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RESEARCH ARTICLE

Effect of Knockdown TAGLN on the Migration Capacity of Wuzhishan Pig's Bone Marrow Mesenchymal Stem Cells [1]

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Summary

Mesenchymal stem cells (MSCs) are multipotent adult stem cells, can easily be isolated from the bone marrow and subsequently expand *in vitro*. Bone marrow MSCs (BMMSCs) appear to be poorly immunogenic, and are the most widely used MSCs in tissue regenerative medicine, and the migration capacity of BMMSCs is the key determinant for the efficiency of their regenerative therapy. The Wuzhishan pig (WZSP) is characterized by its physiological and general biochemical indices very similar to humans, and may be potential organ donors for human being owing to its size. In this study, the BMMSCs from WZSP were used to study their migration capacity which regulated by TAGLN *in vitro*. The specific short hairpin RNA (shRNA) for TAGLN was designed to knock down TAGLN gene, and the scratch assay and transwell migration assay was employed to estimate effect of knockdown TAGLN on the migration capacity of BMMSCs from WZSP. The results showed that the specific shRNA for knocking down TAGLN efficiently was found for the BMMSCs, and there was a significant effect on the migration capacity of the BMMSCs from WZSP with knockdown TAGLN *in vitro*. In conclusion, TAGLN was an important factor in maintaining the migration capacity of the BMMSCs, which may be benefit for the BMMSCs from WZSP to be used in regenerative therapy for human being.

Keywords: *Bone marrow mesenchymal stem cells, Migration capacity, TAGLN, Wuzhishan pig*

TAGLN Geninin Bloke Edilmesinin Wuzhishan Domuz Kemik İliği Mezenkimal Kök Hücrelerinde Göç Etme Kapasitesi Üzerine Etkisi

Özet

Mezenkimal kök hücreleri (MSCs) kemik iliğinden kolayca izole edilebilen ve takibinde *in vitro* olarak çoğalabilen mültipotent olgun kök hücreleridir. Kemik iliği MSC (BMMSC)'lerinin zayıf immunojenik özellikte olduğu görülmekte olup doku yenileme tedavisinde en sıklıkla kullanılan MSC'lerdir ve BMMSC'lerin göç etme kapasiteleri yenileme tedavisindeki başarının anahtarıdır. Wuzhishan domuzu (WZSP) fizyolojik ve genel biyokimyasal belirtileri açısından insana oldukça benzerdir ve boyutu düşünüldüğünde insana organ nakli bakımından oldukça yüksek potansiyele sahiptir. Bu çalışmada WZSP'den elde edilen BMMSC'lerin TAGLN tarafından düzenlenen göç kapasiteleri *in vitro* olarak çalışıldı. TAGLN için spesifik kısa hairpin RNA (shRNA) TAGLN genini bloke etmek için dizayn edildi. Scratch metodu ve transwell göç metodu bloke TAGLN genin WZSP'den elde edilen BMMSC'lerin göç kapasiteleri üzerine etkisini araştırmak amacıyla uygulandı. Sonuçlar TAGLN genini bloke etmede kullanılan spesifik shRNA'nın BMMSC için etkili olduğunu ve *in vitro* ortamda TAGLN blokajının WZSP'den elde edilen BMMSC'lerin göç kapasiteleri üzerine anlamlı bir etkisinin var olduğunu gösterdi. Sonuç olarak TAGLN BMMSC'lerde göç kapasitesini sağlamada önemli bir faktördür. Bu durum; WZSP'den elde edilen BMMSC'lerin insanlarda yenileme tedavilerinde kullanılmasına olanak sağlayabilir.

