RESEARCH ARTICLE

Combination Effect of Rotator Cuff Repair with Secretome-hypoxia MSCs Ameliorates TNMD, RUNX2, and Healing Histology Score in Rotator Cuff Tear Rats

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Abstract

Objectives: In order to treat a rat model of rotator cuff rupture, this work concentrated on the expression of TNMD and RUNX2, followed by rotator cuff repair and secretome-hMSCs.

Methods: A total of thirty 10-weeks-old male Sprague–Dawley rats were separated into five groups randomly, RC on week 0, lesion treated with a rotator cuff repair and saline (RC + NaCl group, n = 6) for 2 and 8 weeks, and lesion treated with a rotator cuff repair and secretome-hMSCs (RC + secretome-hMSC group, n = 6) for 2 and 8 weeks. The supraspinatus and infraspinatus muscle–tendon units were obtained for histological and biomechanical investigation at 0, 2 and 8 weeks following injury.

Results: The findings showed that, in comparison with the RC + NaCl group, secretome-hMSCs significantly improved tendon repair by upregulating TNMD and RUNX2 expression and histology score.

Conclusion: Combining Secretome-hypoxia MSCs with RC healing may help rats with rotator cuff tears.

Level of evidence: IV

Keywords: Mesenchymal stem cells, Rotator cuff tear, RUNX2, Secretome, TN MD

Introduction

A rotator cuff tear (RCT) is a severe pathological condition of joint or ligament tears that is often alleviating sufferers' quality of life.[1,2] The current RCT treatments for injuries, which include physical therapy, pharmaceutical painkillers, steroid injections, and reconstructive surgery, do not always work well and may not be sufficient, which results in the development of scar tissue.[1,3–5] Nonoperative treatments may merely provide pain relief and have inadequate results.[6] Indeed, RCR postoperative may lead some infection and may require multiple debridements.[7] Therefore, optimal clinical recovery and creative therapeutic approaches are necessary.

Because of their therapeutic potential in promoting cartilage and bone repair, mesenchymal stem cells (MSCs) have been the cornerstone of orthopedists and researchers for the majority of the previous several decades.[8] Interestingly, the secretome-hypoxia MSC (secretome-hMSC) is a group of MSCs that strongly produce large amounts of growth factors, cytokines, and vesicles under hypoxic conditions.[9] S-hMSC may promote immunomodulatory, proliferative, and antiapoptotic activities that result in tendon regeneration, according to earlier research.[10–13]A biomarker for tendon proliferation called tenomodulin (TNMD) describes tendon activity at the molecular level.[14] Loss of TNMD translation can impair tenocyte expansion, which lowers tenocyte density and thickens pathological collagen fibers, both of which result in

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a physiological situation that is less favorable.[2] Runt-related transcription factor 2 (RUNX2) is a transcription factor that controls calcium deposition and osteoblast-specific protein expression to serve a crucial role in the osteogenesis regulation.[2,15]

According to additional recent studies, the hypoxia precondition of MSCs enriches the secretion of powerful bioactive soluble molecules as well as IL-10, TGF-β, PDGF and VEGF, known as Secretome-hypoxia MSCs (secretome-hMSCs), which reduce inflammation and enhance tissue regeneration by paracrine mechanism.[10,11,16,17] Another study found that local ASC-exosome injection in chronic RCTs may minimize lipid infiltration, improve tendon-bone healing, and ameliorate biomechanical aspects of the chronic RCT repair.[18] A novel in vitro study has unfold that secretome-hMSC can improve the phenotype, density, and survival of human tendon cells (hTC), moreover, when compared to no treatment, study in mice has also demonstrated that MSC secretome may enhance the rotator cuff tendon-bone interface's histological repair.[19] According to prior studies, MSC treatment can boost RUNX2 expression in anterior cruciate ligament re-establishment cases, which leads to new bone tissue development and the chondrocytes proliferation in the tendon-bone junction.[20]

