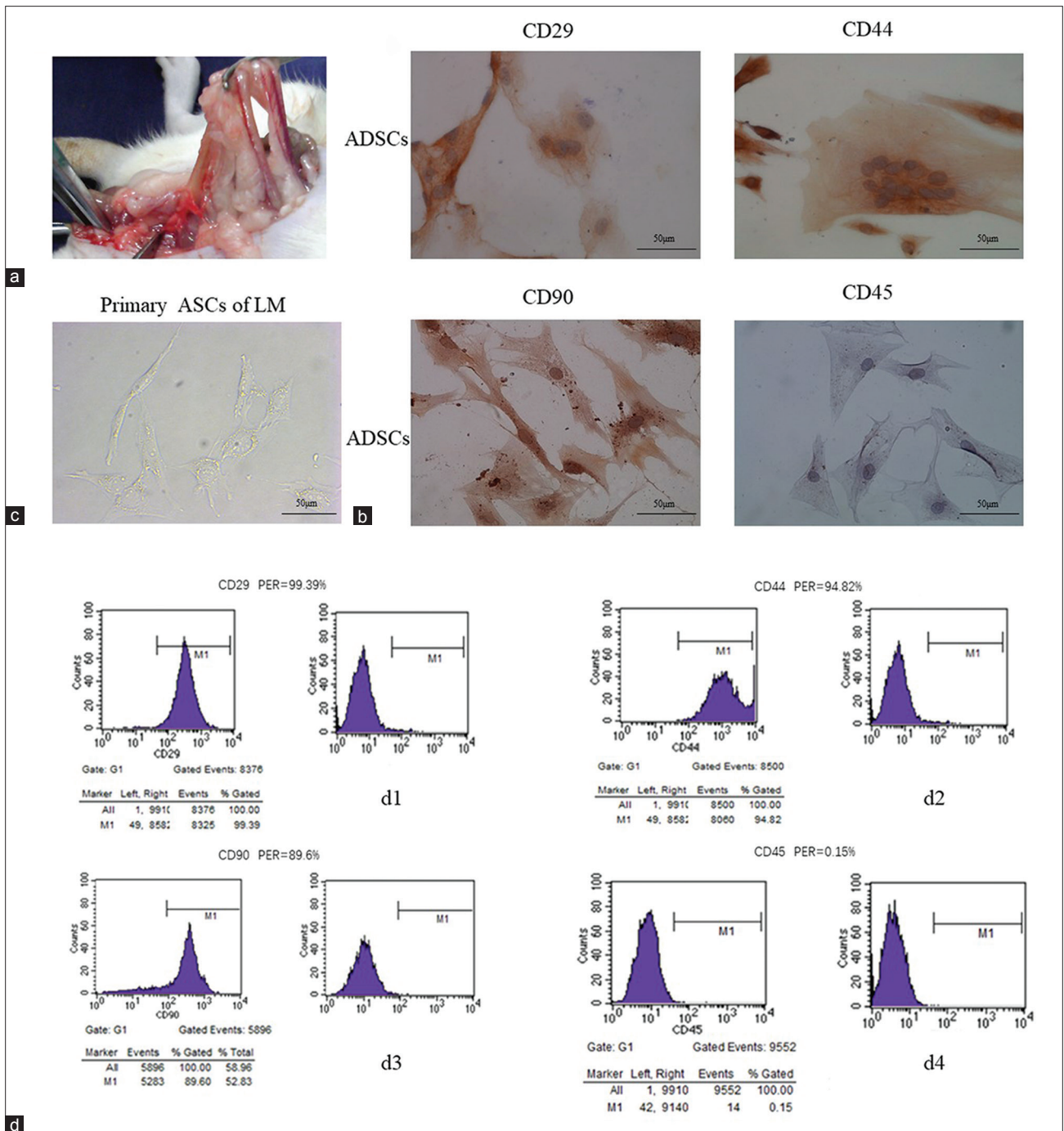
day 7:  $2.031 \pm 0.1476$  [P3] vs.  $1.435 \pm 0.1765$  [P6],  $t = 4.487$ ,  $P = 0.0110$ ; Figure 2a). After 2 weeks of induction, the cells grew with a long spindle morphology and were positive for FSP-1 [Figure 2b], a specific protein expressed by fibroblasts. In addition, the expression levels of *Col-I* and *Col-III* mRNAs in fibroblasts were increased after successful differentiation (*Col-I*:  $0.640 \pm 0.068$  [ADSC] vs.  $1.031 \pm 0.102$  [fibroblasts],  $t = 5.524$ ,  $P = 0.0052$ ; *Col-III*:  $0.692 \pm 0.131$  [ADSC] vs.  $0.954 \pm 0.074$  [fibroblasts],  $t = 3.016$ ,  $P = 0.0390$ ; Figure 2c).