Anahtar sözcükler: *Kemik iliği mezenkimal kök hücreleri, Göç kapasitesi, TAGLN, Wuzhishan domuzu*

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent adult stem cells, and can easily be isolated from the bone marrow (BM) and subsequently expand *in vitro*. MSCs can differentiate into mesodermal lineages (osteogenic, adipogenic, and chondrogenic lineages), and also can differentiate towards endodermal or ectodermal derivatives. In addition, MSCs can secrete many bioactive molecules which affect local cellular environment with strong anti-inflammatory and immunosuppressive properties [1]. The bone marrow MSCs (BMMSCs) are the best characterized adult stem cells, which has a great therapeutic potential in tissue engineering, regenerative medicine and autoimmune diseases [2]. BMMSCs constitutively express low levels of major histocompatibility complex (MHC) class I molecules and no MHC class II molecules, thus appear to be poorly immunogenic. There is no expression of co-stimulatory molecules in BMMSCs, including CD40, CD80, or CD86 which are involved in the activation of T cell for transplant rejection. It is due to the easy isolation of BMMSCs and the reports of differentiation into extra-mesodermal cell types, which has made BMMSCs the most widely used MSCs in cell therapy for pre-clinical and clinical trials of a variety of diseases [3].

TAGLN is a gene that encodes the Transgelin protein in humans, is also known as SM22 and WS3-10. Transgelin is ubiquitous in vascular and visceral smooth muscle, and is an early marker of smooth muscle differentiation. Transgelin is also an actin cross-linking/gelling protein which is involved in calcium interactions and regulates contractile properties. Transgelin contains a C-terminal calponin-like module and an upstream positively charged amino acid region which is required for actin binding [4]. It is by stabilizing the cytoskeleton through binding to actin that Transgelin plays a role in cell differentiation, cell migration, cell invasion and matrix remodeling [5,6]. Transgelin is upregulated in repopulating mesangial cells, and promotes their migratory and proliferative repair response after injury [7].

The Wuzhishan pig (WZSP) is characterized by its small adult size, and has a mature body weight of only approximately 30 kg with physiological and general biochemical indices very similar to human being. The heart, small intestine and guts of WZSP are similar to those of human body, and the pig also is a unique pharmacology and toxicology model for the investigation of human health [8]. However, it is not clear whether knockdown TAGLN has an effect on the migration capacity of BMMSCs from WZSP. In this study, the specific short hairpin RNA (shRNA) for TAGLN gene was designed to explore the influence for expression of TAGLN in BMMSCs through the quantitative real time PCR (qRT-PCR) and Western blot analysis. In addition, the effect of Transgelin on the migration capacity of BMMSCs from WZSP was evaluated through scratch assay and transwell migration assay.

MATERIAL and METHODS

Culture of Porcine BMMSCs

The BMMSCs were isolated from femur and tibia of WZSP, and flow cytometric analysis, adipogenic and osteogenic differentiation had been used to evaluate the character as described previously [9]. The BMMSCs were cultured in D-MEM/F-12 (Gibco) medium with 10% (v/v) fetal bovine serum (FBS, Gibco) containing penicillin/streptomycin (50 IU/ml, 50 μg/ml), and incubated at an atmosphere of 5% CO_2 in air at 37°C. Media were changed every other day. All animals were handled according to the animal protocols approved by the Chinese Academy of Agricultural Sciences Institutional Animal Care and Use Committee.

Design and Assessment of Specific Short Hairpin RNA for TAGLN Gene

Specific shRNA sequences for TAGLN gene were designed based on TAGLN gene order from NCBI Gene database (Gene ID 6876) and design principle for shRNA [10], and synthesized by Shanghai GenePharma Co, Ltd. *(Table 1)*. The negative control was also designed and synthesized, which had no homology to TAGLN gene sequences with the same composition of nucleic acids to the specific shRNA sequences. The expression vectors encoding different shRNA included TAGLN-sus-246, TAGLN-sus-473, TAGLN-sus-496 and TAGLN-sus-626. The above four expression vectors and non-specific transfection vector of TAGLN-NC (Control) were utilized to transfect the BMMSCs using X-tremeGENE HP DNA transfection reagent following the manufacturer's protocol (Liposomal transfection reagent kit, Roche). The expression of TAGLN mRNA was detected by qRT-PCR assay after post-transfection for 24 h, and the expression vector with best interference effect was selected and used in the following experiments. The best vector (TAGLN-sus-473) was recovered after digested with the restriction endonuclease ApaLI (New England Biolabs).