In this investigation, we primarily concentrated on the mechanisms behind the impacts of rotator cuff repair with secretome-hMSCs on regulating tenogenesis-gene expression, particularly TNMD and RUNX2 related with healing histology score in the RCT rat model. Despite these facts, the effectiveness of secretome-hMSCs as a treatment modality for RCT conditions remains uncertain. Consequently, additional research is still needed. We hypothesized that the secretome-hMSCs play critical roles in facilitating rotator cuff repair by regulating the expression of genes encoding tenogenesis. This research assists to clarify the interaction between secretome-hMSCs and tenocytes, which will enhance therapeutic rotator cuff repair strategies.

Materials and Methods

Animal and Experimental Design

Animals were maintained and handled following the guidelines. The sample size in this study is calculated using the resource equation formula that is E = N-T (T = number of treatments; N = number of repetitions), E = 10-20. In this study, two treatments were carried out in the five groups based on the time of treatments, namely, RC week 0, RC + secretome-hMSCs (the treatment group was given a secretome-hMSCs) for 2 and 8 weeks and RC + NaCl (the control group was provided with NaCl) for 2 and 8 weeks. Consequently, the sample quantity employed was N = 12/2 = 6 in each group. However, the addition of repetition is required as an alternative. As a result, the total number of samples required was 14 samples with 7 samples for each treatment.

MSC Culture and Secretome-hMSC Collection

Based on our prior research, MSCs were extracted from the female rat umbilical cord during 19 days of pregnancy.[21] The umbilical cord was separated and cultured as in previously mentioned techniques[17,22,23] with a slight modification. Every three days, the medium was renewed. MSCs were incubated in DMEM (Gibco, USA) after the fourth passage when they reached 70% confluence in a flask containing a complete medium. They were then subsequently positioned in an hypoxic chamber (Thermo Forma, Waltham, MA, USA) for 24 hours. The hermetic moist hypoxic chamber was continually fueled with 5% CO2, 10% H2, and 85% N2 while being kept at a temperature of 37°C. The chamber's oxygen concentration was ~0.5%. Secretome-hMSCs were filtrated after incubation from hMSC medium using tangential flow filtration (Formulatrix, USA).

Mesenchymal Stem Cell Characterization and Validation

Plastic adherent stromal cell populations in the fourth passage have been identified through flow cytometry testing to corroborate the MSC-like cell membrane antigens. The cells were then trypsinized, pelleted, and incubated for 30 min at dark room with anti-rat FITC-CD90.1, PE-CD29, perCP-Cy5.5.1-CD31, and CD45 conjugated antibodies [BD Bioscience, San Jose, CA, USA]. In addition, a conjugated anti-IgG specific to an isotype [BD Bioscience, San Jose, CA, USA] was employed as the negative control. After that, PBS was used to wash the cells twice. The experiments were conducted using a BD Accuri C6 Plus flow cytometer, and post-acquisition analysis of the data was completed using BD Accuri C6 Plus software.

In the fifth passage, we added the osteogenic differentiation validation. The cells were grown in DMEM with 10% FBS, 1% penicillin/streptomycin [100 U/mL and 100 µL, respectively] and 0.25% amphotericin B [62.5 µg/mL] at 37°C and five percent CO_2 up to 95% their confluence. Next, the standard medium was substituted with medium that is supplemented with 20% Rat MesenCult Osteogenic Differentiation 5X Supplement [Stem Cell Technologies, Singapore], 1% L-glutamine [GibcoTM Invitrogen, NY, USA], 1% penicillin/streptomycin, and 0.25% amphotericin B. They, on the other hand, have been grown in an adipogenic differentiation medium, that is composed of Rat MesenCult™ MSC Basal Medium [Stem Cell Technologies], Rat MesenCult™ Adipogenic Differentiation Supplement [Stem Cell Technologies], 1% L-glutamine, 1% penicillin/streptomycin, and 0.25% amphotericin B for triggering adipogenic differentiation. Every 3 days, the medium was replaced. Alizarin Red and oil red O staining, respectively [Sigma-Aldrich, Louis St, MO], followed by 21 days of induction, were used for demonstrating calcium and lipid accumulation.