### Basic fibroblast growth factor and epidermal growth factor promote cell proliferation of ADSCs-induced fibroblasts at an optimized concentration

The growth curves showed that cells proliferated in culture, and there were significant differences between the groups containing growth factors and groups without growth factors [Figure 3]. In groups containing bFGF, the growth of fibroblasts was enhanced markedly by 10 and 100 ng/ml bFGF, and the number of cells was increased dramatically [Figure 3a]. However, in the 100 ng/ml bFGF group, fibroblast numbers did not increase further, indicating that 10 ng/ml bFGF was a more suitable culture condition to maintain and promote the proliferation of fibroblasts (0 ng/ml,  $1.710 \pm 0.205$ ; 1 ng/ml,  $2.318 \pm 0.260$ ; 10 ng/ml,  $2.899 \pm 0.1057$ ; and 100 ng/ml,  $2.360 \pm 0.2001$ ;  $t_{0-1} = 3.1806$ ,  $P_{0-1} = 0.0335$ ,  $t_{1-10} = 3.586$ ,  $P_{1-10} = 0.0230$ ,  $t_{10-100} = 4.127$ , and  $P_{10-100} = 0.0140$ ;  $F = 17.64$ ,  $P = 0.0007$ ). In groups containing 10 ng/ml EGF, the growth of fibroblasts was enhanced markedly, and the number of cells was increased dramatically [Figure 3b] compared with the 100 ng/ml EGF group, indicating that 10 ng/ml EGF was a more suitable culture condition to maintain and promote the proliferation of fibroblasts (0 ng/ml,  $1.667 \pm 0.232$ ; 1 ng/ml,  $2.203 \pm 0.208$ ; 10 ng/ml,  $2.697 \pm 0.126$ ;  $2.455 \pm 0.054$ ;  $t_{0-1} = 2.9795$ ,  $P_{0-1} = 0.0408$ ,  $t_{1-10} = 3.518$ ,  $P_{1-10} = 0.0240$ ,  $t_{10-100} = 3.057$ , and  $P_{10-100} = 0.0370$ ;  $F = 11.66$ ,  $P = 0.0027$ ). The combination of 10 ng/ml bFGF and 10 ng/ml EGF had the optimal effect on promoting fibroblast proliferation among the various concentrations with the number of cells increased dramatically (0 ng/ml,  $1.703 \pm 0.254$ ; 1 ng/ml,  $2.550 \pm 0.244$ ; 10 ng/ml,  $3.011 \pm 0.102$ ; and 100 ng/ml,  $2.620 \pm 0.115$ ;  $t_{0-1} = 4.165$ ,  $P_{0-1} = 0.0140$ ,  $t_{1-10} = 3.019$ ,  $P_{1-10} = 0.0390$ ,  $t_{10-100} = 4.405$ , and  $P_{10-100} = 0.0110$ ;  $F = 24.64$ ,  $P = 0.0002$ ; Figure 3c).

### Type I and III collagens expression in ADSCs-induced fibroblasts under the optimized concentration of combined basic fibroblast growth factor and epidermal growth factor treatment

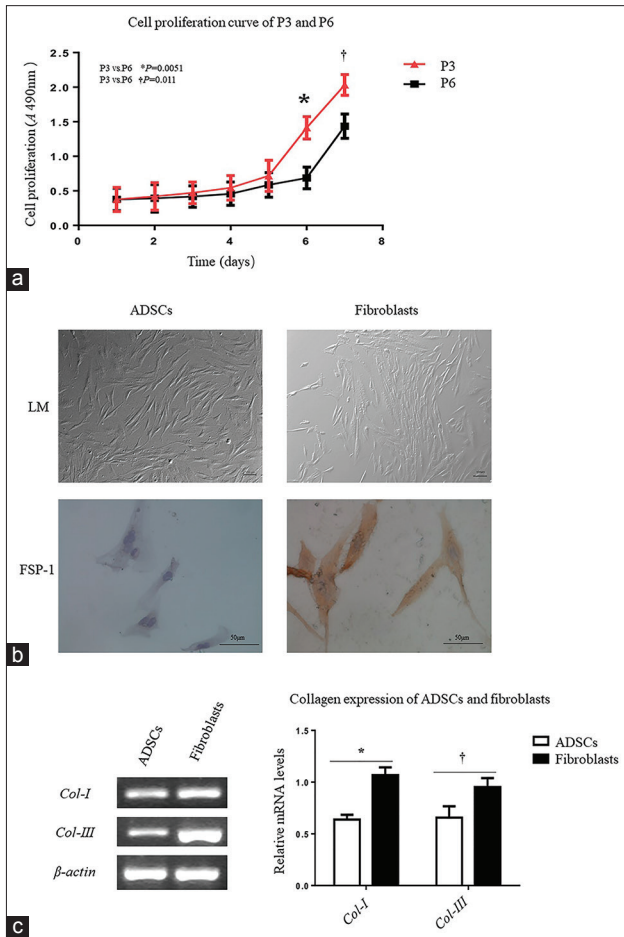
To evaluate the effects of the various experimental conditions of bFGF and EGF on the capacity of the fibroblastic cells to express *Col-I* and *Col-III*, RT-qPCR was used to measure the mRNA levels of *Col-I* and *Col-III*. As an effect of the various concentrations of bFGF and EGF, the expression of *Col-I* and *Col-III* in fibroblasts was significantly different [Figure 4a]. In the 10 ng/ml bFGF group, expression of *Col-I* was higher



