

Comparative Analysis of Tenogenic Gene Expression in Adipose-derived Stem Cells in Response to Various Biochemical Stimuli

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INTRODUCTION: Tendon injuries including strains, tears, and ruptures, became more common in sports and daily life, which might lead to disability in millions of patients worldwide. Due to the insufficient blood supply and low cellularity of tendons, these tissues have limited healing capacity; therefore, it is difficult to regain the original structure and function of the tendon after damage. Current surgical treatments, such as autografts, allografts, and tendon prostheses are often required for tendon repair. However, these methods are limited owing to the high incidence of recurrent tears, donor site morbidity, and inferior graft integration.

Tissue engineering is emerging as a promising strategy for tendon repair. However, tendon tissue engineering is hampered by difficulties in obtaining appropriate seed cells, in determining the best condition for proliferation and differentiation, and in selecting optimal cell carriers for the degenerated tendon. Although tenocytes have been effectively utilized for this purpose, harvesting these cells from autologous tendons is difficult due to the limited cell number and donor site morbidity. In comparison, adipose-derived stem cells (ADSCs) are preferable seed cells that can be easily harvested with minor donor site injury. ADSCs have already been applied for tendon regeneration, demonstrating that these cells may be practical seed cells for tendon tissue engineering. As mentioned, however, establishing an efficient strategy to differentiate the ASCs is still challenging.

In this study, we investigated the tenogenic gene expression of ADSCs within various tenogenic differentiation media, such as growth/differentiation factor 5 (GDF5), bone morphogenetic protein 12 (BMP12), transforming growth factor beta-1 (TGFβ-1), and transforming growth factor beta-3 (TGFβ-3).

METHODS: Adipose-derived stem cell preparation and Media preparation: Human ADSCs were achieved from the detached fat tissue during total hip arthroplasty after informed consent and approval of our institutional review board. low glucose-Dulbecco's Modified Eagles Medium/Nutrient Mixture F-12 (DMEM/F12) + 10% Fetal Bovine Serum (FBS) + 1% Penicillin-Streptomycin + ascorbic acid (50μg/mL) was used as a cell growth media, and split sub-confluent cultures (70-80 % confluency, density of $1-2 \times 10^3$ cells/cm², 0.25% trypsin/EDTA for cell detachment) was performed. For tenogenic differentiation, GDF5 (50ng/mL), BMP12 (50ng/mL), TGFβ-1 (10ng/mL), and TGFβ-3 (10ng/mL) was respectively added to the aforementioned growth media. **Proliferation analysis:** We seeded 2×10^4 of pre-cultured adipose-derived stem cells into tissue culture plates and placed the plates on a bidirectional rotator with 50Hz of oscillation at 37°C for 24 hours for homogenous cell distribution. Cell number was determined after 1, 5, and 7 days of differentiation to calculate the proliferation rate of both cells using Cell Counting Kit-8. **RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (qPCR):** Total RNA was isolated from cultured cells after 1, 7, 14, and 21 days of cell seeding using the RNeasy Mini Kit (Qiagen, Germany). Expression of tenocyte phenotype was completed with five sets of primers for Type I and III collagen, scleraxis (SCX), tenascin C (TNC), and tenomodulin (TNMD). SCX represented the early phase of tenocyte differentiation, while TNC represented the delayed phase. TNMD is believed to be a marker for mature tenocytes. qPCR was carried out using SYBR Green PCR master mix (Biotool, USA) on an Applied Biosystems ViiA 7 Real-Time PCR System. All PCRs were performed in triplicates. PCR cycle parameters were 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 15 s. At the end of the program, a melt curve was produced by taking readings every 1.6°C from 95 to 60°C. The reference gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used to normalize gene expression, and relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. **Statistics:** Data were presented as means ± STDEV. Statistical analysis was performed by one-way repeated measures ANOVA test in gene expression between the control and treated groups. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS: On adhesion assay, the initial cell seeding efficiency was comparable in all media. On proliferation assay, cells proliferated, and the cell number increased significantly in all media, with the highest fold in control media. To assess the effects of each biochemical stimulus on the tenogenic differentiation potential of ADSC, cells were treated, and gene expression was measured by qPCR. In qPCR, all five groups demonstrated substantial expression of COL1A1 and COL3A1. Compared to the control group, the cells treated with TGFβ-1 and TGFβ-3 showed increased expression of SCX and TNC in 7, 14, and 21 days, while the ADSCs treated with GDF5 and BMP12 showed a non-specific increase in SCX and a decrease in TNC, a tendon-related gene.

DISCUSSION: We investigated the cell adhesion, proliferation, and gene expression of the tenogenic differentiated adipose-derived stem cell using different growth factors. The proliferation assay result showed substantial cell growth in all media with each factor. Time-based expression of tendon-specific genes in qPCR suggested that cells treated with TGFβ-1 and TGFβ-3 were considered to be optimal for tenogenic differentiation.