Rotator Cuff Repair and Secretome-hMSC Treatment

A combination of intramuscular zolazepam (0.05–0.3 ml/kg, Zoletil, Virbac SA, Carroscedex, France) and intraperitoneal xylazine hydrochloride (0.15 ml/kg, Rompun, Bayer HealthCare, Leverkusen, Germany) injection were used to anesthetize the rats. According to a prior description, a bilateral, two-tendon (infraspinatus and supraspinatus) RCT rat had been developed.[1] In the RC + secretome-hMSC group, 0.5 ml of secretome-hMSCs was injected into the larger tuberosity's bony groove locally above the repair lesion area during surgery before the skin was closed. While in the RC + NaCl group, 0.5 ml of saline was given. The surgical wound was then closed layer by layer according to the standard with 4-0 nylon sutures. All surgical procedures are carried out under

strict aseptic conditions. At week 0, 2, and 8 after operation, the supraspinatus and infraspinatus muscle-tendon components, alongside roughly 1.5 cm of the proximal humerus, were cut off for histological analysis after the rats received a cocktail lethal dose of ketamine, xylazine and acepromazine (50 mg/kgBW, 10 mg/kgBW, and 2 mg/kgBW, respectively) [Figure 1].

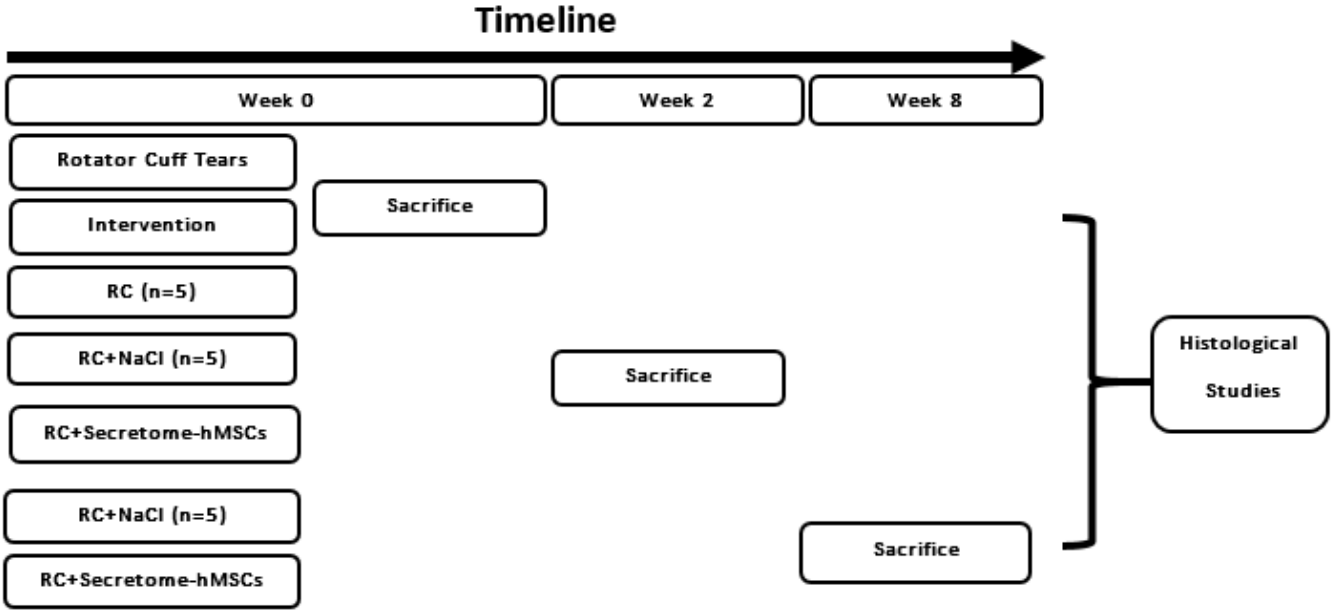

Figure 1. Schematic representation of the experiments. RC: rotator cuff repair surgery; secretome-hMSC: secretome-hypoxia mesenchymal stem cells