**Figure 1:** Isolation and identification of ADSCs. (a) ADSCs were derived from rat groin adipose tissue. (b) Immunohistochemical staining ( $\times 400$ ) of the third passage ADSCs showed that typical ADSC surface markers CD29, CD44, and CD90 were positive and CD45 was negative. (c) Light microscopy image ( $\times 400$ ) of the third passage ADSC morphology. Cells were multinucleated with a polygonal irregular shape. (d) Flow cytometry for phenotypic identification of ADSCs. d1: CD29 was highly expressed (99.39%); d2: CD44 was highly expressed (94.82%); d3: CD90 was highly expressed (89.6%); d4: CD45 was weakly expressed (0.15%). ADSCs: Adipose mesenchymal stem cells; LM: Light microscope; PER: Positive expression rate.

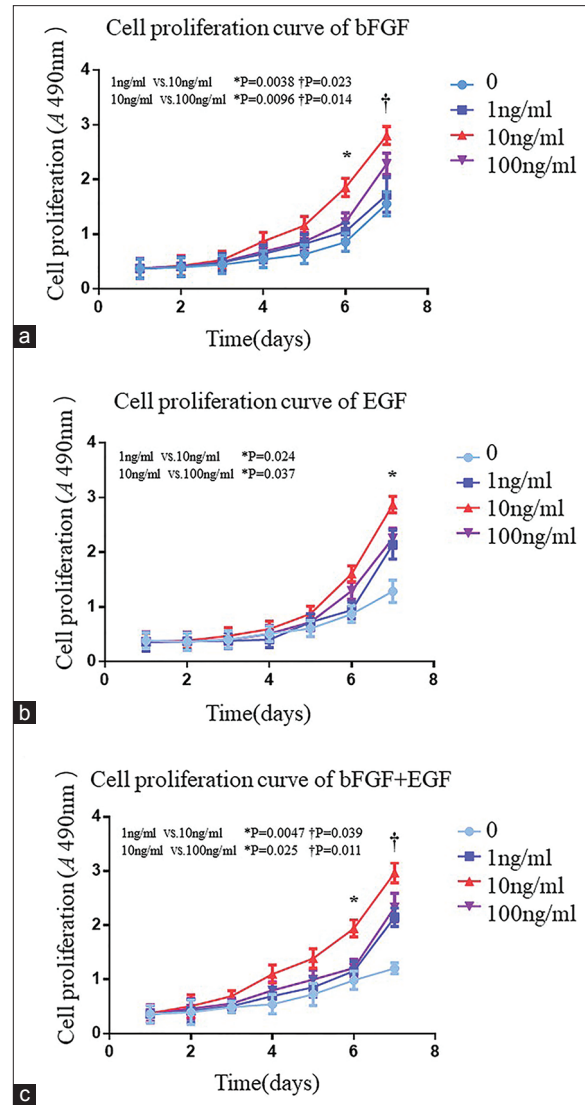
than that in the other two bFGF groups ( $0.699 \pm 0.074$  vs.  $1.608 \pm 0.114$  vs.  $1.500 \pm 0.015$ ,  $F = 79.13$ ,  $P = 0.0025$ ; Figure 4b). Among the EGF groups, there was slightly higher expression of *Col-1* in the 10 ng/ml group than that in the other concentrations ( $0.219 \pm 0.018$  vs.  $0.892 \pm 0.022$  vs.  $0.350 \pm 0.048$ ,  $F = 245.50$ ,  $P = 0.0005$ ; Figure 4b). The *Col-1* mRNA expression of group 10 ng/ml bFGF mixed with

