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Research Article

Effect of recombinant Sox9 protein on the expression of cartilage-specific genes in human dermal fibroblasts cell culture

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Abstract

Introduction: Damage to the hyaline layer of large joints resulting from injuries or age-related changes restricts their mobility. The repair of these disorders is an actual issue in medicine. One of the promising therapies is the usage of cell engineering constructs based on a biodegradable scaffold and a modified cell culture. A frequently used method to modify the proliferation of cell culture for tissue engineering of hyaline cartilage, which makes it possible to introduce an experimental technique into clinical practice, is the application of recombinant proteins that affect chondrogenesis and lead to increase synthesis of extracellular matrix proteins. The goal of this work was to elucidate the effect of the key transcription factor in the chondrogenesis process – Sox9 protein – on the expression of genes responsible for chondrogenesis (*Tgfβ3*, *Sox9*, *Acan*, *Comp*, *Col2a1*).

Materials and methods: Human dermal fibroblasts were used as a cell culture; recombinant Sox9 was added at each change of medium; the modification was carried out for 21 days, and difference in gene expression was determined by real-time PCR and -ΔΔCt method.

Results and discussion: To assess the effectiveness of fibroblast modification, we analyzed the changing of expression of genes responsible for chondrogenesis (*Tgfβ3*, *Sox9*, *Col2a1*, *Acan*, *Comp*). We studied the direct effect of different concentrations of the recombinant Sox9 protein on the proliferation of dermal fibroblasts in the chondrogenic direction. We showed that the addition of the recombinant Sox9 protein in various concentration did not significantly change the expression of both the genes encoding proteins of the extracellular matrix of hyaline cartilage (*Acan, Col2a1, Comp*) and the genes encoding chondrogenesis inducers (*Tgfβ3, Sox9*).

Conclusion: As a result of the experiments, it was shown that the recombinant Sox9 protein has practically no effect on chondrogenic differentiation and does not significantly change the expression of chondrogenesis genes.

Keywords

Sox9, chondrogenesis, fibroblasts, gene expression, recombinant proteinroloquinolones.

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Introduction

The hyaline cartilage covers the surfaces of the epiphyses of the bones articulating in the joint. The movement of the joint is provided by the sliding of hyaline layers. This tissue consists of extracellular matrix proteins (the most important proteins are type 2 collagen and aggrecan), a large amount of water (more than 2/3 by weight) and a small number of differentiated cells (Komarraju et al. 2020). Hyaline cartilage has a limited ability for recovery, and its defects only increase over time and lead to further tissue degradation and dysfunction of the joint (Bozhokin et al. 2017). The factors which promote a start of articular surface damage are: age-related changes (Anderson and Loeser 2010; Lotz and Loeser 2012), excessive exercise (Flanigan et al. 2010), genetic predisposition (Zabello et al. 2015), overweight (Kulkarni et al. 2016), inflammatory diseases of the joints, defects of the ligamentous apparatus, as well as native avascular and less cellular hyaline cartilage structure (Komarraju et al. 2020; Kulyaba et al. 2020).

Tissue engineering is one of actively developed methods that allows in the long term to delay and ideally avoid the need for joint arthroplasty (Makris et al. 2015; Bozhokin et al. 2016; Jeuken et al. 2016). This area of science includes a developing and implantation of a tissue-engineering structure based on a biodegradable scaffolds, containing a modified cell culture in the damaged area of the articular surface (Lammi et al. 2018). It is noteworthy that the restoration of hyaline cartilage using a tissue engineering method suggests the possibility of allogeneic cell products usage, for example, dermal fibroblasts or mesenchymal stem cells (Bozhokin et al. 2022). The advantages of these cell cultures are their availability (cell culture can be obtained from a specialized cell bank), the possibility of reprogramming (Makris et al. 2015), and the non-immunogenicity (Zorina et al. 2010).

The most important transcription factor that activates extracellular matrix genes in proliferating chondrocytes is the Sox9 protein (Song and Park 2020). Sox9 binds DNA via highly conservative high mobility group (HMG) domain, which contains nuclear localization sequences (NLS) and the nuclear export sequences (NES) (Jo et al. 2014). Sox9 also has a self-dimerization domain and a transactivation domain at the C-terminus. Sox9 forms dimers that recruit SoxD (Sox5/6) dimers to activate type 2 collagen gene (*Col2a1*) transcription via a conserved enhancer sequence within the first intron (Ikeda et al. 2004). Sox9 promotes *Acan* (aggrecan) and *Comp* (cartilage oligomeric matrix protein) expression in human chondrocytes (Zhang et al. 2015).