TNMD and RUNX2 Immunohistochemistry Staining

After the treatment, section of the supraspinatus and infraspinatus muscle-tendon tissues were stained for the TNMD and RUNX immunohistochemical evaluation. Paraffin-embedded sections (5 µm) were then deparaffinized and rehydrated, fully washed, antigen retrieval, blocked for 30 minutes with Blocking Buffer (StarrTrek, Biocare Medical, CA, USA) and overnight-incubated using a rabbit-anti-rat RUNX2 (1:200 dilution; Elabscience Cat. No. E-AB-53343) and TNMD polyclonal antibody (1:200 dilution; ABclonal Cat No. A2851) in the humid chamber. After washing, the slides were incubated for 60 minutes at room temperature with an HRP-conjugated secondary antibody (StarrTrek, Biocare Medical, CA, USA).[24] Applying diaminobenzidine (DAB), immunostaining was accomplished. As a counterstain, Mayer's hematoxylin was used. The representative images were acquired by an Olympus bright-field microscope with 4x and 40x objective magnification and were then calculated by ImageJ software.

Safranin O Staining

The safranin solution was applied to the paraffin-embedded tissue slides for 10 minutes, and they were subsequently washed in filtered water. The sections were then further stained for 15 seconds in a fast green solution (0.04 g of fast green chroma, 0.2 mL of 100% acetic acid, and 100 mL of distilled water), and rinsed in distilled water. Once the remaining fast green had been omitted the slides were soaked in 100% alcohol until the safranin O staining was apparent. A cover slip was finally placed on top of the dyed tissue after two (5-minute) xylene washings.

Histological Examination and Grading

Safranin O–stained tissue slides were evaluated by a light microscope (Olympus BH-2, Olympus Optical) and scored according to the Bern system. Three observers (one pathologist and two fundamental scientists with expertise analyzing cartilage tissue slide histology). The Bern score takes into account cellular morphologies, histological density, and matrix staining consistency and intensity. A total score was calculated by adding the scores of each category for each section.

Statistical Analysis

We employed the Kolmogorov-Smirnov test to ascertain the data's normal distribution and Levene's test to look into the homogeneity of the data. A T-dependent with a p-value less than 0.05 was regarded as statistically significant when comparing two groups, and comparisons between multiple groups were assessed using a one-way ANOVA test. IBM Corp.'s SPSS 22.0 was used to conduct the statistical comparisons.

Results

MSC Differentiation and Characterization

Secretome-hMSCs have been isolated from the fourth passage UC-MSC culture media. Through a microscopic viewpoint, the confirmation of the MSC culture morphology resulted in a representation of adherent cells with spindle-like cell forms, whereas the outcomes of osteogenic differentiation test confirmed that MSCs might transform into osteocytes reflected by the red calcium deposits in the MSC population exhibited by Alizarin Red staining [Figure 2 A and B]. The populations were also verified using flow cytometry to demonstrate that MSCs showed numerous MSC membrane antigens, which is consistent with their osteogenic capacity. According to the analysis, MSCs exhibited CD90 (99.80%) and CD29 (94.20%) but not CD45 (1.60%) or CD31 (6.60%) [Figure 2C].