10 ng/ml EGF was the highest in the double growth factor groups ( $0.858 \pm 0.023$  vs.  $1.883 \pm 0.099$  vs.  $1.840 \pm 0.089$ ,  $F = 110.50$ ,  $P = 0.0015$ ; Figure 4b) and also the highest among all 10 ng/ml growth factor groups as well ( $1.608 \pm 0.114$  vs.  $0.892 \pm 0.022$  vs.  $1.883 \pm 0.099$ ,  $F = 67.45$ ,  $P = 0.0032$ ; Figure 4b). In addition, the expression of *Col-1* in the 1 ng/ml mixed growth factors' group was obviously higher than the

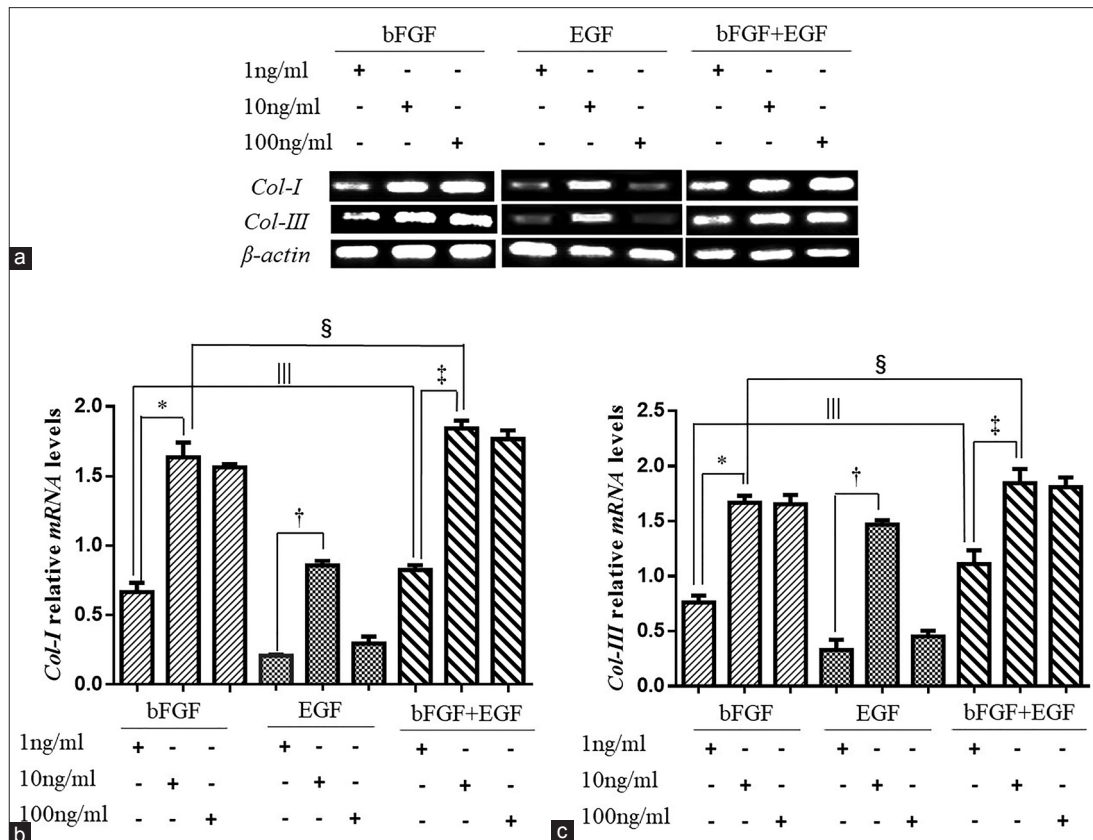


**Figure 2:** Identification of ADSCs before and after differentiation to fibroblasts. (a) Cell proliferation was evaluated by CCK8 assays in 7 days in 96-well plates by comparing the third passage and the sixth passage ADSCs. The growth curve was indicating that the third passage ADSCs had better proliferation activity (day 6:  $1.413 \pm 0.163$  [P3] vs.  $0.686 \pm 0.157$  [P6],  $t = 5.547$ ,  $P = 0.0051$ ; day 7:  $2.031 \pm 0.1476$  [P3] vs.  $1.435 \pm 0.1765$  [P6],  $t = 4.487$ ,  $P = 0.0110$ ). (b) The third passage ADSCs were cultured in induction medium for 2 weeks. The induced cells grew as long spindle cells with compact cytoplasm that was different from the loose cytoplasm of ADSCs under a light microscope ( $\times 400$ ). FSP-1 was only expressed after ADSCs induction into fibroblasts ( $\times 400$ ). (c) Expression of Type I and III collagens was evaluated at the mRNA level. Total RNA of cells was extracted before and after induction from ADSCs. The RNA samples were reverse transcribed, and cDNA of the following genes was amplified by RT-qPCR. The mRNA expression levels of Type I and III collagens in fibroblasts were increased after successful differentiation (*Col-I*:  $0.640 \pm 0.068$  [ADSC] vs.  $1.031 \pm 0.102$  [fibroblasts],  $t = 5.524$ ,  $*P = 0.0052$ ; *Col-III*:  $0.692 \pm 0.131$  [ADSC] vs.  $0.954 \pm 0.074$  [fibroblasts],  $t = 3.016$ ,  $^{\dagger}P = 0.0390$ ). ADSCs: Adipose mesenchymal stem cells; P3: The third passage ADSCs; P6: The sixth passage ADSCs; CCK8: Cell Counting Kit-8; SD: Standard deviation; LM: Light microscope; FSP-1: Fibroblast-specific protein-1; cDNA: Complementary DNA; RT-qPCR: Reverse transcription quantitative real-time polymerase chain reaction; *Col-I*: Type I collagen; *Col-III*: Type III collagen.

expression of *Col-I* in 1 ng/ml EGF group and 1 ng/ml bFGF group ( $0.699 \pm 0.074$  vs.  $0.892 \pm 0.022$  vs.  $0.858 \pm 0.023$ ,