In recent years, in order to obtain a more effective replacement of a superficial cartilage defect, researchers have usually modified cell cultures, for example, by use of various recombinant proteins that affect chondrogenesis (Crecente-Campo et al. 2017; Wang et al. 2017; Bozhokin et al. 2018; Cheng et al. 2018). However, we have not found any works studying the chondrogenic effect of this recombinant protein on the culture of fibroblasts or chondrocytes when added to a nutrient medium.

In this work, we studied the effect of the recombinant Sox9 protein on the induction of the expression of chondrogenesis genes in human dermal fibroblasts.

Materials and methods

Cell culture

The study was performed on a dermal fibroblasts cell culture of DF2 (DF2 cells were obtained from the shared research facility “Vertebrate Cell Culture Collection” supported by the Ministry of Science and Higher Education of the Russian Federation (Agreement №075-15-2021-683)). Cells were cultured until the third passage under standard conditions (37 °C, 5% CO_2) in DMEM (Gibco, USA) supplemented with fetal bovine serum (10%; Gibco, USA) and a mixture of antibiotics penicillin (50000 units/ml) and streptomycin (50 µg/ml) (Gibco, USA).

Cell modification

Fibroblasts were modified with using the recombinant Sox9 protein (Sigma, USA) at concentrations of 1, 5, and 20 ng/µl. Bovine serum albumin (BSA; Serva, Germany) was used as a control at concentrations of 2 and 20 ng/µl. Proteins were added at each change of a culture medium. Fibroblasts were incubated for 7, 14, and 21 days, then separated from the culture plastic using Tryple Express Enzyme (Thermofisher, USA) and frozen at –80 °C.

Isolation of RNA and obtaining cDNA

Total RNA was isolated from cells using Extract RNA reagent (Evrogen, Russia). RNA was used in a reverse transcription reaction using a MMLV RT kit (Evrogen, Russia) and a T100 Thermal Cycler (BioRad, USA).

Selection of primers for RT-PCR

To assess the effectiveness of fibroblast modification, we analyzed the changing of expression of genes responsible for chondrogenesis (*Tgfβ3*, *Sox9*, *Col2a1*, *Acan*, *Comp*) by real-time PCR (RT-PCR) (Table 1). Nucleotide sequences were taken from the Ensembl database (https://www.ensembl.org/index.html). Primers were selected according to the general design parameters and capture of exon-exon regions to prevent amplification of genomic DNA. The specificity of the amplicon and primer pairs was checked with the BLAST alignment tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. List of analyzed genes and primers used for real-time PCR

Gene	Forward primer	Reverse primer	Amplified region length, bp	Function and role
Gapdh	5'-atgttccaatatgattccacc	5'- atggtggtgaagacgcca	179	housekeeping gene (catalyses the sixth step of glycolysis)
Acan	5'- ttcgggcagaagaaggac	5'- ttctgtagtctgcgtttgta	170	participation in chondrogenesis, extracellular matrix protein
Col2a1	5'- cattgcctatctggacgaa	5'- cccacttaccggtatgttt	149	participation in chondrogenesis, extracellular matrix protein
Comp	5'- gtgactgtgcccaactca	5'- tttggtcgtcgttcttctg	202	participation in chondrogenesis, extracellular matrix protein
Sox9	5'- catcttcaaggcgctgca	5'- tcgcttcaggtcagcctt	157	participation in chondrogenesis, transcription factor
Tgfβ3	5'- cagtggagaaaaatagaacca	5'- agattcttgccaccgatata	160	participation in chondrogenesis, cytokine

RT-PCR

RT-PCR was performed using the qPCRmix-HS SYBR kit (Evrogen, Russia) and previously selected and synthesized primers (Table 1). The reaction was set up on a CFX96 Touch device (BioRad, USA) according to the following scheme: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 3 min, 58 °C for 20 s and 72 °C for 20 s.

Gene expression values were assessed in terms of fold induction relative to the untreated cell population (control) using method 2-ΔΔCt, which shows the amount of amplification product relative to the *Gapdh* housekeeping gene calibration sample (Livak and Schmittgen 2001).

Results and discussion

Total RNA was isolated from all obtained cell samples with concentration ranged from 2.6 ng/μl to 32.01 ng/μl. Reverse transcription of the isolated RNA was performed to obtain cDNA.

The optimal primer annealing temperature according to the results of preliminary experiments was 58 °C. At this temperature, there was no nonspecific binding of primers, and only one amplification product was noted on the gel electrophoregram.

After PCR optimization, a semi-quantitative polymerase chain reaction was performed. Data on the threshold cycle of each of the samples were obtained (Fig. 1). We did not find any substantial change in *Tgfβ3* and *Sox9* expression independent of periods of observation or recombinant protein concentration. The expression of *Col2a1*, *Acan* and *Comp* slightly increased on 7th day independent of concentration of recombinant Sox9, but then it returned to basic level on 14th and 21st day (Fig. 2).