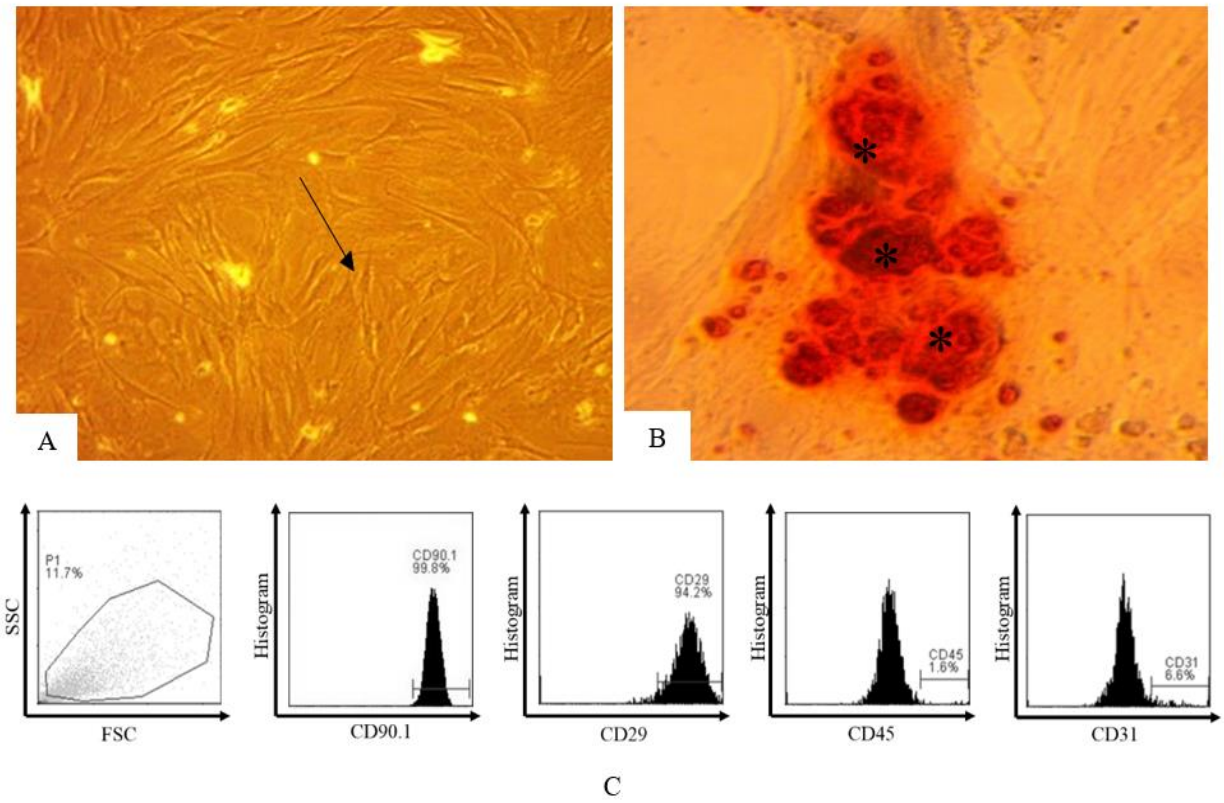

Figure 2. MSC validation. (A) Isolated MSCs with 80% confluent displayed spindle-like cells (shown by arrows) at 100x magnification. (B) Osteogenic differentiation using Alizarin Red staining appears in the MSC population at 100x magnification. (C) Flow cytometry analysis of the expression of CD90, CD29, CD45, and CD31

Combination of Rotator Cuff Repair with Secretome-hypoxia MSCs Increase TNMD and RUNX2 Expression on RCT Rats

The IHC analysis results showed that the RCT conditions reduced the TNMD and RUNX2 presence among tendon-bone tissue [Figure 3A]. Administration of secretome-hMSC combined with RC surgery increases the expression of TNMD and RUNX2 over time. In the second week, the TNMD and RUNX2 expression of the RC + NaCl group were the same as the RC + secretome-hMSC mice ($P > 0.05$).

At week 8, meanwhile, the RC + secretome-hMSCs rats had notably stronger levels of TNMD and RUNX2 expression in comparison to the RC + NaCl group (p 0.05) [Figure 3B and C].

Combination of Rotator Cuff Repair with Secretome-Hypoxia MSCs Increases Histological Score of RCT

The histological scores of RCT rats were analyzed from safranin O staining result using the Bern score method which increased over time [25] [Table I].