**Figure 3:** Effects of the various concentrations of bFGF and EGF on cell proliferation of fibroblasts induced from ADSCs. (a) Fibroblasts were cultured in 96-well plates and evaluated every 24 h by CCK-8 assays. OD values at 490 nm of fibroblasts were shown. Only the OD value in the 10 ng/ml bFGF group (red curve) was increased significantly (day 6:  $0.754 \pm 0.114$ ,  $1.069 \pm 0.155$ ,  $1.855 \pm 0.166$ ,  $1.216 \pm 0.170$ ,  $t = 2.835$ ,  $P = 0.0470$ ,  $t = 5.994$ ,  $P = 0.0038$ ,  $t = 4.658$ ,  $P = 0.0096$ ;  $F = 27.52$ ,  $*P = 0.0001$ ; day 7:  $1.710 \pm 0.205$ ,  $2.318 \pm 0.260$ ,  $2.899 \pm 0.106$ ,  $2.360 \pm 0.200$ ,  $t = 3.1806$ ,  $P = 0.0335$ ,  $t = 3.586$ ,  $P = 0.0230$ ,  $t = 4.127$ ,  $P = 0.0140$ ,  $t = 3.586$ ,  $P = 0.0340$ ;  $F = 17.64$ ,  $^{\dagger}P = 0.0007$ ). (b) Effect of EGF on fibroblasts. Only the OD value in the 10 ng/ml EGF group (red curve) was increased significantly during the past 2 days ( $1.667 \pm 0.232$ ,  $2.203 \pm 0.208$ ,  $2.697 \pm 0.166$ , and  $2.343 \pm 0.252$ ;  $t = 2.9795$ ,  $P = 0.0408$ ;  $t = 3.215$ ,  $P = 0.0324$ ; and  $t = 2.031$ ,  $P = 0.1100$ ;  $F = 11.66$ ,  $*P = 0.0027$ ). (c) Effects of bFGF mixed with EGF on fibroblasts. The OD value was increased obviously in the intermediate concentration group (red curve) (day 6:  $1.204 \pm 0.100$ ,  $2.149 \pm 0.170$ ,  $2.909 \pm 0.182$ , and  $2.330 \pm 0.262$ ;  $t = 8.590$ ,  $P = 0.0011$ ;  $t = 5.678$ ,  $P = 0.0047$ ; and  $t = 3.457$ ,  $P = 0.0250$ ;  $F = 45.15$ ,  $*P < 0.0001$ ; day 7:  $1.703 \pm 0.254$ ,  $2.550 \pm 0.244$ ,  $3.011 \pm 0.102$ , and  $2.620 \pm 0.115$ ;  $t = 4.165$ ,  $P = 0.0140$ ;  $t = 3.019$ ,  $P = 0.0390$ ;  $t = 4.405$ ,  $P = 0.0110$ ;  $F = 24.64$ ,  $^{\dagger}P = 0.0002$ ). ADSCs: Adipose mesenchymal stem cells; CCK8: Cell Counting Kit-8; SD: Standard deviation; OD: Optical density; bFGF: Basic fibroblast growth factor; EGF: Epidermal growth factor.