The key step for development of techniques for the replacement of hyaline cartilage defects using the method of cell engineering is the effective modification of cells (Shestovskaya et al. 2021). Many researchers develop tissue engineering constructs based on autologous mesenchymal stem cells or chondrocytes, which greatly complicates and increases the cost of preclinical research and clinical application of this method (Tsumaki 2013; Tam et al. 2014; NCT02291926; NCT01050816). Fibroblasts, in our opinion, are some of the promising sources for cell engineering of hyaline cartilage, since this culture can be easily and minimally invasively isolated, non-immunogenic, and subject to modification for the formation of articular cartilage regenerate, which was shown in (Yu et

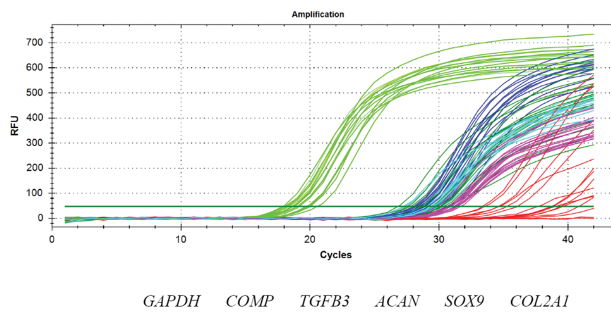

Figure 1. Amplification plot showing PCR profiles

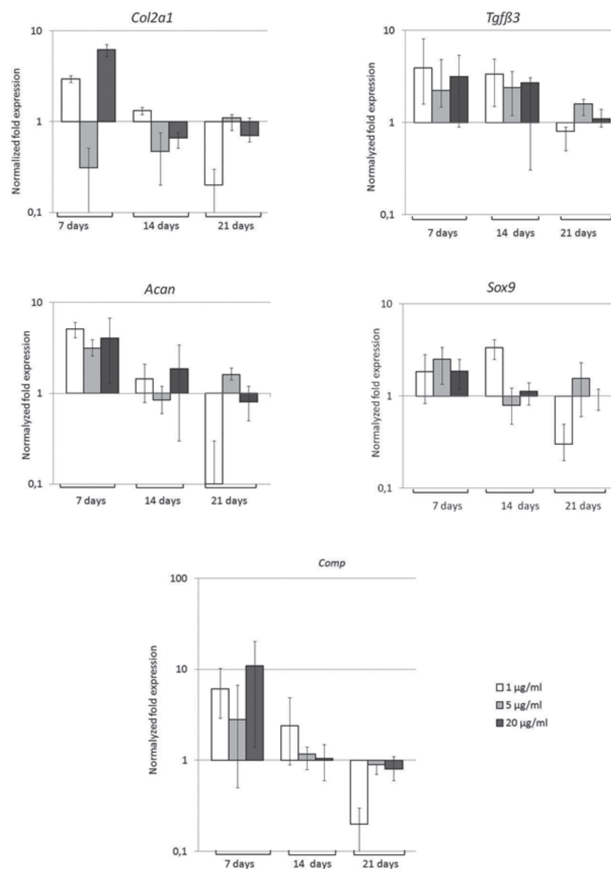

Figure 2. Changes in relative expression of *Tgfβ3, Sox9, Col2a1, Acan,* and *Comp* genes in human dermal fibroblasts calculated by the 2-ΔΔCt method at different concentrations of the recombinant Sox9 protein.

al. 2022). For cell modification, we used the recombinant Sox9 protein, which is the most important transcription factor for chondrogenesis (Song and Park 2020). Tam et al. (2014) reported positive results using Sox9-modified (viral transduction) fibroblasts to repair both bone and, to a lesser extent, hyaline cartilage. We studied the direct effect of different concentrations of the recombinant protein on the proliferation of dermal fibroblasts in the chondrogenic direction. As a result, we showed that the addition of the recombinant Sox9 protein in the concentration range from 1 to 20 ng/ml of the nutrient medium did not significantly change the expression of both the genes encoding proteins of the extracellular matrix of hyaline cartilage (*Acan*, *Col2a1*, *Comp*) and the genes encoding chondrogenesis inducers.

The results obtained by Zhang et al. (2017) show that a modified SOX9, fused with supercharged GFP (scSOX9) has the capability of entering cells and inducing MSC to differentiate into chondrocytes *in vitro*. The absence of chondrogenic differentiation in our experiments can be explained by the fact that the recombinant Sox9 protein without modifications is not able to penetrate into the cell and trigger the induction of chondrogenesis.

Conclusion

Thus, the direct effect of the recombinant Sox9 protein on the expression of genes associated with chondrogenic differentiation in the culture of dermal fibroblasts is insignificant and is not suitable as a tool for activating directed differentiation.

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