The linearized shRNA vector with no specific transfection to the BMMSCs was named as shRNA-NC (Control), and the linearized shRNA vector with best interference effect to the BMMSCs was shRNA-473. The experimental group was the BMMSC treated with the transfection vector of shRNA-473, and the control group was the BMMSC treated with shRNA-NC. The G418 (200 μg/ml) was added to the culture medium for the BMMSCs after the cells had been transfected with shRNA-NC and shRNA-473 for 36 h, and then the culture media were changed once every two days. After elimination of non-transfected cells and selection for 14 days, the stable cell lines transfected with shRNA-NC and shRNA-473 were obtained.

Table 1. *The sequence of specific short hairpin RNA for TAGLN*
Tablo 1. *TAGLN için spesifik kısa hairpin RNA'nın sekansı*

Vector	Sequence Name	Sequences
TAGLN-sus-246	S	5′-CACCGCTGGTGGAGTGGATCATAGTTTCAAGAGAACTATGATCCACTCCACCAGCTTTTTTG-3′
	A	5′-GATCCAAAAAAGCTGGTGGAGTGGATCATAGTTCTCTTGAAACTATGATCCACTCCACCAGC-3′
	Transcript	GCTGGTGGAGTGGATCATAGTTTCAAGAGAACTATGATCCACTCCACCAGCTT
TAGLN-sus-473	S	5-CACCGCGTCACCAAGACTGACATGTTTCAAGAGAACATGTCAGTCTTGGTGACGCTTTTTTG-3′
	A	5′-GATCCAAAAAAGCGTCACCAAGACTGACATGTTCTCTTGAAACATGTCAGTCTTGGTGACGC-3′
	Transcript	GCGTCACCAAGACTGACATGTTTCAAGAGAACATGTCAGTCTTGGTGACGCTT
TAGLN-sus-496	S	5′-CACCGCAGACTGTTGACCTCTTCGAATTCAAGAGATTCGAAGAGGTCAACAGTCTGTTTTTTG-3′
	A	5′-GATCCAAAAAACAGACTGTTGACCTCTTCGAATCTCTTGAATTCGAAGAGGTCAACAGTCTGC-3′
	Transcript	GCAGACTGTTGACCTCTTCGAATTCAAGAGATTCGAAGAGGTCAACAGTCTGTT
TAGLN-sus-626	S	5′-CACCGCCAGGAGCATAAGAGGGAATTTCAAGAGAATTCCCTCTTATGCTCCTGGTTTTTTG-3′
	A	5′-GATCCAAAAAACCAGGAGCATAAGAGGGAATTCTCTTGAAATTCCCTCTTATGCTCCTGGC-3′
	Transcript	GCCAGGAGCATAAGAGGGAATTTCAAGAGAATTCCCTCTTATGCTCCTGGTT

RNA Extraction and qRT-PCR Assay

The MicroElute Total RNA Kit (OMEGA bio-tek, USA) was employed to extract the total RNA, and the RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas, International Inc., Ontario, Canada) was used to synthesize the cDNA. The qRT-PCR was performed using TaKaRa RNA PCR Kit (AMV), and primer sequences of TAGLN (forward: 5′-CCCATCCTGTCTGTCCAAGT-3′, reverse: 5′-CCAGCTCCTC GTCGTACTTC-3′) and GAPDH (forward: 5′-GTGAAGGTCG GAGTGAACG-3′, reverse: 5′-CTCGCTCCTGGAAGATGGTG -3′) were designed and synthesized by Shanghai Sangon Biotech. GAPDH was used as an internal control gene to calculate the relative mRNA expression levels.

Western Blot

The BCA Protein Assay Kit (Thermo Scientific) was used to measure the protein concentration with bovine serum albumin as the standard after the BMMSCs were washed and lysed. Lysates (10 µl/lane) were separated using 12% SDS-PAGE followed by transferring proteins to 0.22 µm PVDF membranes (Millipore Corp, Bedford, MA, USA). The PVDF membranes were blocked in 5% non-fat milk (w/v) at 4°C overnight. Transgelin was detected by Western blot using a Transgelin primary antibody (Abcam, Anti-SM22 alpha antibody, ab14106) to probe the membrane. After washed with TBS-T, the membrane was incubated with second antibody. The immunoreactive bands were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce) and recorded on x-ray films (Fuji Medical, Tokyo, Japan). The GAPDH antibody (MBL International Corp) was used to monitor variation in loading of samples.