**Figure 4:** Different mRNA expression levels of Type I and III collagens in fibroblasts induced from ADSCs in various concentrations of growth factors. (a) Fibroblasts were cultured in 6-well plates. Relative mRNA expression levels of *Col-I* and *Col-III* in the various groups of fibroblasts at the 7<sup>th</sup> day were measured by RT-qPCR. Values were calculated as a percentage of expression relative to  $\beta$ -actin. (b) Relative mRNA expression levels of *Col-I* of different concentration of growth factors. In the bFGF group, expression of *Col-I* of 10 ng/ml was highest ( $0.699 \pm 0.074$ ,  $1.608 \pm 0.114$ , and  $1.500 \pm 0.015$ ,  $F = 79.13$ ,  $*P = 0.0025$ ). Among the EGF groups, the expression of *Col-I* in the 10 ng/ml group was more than other two concentrations ( $0.219 \pm 0.018$ ,  $0.892 \pm 0.022$ , and  $0.350 \pm 0.048$ ,  $F = 245.50$ ,  $^{\dagger}P = 0.0005$ ). In the groups of two kinds of growth factors, the concentration of 10 ng/ml had the most effective promoting effect on the expression of *Col-I* ( $0.858 \pm 0.023$ ,  $1.883 \pm 0.099$ , and  $1.840 \pm 0.089$ ,  $F = 110.50$ ,  $*P = 0.0015$ ). At the same concentration of 10 ng/ml, the expression of *Col-I* of bFGF mixed with EGF group was better than the other two groups ( $1.608 \pm 0.114$ ,  $0.892 \pm 0.022$ , and  $1.883 \pm 0.099$ ;  $F = 67.45$ ,  $^{\S}P = 0.0032$ ). Even at the lowest concentration, 1 ng/ml, the group with two kinds of growth factors had better effect of improving the expression of *Col-I* than the single growth factor group ( $0.699 \pm 0.074$ ,  $0.892 \pm 0.022$ , and  $0.858 \pm 0.023$ ,  $F = 104.90$ ,  $^{\parallel}P = 0.0017$ ). (c) Relative mRNA expression levels of *Col-III* of different concentration of growth factors. In the bFGF group, the *Col-III* expression of concentration of 10 ng/ml was higher than that in the 1 ng/ml bFGF group ( $0.649 \pm 0.048$ ,  $1.686 \pm 0.045$ , and  $1.612 \pm 0.080$ ,  $F = 187.20$ ,  $*P = 0.0007$ ) but was not different compared with the 100 ng/ml bFGF group. In the EGF groups, only the expression of *Col-III* in the 10 ng/ml group was highest ( $0.378 \pm 0.093$ ,  $1.457 \pm 0.023$ , and  $0.490 \pm 0.055$ ,  $F = 173.1$ ,  $^{\dagger}P = 0.0008$ ). *Col-III* expression in the group of bFGF mixed with EGF was the highest at 10 ng/ml ( $1.104 \pm 0.110$ ,  $1.894 \pm 0.121$ , and  $1.801 \pm 0.098$ ,  $F = 30.78$ ,  $^{\S}P = 0.010$ ). The *Col-III* expression at 1 ng/ml of two growth factors' group was still higher than that in the 1 ng/ml bFGF and EGF alone groups ( $0.649 \pm 0.048$ ,  $0.378 \pm 0.093$ , and  $1.104 \pm 0.110$ ,  $F = 35.05$ ,  $^{\parallel}P = 0.0083$ ). (c) At the 10 ng/ml, the group contained two growth factors made the fibroblasts expressed more *Col-III* ( $1.686 \pm 0.045$ ,  $1.457 \pm 0.023$ , and  $1.894 \pm 0.121$ ,  $F = 16.67$ ,  $^{\parallel}P = 0.0237$ ). ADSCs: Adipose mesenchymal stem cells; RT-qPCR: Reverse transcription quantitative real-time polymerase chain reaction; *Col-I*: Type I collagen; *Col-III*: Type III collagen; bFGF: Basic fibroblast growth factor; EGF: Epidermal growth factor.