In the second week, the histological scores of the RC + NaCl group were the same as the RC + secretome-hMSC mice ($p > 0.05$). Meanwhile, at week 8, the histology grades of the RC + NaCl group were considerably lower ($p < 0.05$) relative to RC + secretome-hMSC rats [Table 1].

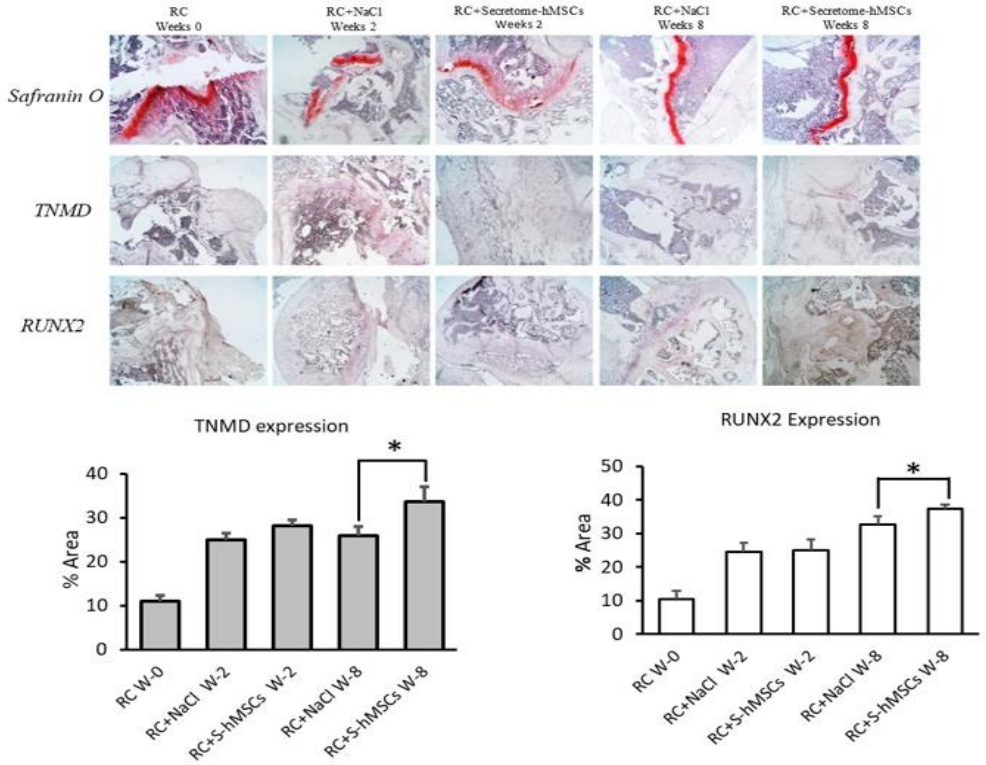

Figure 3. (A) Representative images of safranin O staining, TNMD, and RUNX2 immunohistochemistry in the cartilage tissue. Original magnification: 40×. (B) Percentage area of TNMD histological analysis for each group. (C) Percentage area of RUNX2 histological analysis for each group. Each bar displays the mean value (standard deviation, n = 5). *p < 0.05. RC: rotator cuff repair; S-hMSC: secretome-hypoxia mesenchymal stem cells

Table I. Breakdown of grades of cartilage tissue using the Bern score[19]

Sample	Scoring category			Total score
	A: Safranin O	B: Cell distance	C: Morphology	
RC W-0	1,0±0,0	1,0±0,0	1,0±0,0	3,0±0,0
RC+NaCl W-2	1,6±0,5	1,8±0,4	1,8±0,4	5,2±0,5
RC+Secretome-hMSCs W-2	1,8±0,4	1,8±0,4	1,8±0,4	5,4±0,4
RC+NaCl W-8	2,0±0,5	2,0±0,5	2,0±0,5	6,0±0,5
RC+Secretome-hMSCs W-8	2,8±0,4	2,8±0,4	2,8±0,4	8,4±0,4*