BMMSCs Migration Analyzed by Scratch Assay and Transwell Migration Assay

The migration capacity of BMMSCs was evaluate using scratch assay and transwell migration assay. The BMMSCs were cultured in 60 mm culture plate to confluence and treated with 10 µg/ml mitomycin-C for two hours. The growth arrested BMMSCs were transferred into 6 well-plate in a density of 3×10^6 per well, and the 'scratches' were made using a 10 µl pipette tip along the bottom of the plate after cultured for six hours, and then the BMMSCs were cultured for another 36 h. At the selected time points (0 h, 6 h, 12 h, 24 h and 36 h), the images of the BMMSCs were acquired from the culture dishes under phase contrast microscope.

The BMMSCs at a density of 4×10^6 cells/ml were placed in the apical well of the transwell assembly (6.5 mm diameter inserts, 8.0 µm pore size, Corning Costar, NY) with 100 µl serum-free medium, and 800 µl culture medium containing 10% FBS was filled into lower chambers as a source of chemoattractants. The nucleus of BMMSCs was stained with Hochest 33342 after incubation at 37°C for 12 h, and the number of migrating cells was ascertained through calculating nine fields per well at random under the fluorescence microscope.

Statistical Analysis

The experiment was repeated at least three times and the results were expressed as the mean ± SD. Statistical analyses were performed by Student *t* test. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Assessment of Specific Short Hairpin RNA for TAGLN

After the BMMSCs were transfected by the vectors of TAGLN-NC (Control), TAGLN-sus-246, TAGLN-sus-473, TAGLN-sus-496 and TAGLN-sus-626, the expression of TAGLN mRNA by the BMMSCs was evaluated through qRT-PCR assay. The result showed that the expression of TAGLN

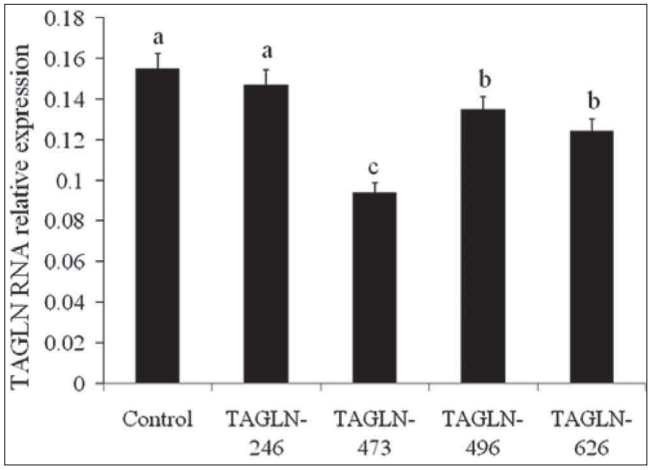

Fig 1. The expression level of TAGLN in BMMSCs through qRT-PCR analysis. The groups of TAGLN-246, TAGLN-473, TAGLN-496, TAGLN-626 and Control were the BMMSCs which were transfected with the vectors of TAGLN-sus-246, TAGLN-sus-473, TAGLN-sus-496, TAGLN-sus-626 and TAGLN-NC. Significant differences (P<0.05) are indicated by different letters

Şekil 1. qRT-PCR analisi ile elde edilen BMMSC'lerin TAGLN'lerinin ekspresyon seviyesi. TAGLN-246, TAGLN-473, TAGLN-496, TAGLN-626 ve kontrol grupları TAGLN-sus-246, TAGLN-sus-473, TAGLN-sus-496, TAGLN-sus-626 ve TAGLN-NC ile transfekte edilen BMMSC'leridir. Anlamlı farklılıklar (P<0.05) farklı harfler ile belirtilmiştir

in the BMMSCs transfected with the vectors of TAGLN-sus-473 and TAGLN-sus-496 TAGLN-sus-626 were lower than that transfected with the vector of TAGLN-NC (Control) (P<0.05), and the specific shRNA for TAGLN induced TAGLN knockdown in the BMMSCs efficiently *(Fig. 1)*. The vector of TAGLN-sus-473 was the most efficient one among the four experimental groups in knockdown TAGLN.