$F = 104.90$ ,  $P = 0.0017$ ; Figure 4b). The increasing trend of *Col-III* expression in the various concentration groups was similar to that of *Col-I* expression. In the 10 ng/ml bFGF group, *Col-III* expression was higher than in the 1 ng/ml bFGF group and 100 ng/ml bFGF group ( $0.649 \pm 0.048$  vs.  $1.686 \pm 0.045$  vs.  $1.612 \pm 0.080$ ,  $F = 187.20$ ,  $P = 0.0007$ ; Figure 4c) but was not different compared with the 100 ng/ml bFGF group. In EGF groups, there was obviously higher expression of *Col-III* in the 10 ng/ml group than that in the other concentrations ( $0.378 \pm 0.093$  vs.  $1.457 \pm 0.023$  vs.  $0.490 \pm 0.055$ ,  $F = 173.10$ ,  $P = 0.0008$ ; Figure 4c). *Col-III*

expression in the group of bFGF mixed with EGF was the highest at 10 ng/ml each ( $1.104 \pm 0.110$  vs.  $1.894 \pm 0.121$  vs.  $1.801 \pm 0.098$ ,  $F = 30.78$ ,  $P = 0.0100$ ;  $1.686 \pm 0.045$  vs.  $1.457 \pm 0.023$  vs.  $1.894 \pm 0.121$ ,  $F = 16.67$ ,  $P = 0.0237$ ; Figure 4c), and *Col-III* expression at 1 ng/ml was still higher than that in the 1 ng/ml bFGF or EGF alone groups ( $0.649 \pm 0.048$  vs.  $0.378 \pm 0.093$  vs.  $1.104 \pm 0.110$ ,  $F = 35.05$ ,  $P = 0.0083$ ; Figure 4c). The ratio of *Col-I* expression to *Col-III* expression of group with 10 ng/ml bFGF mixed with EGF was just less than one and compared with other groups with no significant difference.

## DISCUSSION

In this study, we found that the cell proliferation did not have a linear relationship with the concentrations of bFGF and EGF. However, the most effective concentration was 10 ng/ml. bFGF significantly promoted cell proliferation and collagen expression of fibroblasts. EGF mildly promoted fibroblast proliferation and had a slight effect on collagen expression. When bFGF was combined with EGF, we found significant promotion of fibroblast proliferation and expression of collagens. Therefore, bFGF plays more important roles in expression of *Col-I* and *Col-III*. This study revealed that the combined use of bFGF/EGF significantly improved fibroblast proliferation and differentiation.

Optimized concentrations of growth factors can simulate a microenvironment in tissue engineering, which is closer to the microenvironment *in vivo*. Growth factors such as bFGF are present in the microenvironment of tissue repair after tissue damage and play an important role in repair of the pelvic ligament. bFGF and EGF are the most widely studied growth factors.<sup>[17,18]</sup> bFGF stimulates the proliferation of fibroblasts. Moreover, Wang *et al.*<sup>[19]</sup> showed that bFGF promotes collagens' expression. EGF has been shown to affect cell proliferation and collagen expression in wound repair and reconstruction of tissue.<sup>[13,17]</sup> For different organs, there may be different kinds of EGF receptors with different effects on cell proliferation and protein expression.<sup>[20-22]</sup> Many studies<sup>[22-24]</sup> have focused on how growth factors mediate signaling pathways that affect cell proliferation and protein expression, while there were few studies on the physiological concentrations of growth factors. Accumulating studies have focused on new materials and various growth factors to make the microenvironment of seed cells in tissue engineering closer to the microenvironment *in vivo*. Therefore, construction of the microenvironment in tissue engineering *in vitro* with optimal concentrations of growth factors is important. The optimized concentration of combined bFGF and EGF promoted the proliferation of fibroblasts, which are the main cells in repair of pelvic ligaments, contributing to efficient construction of tissue with seed cells for tissue engineering.

*Col-I* and *III* play a very important role in the repair of defects in female pelvic floor tissue, which obviously influence the elasticity and toughness of ligaments in pelvic floor tissue.<sup>[25]</sup> For ideal repair of pelvic floor ligament tissue, high expression of *Col-I* will lead to more tenacity, and high expression of *Col-III* will result in greater elasticity of the ligament.<sup>[26]</sup> Both *Col-I* and *Col-III* increased in the early time after pelvic floor damaged in a very short time, while the flawed tissue stimulated and promoted the regeneration of surroundings which might gather a lot of inflammatory cells with various kinds of growth factors. However, the collagens would decrease in the next period, in which inflammatory cytokines stimulated cells to release a large amount of proteases to the extracellular matrix. Some proteases, such as fibrinolytic enzyme, kinase releasing enzyme, and matrix metalloproteinases, could inhibit