Values are expressed as mean ± SD (n=5). *P<0.05 when compared with the control group. SD: standard deviation

Discussion

This study's main outcome is that secretome-hMSC in combination with the rotator cuff enhances RUNX2 and TNMD expression, and the histology score promotes tendon and ligament healing in preclinical trial. Due of tenocytes' restricted capacity for proliferation, the tendon itself recovers poorly in RCT and leads to the destruction of histology and tendon function leading to therapeutic failure.[26] Interestingly, recent research clearly and extensively show that there are various active protein components including cytokines, chemokines, and growth factors that have the potential assessment to overcome various types of diseases, including contributing to cartilage and bone repair through cell signaling and communication mechanisms.[1,27–32] As previously reported, an essential step towards the tendon injuries regeneration by MSCs is the interaction between tenocytes and MSCs through their bioactive molecules in inducing specific biomarkers for tenocyte proliferation and tenogenic differentiation.[4,8,19] However, the precise mechanism after MSCs to stimulate tendon healing and RC repair still needs further research.

Thus, in this context, we focused on the expression of TNMD and RUNX2 followed by the combination of rotator cuff repair and secretome-hMSCs as a treatment for RCTs.

In this study, we demonstrated that TNMD expression was increased after secretome-hMSC administration. We hypothesized that secretome-hMSC serve an important role in driving the tenocyte proliferating-genes transcription to enhance tenogenesis. To date, multiple other investigations reported that comprehensive secretome-hMSC screening by ELISA demonstrated the presence of Ang-1, FGF-2, HGF, IL-6, IL-10, INF-γ, PDGF, SDF-1, TGF-β, and VEGF in cell culture supernatants.[11,33–35] Moreover, a current study has shown that during tendon healing, FGF-2 may promote the proliferation of tenogenic progenitor cells to repair injury sites by stimulating tendon proliferation genes and tenocyte markers, especially TNMD.[36] Additionally, exosomes released by MSCs have been shown to trigger tenogenic differentiation in a TGF-β dependent manner, according to a recent study.[4] TNMD gene is specifically expressed in dense connective tissue and detected in mature tenocytes.[14] The discovery of these marker genes enabled us to investigate in depth the cellular and molecular processes that occur during osteotendinous junction formation.[14,37] Although the exact molecular mechanism is still being investigated, these results indicate that secretome-hMSCs are thought to be able to increase the TNMD gene related to tenocyte proliferation and cartilage tissue regeneration through paracrine mechanisms, especially FGF-2 and TGF-β.

On the other hand, the RUNX2 gene that contributes in repairing the damaged tendon–ligament connections also experienced a rise in area expression based on the results of histological investigation following secretome-hMSCs. We assumed that MSCs secreted bioactive paracrine factors for inducing RUNX-2 transcriptional activation. Numerous previous research have shown that MSCs control RUNX2 through the JNK signaling pathway.[37–40] The phosphorylation of c-Jun amino N-terminal protein kinases (JNKs) involves transcriptional activation domain on N-terminal Ser-63 and Ser-73 sites.[40,41] Once the upstream and downstream effectors of JNK signaling pathway are activated, the the Runx2 osteogenic gene was transcribed which lead to osteogenic differentiation and bone production.[40,42] Taken together, the observation of histological scores also resulted in an improvement after being treated with secretome-hMSCs [Table 1]. This study demonstrated that secretome-hMSCs promote RCT repair through paracrine FGF-2, TGF-β, and JNK pathway phosphorylation mechanisms characterized by elevated TNMD, RUNX2, and histology scores. However, this study has a limitation in that it does not measure the expression of FGF-2, TGF-β, and JNK phosphorylation. Therefore, future research can further examine the limitations of this study.

Conclusion

Secretome-hypoxia MSCs combined with rotator cuff repair may increase the RUNX2 and TNMD protein expression and improve the histological score in RCT rat models compared with rotator cuff repair and saline.

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