It was found in *Fig. 2A* that the linearized shRNA vectors for shRNA-NC (Control) and TAGLN-sus-473 (shRNA-473) were reclaimed efficiently. As illustrated in *Fig. 2B* that all BMMSCs were observed with fluorescence, and the stable cell lines were obtained after transfected with the vectors of shRNA-NC and shRNA-473 through selection for 14 days, and non-transfected cells were removed. It was showed in *Fig. 2C* that there was a significant difference between the group of shRNA-NC (Control) and shRNA-473 by qRT-PCR analysis (P<0.05), and TAGLN was knocked down effectually in the BMMSCs which were transfected with the shRNA vector of shRNA-473. There was an obviously decreasing expression of Transgelin in the group of shRNA-473 comparing with that in the group of shRNA-NC (Control) through Western blot analysis, but the GAPDH protein was expressed equally in both the groups of shRNA-NC (Control) and shRNA-473 *(Fig. 2D)*.

The Role of Transgelin on BMMSCs Migration

The scratched BMMSCs transfected with the vector of shRNA-NC (Control) confluenced after cultured for about 36 h, but the scratched BMMSCs transfected with the vector of shRNA-473 did not confluence after cultured for about 36 h *(Fig. 3)*. The scratched BMMSCs transfected with the vector of shRNA-473 migrated more slowly than that transfected with the vector of shRNA-NC. Therefore the scratch assay analysis suggested that TAGLN knockdown degraded the migration capacity of BMMSCs significantly.

It was demonstrated through the transwell migration assay that the number of BMMSCs that migrated across the filters with TAGLN knockdown was 85.30±7.13 (shRNA-473), but the number that migrated across the filters with the normal TAGLN expression was 120.53±16.32 (Control), so the BMMSCs with TAGLN knockdown had inferior ability of transwell migration comparing with the normal BMMSCs *(Fig. 4)*. It was obvious that Transgelin may play a key role in BMMSCs migration.

DISCUSSION

MSCs are multipotent stromal cells which can easily be isolated from the bone marrow. BMMSCs can differentiate into osteoblasts, adipocytes, chondrocytes, myocyates and many other tissues, and characteristically lack hematopoietic antigens, MHC class II, and endothelial antigens, which enable BMMSCs to be used as cell-based regenerative therapy for large bone defects, maxillofacial skeletal reconstruction, cardiovascular and spinal cord injury and other defects without immune rejection [11]. *In vitro* studies manifest that MSCs can inhibit a variety of immune cell functions and cell proliferation of T cells, B cells, natural killer cells and dendritic cells [12]. BMMSCs may play a key role in cell-based regenerative therapy for bone marrow, skin, heart, and corneal transplantation, graft versus host disease, hepatic and renal failure, lung injury, multiple sclerosis, rheumatoid arthritis, diabetes and lupus diseases.

In this study, the BMMSCs from WZSP were used to explore their migration capacity. WZSP is considered useful for medical and veterinary research due to its physiological and general biochemical indices similar to human being and its small size. It was reported that there was a small amount of porcine endogenous retrovirus and a lack of type C retroviruses in the WZSP genome, and pigs strongly resemble human being through investigating gene evolution in the pig and the pig counterparts of human druggable domain and disease related genes, which indicated that this pig may be used as potential organ donors for human being with low risk of pig-to-human infection during xenotransplantation [8].