the formation of collagen or promote the degradation of collagen obviously. The final result was collagens reduced in the sacrospinous ligament of patients with pelvic organ prolapse disease, and what was more significant for *Col-III*. The ratio of *Col-I* to *Col-III* was calculated and compared in women with pelvic floor dysfunction or not and found that the ratio of *Col-I* to *Col-III* increased.<sup>[26-30]</sup> Therefore, it was very important to replenish collagen in the process of pelvic floor tissue repair. By supplementing *Col-I* and *III* exogenously and resisting the degradation of local tissues, the collagen content of local tissues was improved, and local tissue mechanical properties were enhanced. In this study, certain kinds of growth factors induce fibroblasts to secrete of *Col-I* and *Col-III* to the microenvironment, which might improve the efficiency of tissue engineering.<sup>[31]</sup> By secreting collagens, surrounding cells can regenerate to repair tissue defects with a better microenvironment. The optimized concentration of combined bFGF and EGF increases the expression of *Col-I* and *Col-III* effectively. Maintaining the optimal concentrations of bFGF and EGF in culture might contribute to the stability of the tissue engineering microenvironment.

At the early stage of growth factor treatments, the various concentrations did not have obviously different effects on cell proliferation, and the small growth curve may be the result of the excessively low initial densities of cells. The effects on cell proliferation by treatment with 10 ng/ml bFGF, EGF, and both bFGF and EGF at the 7<sup>th</sup> day were similar, which might have been caused by contact inhibition of cells, and the cell densities of the three groups reached their maximum in 96-well plates. Therefore, there was no significant difference on the optical density value of the 7<sup>th</sup> day of fibroblasts among different growth factor groups. The mRNA levels of *Col-I* and *Col-III* in cells treated with 10 ng/ml bFGF, EGF, and both bFGF and EGF were similar, which might be caused by cells reaching their maximum densities in six-well plates. In addition, the reason for the significant increase of collagen expression in the 10 ng/ml EGF group may be that the number of cells was highest at this concentration. Because cell activity *in vivo* is regulated by interactions of several kinds of growth factors from the microenvironment, the interactions of these factors need further study at molecular and cellular levels.

In conclusion, the effects of bFGF and EGF on cell proliferation and protein expression are not stronger as their concentrations increased linearly. The optimal concentration of both bFGF and EGF for cell proliferation and collagen expression of fibroblasts was 10 ng/ml at which fibroblasts grew faster and secreted more *Col-I* and *Col-III* into the extracellular matrix, which might contribute to microenvironment stability. In tissue engineering for pelvic floor reconstruction *in vitro*, the effective concentrations and combination of bFGF and EGF can make the microenvironment more efficient and advantageous. The optimal concentration of growth factors should be considered in research to rebuild or repair organs.

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## Conflicts of interest

There are no conflicts of interest.

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# 不同浓度碱性成纤维细胞生长因子和表皮生长因子对促进成纤维细胞增殖和胶原蛋白表达的作用与盆底组织再生相关性研究

## 摘要

**背景:** 在盆底韧带组织工程的研究中, 成纤维细胞是主要的种子细胞。碱性成纤维细胞生长因子 (bFGF) 和表皮生长因子 (EGF) 研究广泛、但浓度多样。本研究旨在优化bFGF和EGF的作用浓度, 并探索其对成纤维细胞的增殖和胶原蛋白表达的作用。

**方法:** 提取大鼠脂肪进行脂肪间充质干细胞原代培养, 并将其诱导分化为成纤维细胞, 采用流式细胞术和免疫组化方法进行细胞表型鉴定。生长因子被分为三组: 1) bFGF单作用组, 2) EGF单作用组, 3) bFGF+EGF联合作用组, 浓度均分为0,1,10, 和100 ng/ml四种进行作用。以CCK8检测法检测细胞增殖, 通过RT-PCR技术检测成纤维细胞I型和III型胶原蛋白mRNA的表达水平。数据分析采用SPSS软件和GraphPad Prism软件进行单因素方差分析和多样本t检验。

**结果:** 脂肪间充质干细胞提取成功并进行原代培养, 并完成CD29, CD44, CD90和CD45的细胞表型流式鉴定, 成纤维细胞诱导分化成功, 且伴随I型和III型胶原蛋白表达增高( $F=1.29, P=0.0390$ )和FSP-1的表达阳性。bFGF组、EGF组和bFGF+EGF组中浓度为10ng/ml的实验组中促进成纤维细胞增殖作用最强(all  $P<0.05$ ), 10ng/mlbFGF联合EGF作用组中的I型和III型胶原蛋白的mRNA表达水平最高(all  $P<0.05$ )。

**结论:** bFGF和EGF的优化作用浓度为10ng/ml, 该浓度作用下成纤维细胞增殖更快, I型和III型胶原蛋白分泌更多, 有助于在体外重建组织工程中维持盆底组织细胞微环境的稳定性。