SIGNIFICANCE: Our study suggested an optimal tenogenic inducer to differentiate the adipose-derived stem cells to tenocytes using various biochemical stimuli and timing for implantation, which can be used as a tissue-engineered approach for the regeneration of tendinopathy patients.

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Adhesion and Proliferation assay

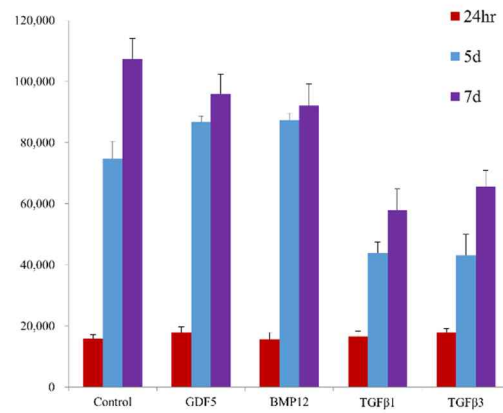


Figure 1. (A) Cell adhesion and proliferation assay of adipose-derived stem cells on various biochemical stimuli until 7 days

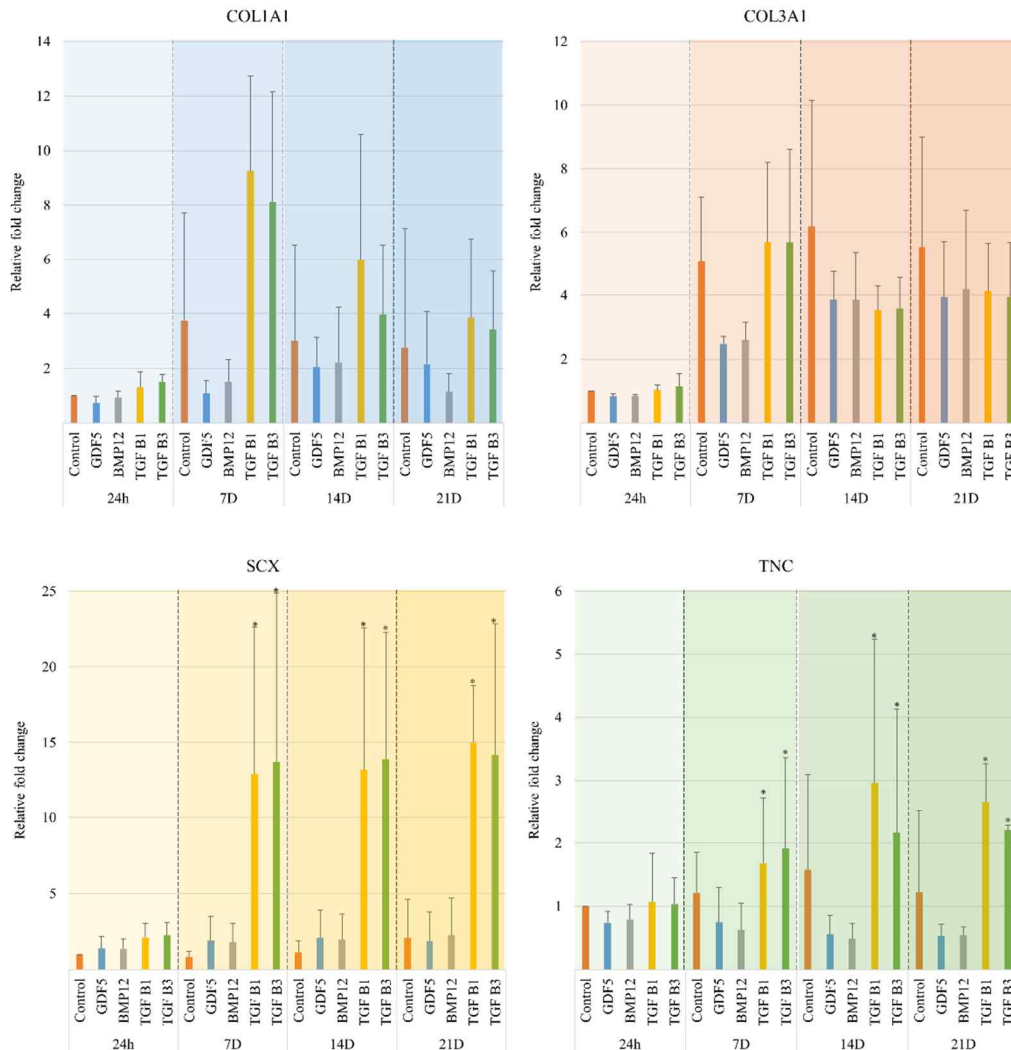


Figure 2. Effect of GDF5, BMP12, TGFβ-1, and TGFβ-3 on tenogenic gene expression in ADSC on 24 hours, 7 days, 14 days, and 21 days. * Data were compared to the GFP/BSA group