RNA interference (RNAi) is a general method to silence gene expression in many organisms. It is possible that creation of continuous cell lines and transgenic animals through shRNAs expression method in which suppression of a target gene is stably maintained [13]. In this study, the specific shRNAs for TAGLN was found to knock down

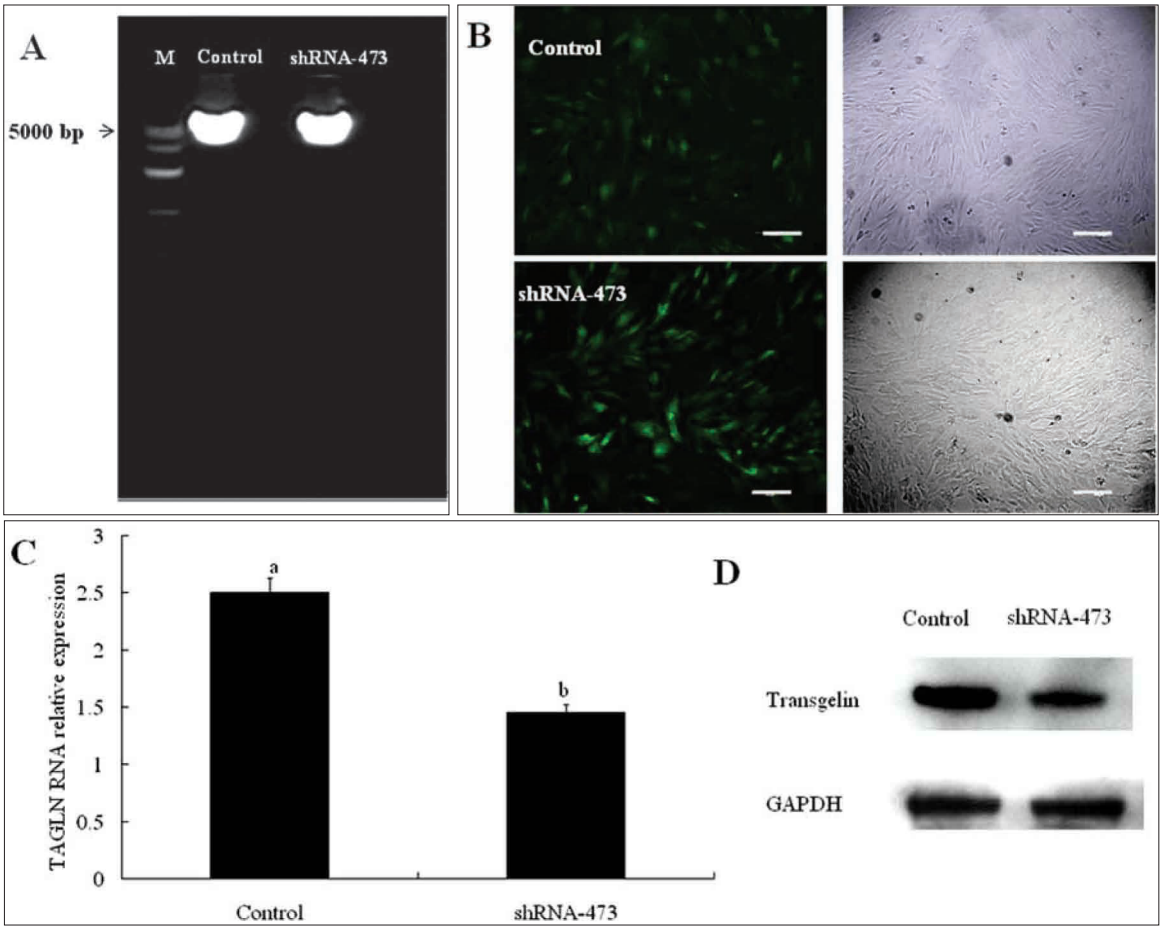

Fig 2. Effect of specific shRNA for TAGLN on knockdown TAGLN. **A-** The linearized shRNA vectors for shRNA-NC (Control) and shRNA-473 were reclaimed. *M:* marker, **B-** All BMMSCs were observed with fluorescence, and the stable cell lines transfected with the vectors of shRNA-NC (Control) and shRNA-473 were obtained. *Bar:* 100 μm, **C-** The expression level of TAGLN mRNA in the BMMSCs through qRT-PCR analysis. Control and shRNA-473 were the BMMSCs which were transfected by the vectors of shRNA-NC and shRNA-473, **D-** The expression of Transgelin in the BMMSCs through Western blot analysis using a specific Transgelin antibody. GAPDH antibody was used to monitor variation in loading of samples. Significant difference ($P<0.05$) is indicated by different letters

Şekil 2. Spesifik shRNA için TAGLN'nin TAGLN blokajındaki etkisi. **A-** shRNA-NC (Kontrol) ve shRNA-473 için linear shRNA vektörler dizayn edildi. *M:* markır, **B-** Tüm BMMSC'ler florasans ile gözlemlendi ve stabil hücre kültürleri shRNA-NC (Kontrol) vektörleri ile transfekte edildi ve shRNA-473 elde edildi. *Bar:* 100 μm, **C-** qRT-PCR analisi ile BMMSC'lerdeki TAGLN mRNA ekspresyon seviyesi. Kontrol ve shRNA-473 shRNA-NC ve shRNA-473 vektörleri ile transfekte edilen BMMSC'lerdir, **D-** spesifik Transgelin antikor kullanılarak yapılan Western Blot analizinde BMMSC'lerdeki Transgelin ekspresyonu. GAPDH antikoru örnekleri yüklemedeki varyasyonu takip etmek için kullanıldı. Anlamlı farklılık ($P<0.05$) farklı harflerle belirtildi

TAGLN expression in the BMMSCs from WZSP. The results showed that specific shRNAs for TAGLN can reduce the expression of TAGLN gene effectively *(Fig. 2)*. The TAGLN expression by the BMMSCS transfected with shRNA-473 was significant lower than that transfected with shRNA-NC (Control) through the qRT-PCR assay and Western blot analysis. Therefore the design of specific shRNAs for TAGLN was suitable for knockdown TAGLN gene in the BMMSCs, and a BMMSCs line with stable TAGLN gene silencing was established. However, the TAGLN gene was not knocked down completely, which may be owing to the liposomal transfection method.

Transgelin is an actin cross-linking protein, which plays a key role in cell differentiation, cell migration cell invasion and matrix remodeling through stabilizing the cytoskeleton [5,6]. Daniel et al.[7] reported that Transgelin is upregulated in repopulating mesangial cells and promotes their migratory. However, it was reported that Apigenin induced up-regulation of Transgelin and inhibited invasion and migration in colorectal cell lines [14]. In this study, the results showed that knockdown TAGLN in the BMMSCs lead to lower migration capacity of the BMMSCs (*Fig. 3* and *Fig. 4*), and it was essential for the BMMSCs to maintain high level expression of Transgelin so as to keep their migration capacity.

It has been shown that BMSCs are able to migrate to the sites and start the differentiation process in the injury and recovery process, suggesting that BMSCs possess migratory capacity. The efficiency and efficacy of BMSCs therapy is limited by the fact that very few transplanted

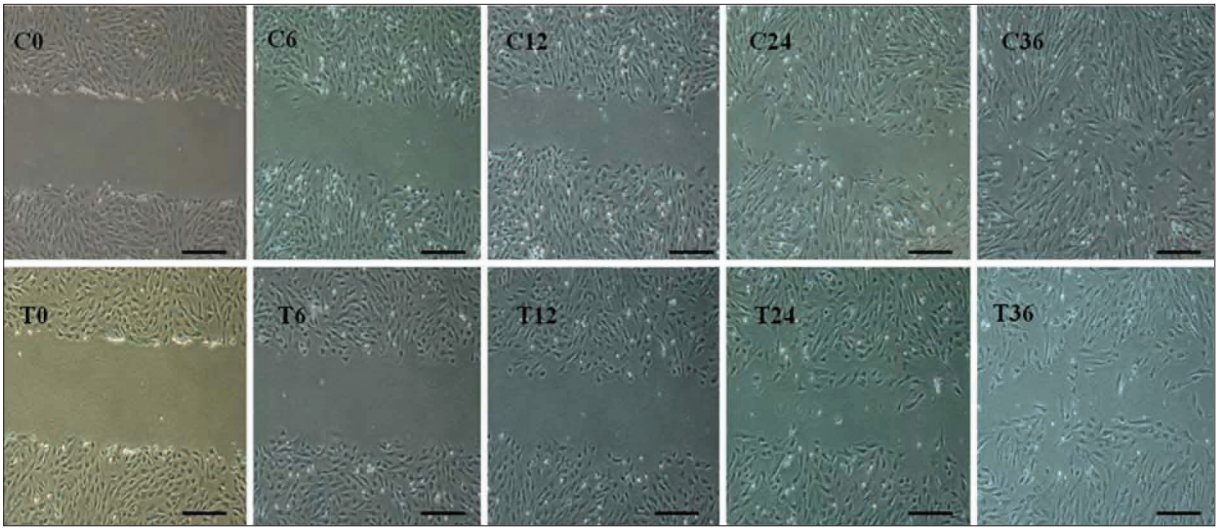

Fig 3. BMMSCs migration capacity analyzed by scratch assay. C0, C6, C12, C24 and C36 were the scratched BMMSCs transfected with the vector of shRNA-NC (Control) after 0 h, 6 h, 12 h, 24 h and 36 h respectively. T0, T6, T12, T24 and T36 were the scratched BMMSCs transfected with the vector of shRNA-473 after 0 h, 6 h, 12 h, 24 h and 36 h respectively. *Bar:* 50 μm

Şekil 3. Scratch analizi ile yürütülen BMMSC göç kapasite analizi. C0, C6, C12, C24 ve C36, sırasıyla 0, 6, 12, 24 ve 36. saatlerde shRNA-NC (Kontrol)'nin vektörü ile transfekte edilen çizilmiş BMMSC'lerdir. T0, T6, T12, T24 ve T36 sırasıyla 0, 6, 12, 24 ve 36. saatlerde shRNA-473 vektörü ile transfekte edilen çizilmiş BMMSC'lerdir. *Bar:* 50 μm

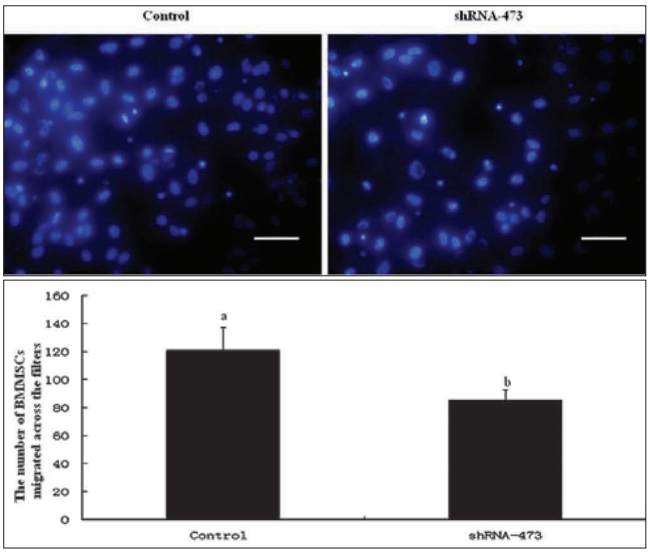

Fig 4. The transwell migration results of the BMMSCs. The shRNA-473 was the BMMSCs with TAGLN knockdown. *Bar:* 100 μm. Significant difference (P<0.05) is indicated by different letters

Şekil 4. BMMSC'lerin transvel göç sonuçları. shRNA-473 TAGLN boklanmış BMMSC'lerdir. *Bar:* 100 μm. Anlamlı farklılık (P<0.05) farklı harflerle belirtildi

cells home to the injured tissues, and functional recovery is often inadequate [15]. The migration potential of BMSCs is one of the determinants of the efficiency in tissue repair *in vivo* [16]. Our results indicated that it needed more time for the BMMSCs with TAGLN knockdown to confluence by scratch assay, and less number of the BMMSCs with TAGLN knockdown migrated across the filters through transwell migration assay (*Fig. 3* and *Fig. 4*). Therefore, TAGLN was an important gene for BMMSCs based transplant therapy.

In conclusion, WZSP may be used as potential organ donors for human being due to the low risk of pig-to-human infection during xenotransplantation. Our results showed that the specific shRNA for knocking down TAGLN efficiently was found for the BMMSCs from WZSP. The results also demonstrated that as an actin cross-linking/gelling protein, Transgelin is involved in maintaining the migration capacity of BMMSCs, which may be benefit for the BMMSCs from WZSP to be used in regenerative therapy for human being.

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