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Research Article

MiR124 suppresses collagen formation of human tendon derived stem cells through targeting *egr1*

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ABSTRACT

Collagen formation is used as a crucial indicator of tenogenic differentiation of human tendon derived stem cell (hTDSC). Early growth response-1(*egr1*), a transcriptional factor, has been demonstrated to regulate tendon differentiation and promote tendon repair. Considering that the therapeutic options for tendon injuries remain limited, investigating the regulation of *egr1* could facilitate the understanding of tendon development at molecular level so as to find a promising therapeutic target. MicroRNAs (miRNA) have been considered as epigenetic regulators to mediate multiple biological activities including stem cell differentiation. In the present study, biological experiments confirmed the prediction that miR124–3p (miR124) could have direct binding with *egr1*. We also found that miR124 suppressed collagen formation during the tendon differentiation of hTDSC while anti-miR124 promoted it. Furthermore, *egr1* knockdown abolished the promotive effect of anti-miR124, suggesting that miR124 prevents tendon differentiation via suppressing *egr1* expression. Therefore, miR124 may be a promising therapeutic target for tendon injury.

1. Introduction

Tendons are connective tissues that transmit the mechanical force from muscles to bones and tendon injuries usually result in chronic inflammation or acute rupture. Till now, there is no promising therapy for the tendon injuries, and the mismatch of the biochemical and mechanical properties between healed and intact tendon tissues compromises the tendon healing which is the major obstacle in clinical practice [1]. This mismatch may be due to the low proliferation and differentiation ability of local tendon cells [2]. Recently, stem cells therapy has attracted increasingly more attention in the field of tissue engineering. For the sake of promoting tendon repair, multipotent stem cell have been successfully isolated from tendon [3,4]. This tendon derived stem cell (TDSC) have successfully promoted tendon healing in animal models [5]. During the tenogenic differentiation of TDSCs, the collagen synthesis ability is one of the most essential properties, of which collagen type I is a major component of extra cellular matrix in tendon structure [6]. Collagen type I is widely used as scaffold material in the field of engineering and manipulation of collagen production is essential in autologous transplantation [7]. Thus, production of collagen type I during the tenogenic process is very important for tissue regeneration.

Several transcriptional factors, such as scleraxis bHLH transcription factor (*scx*), mohawk homeobox (*mkx*) and tenomodulin (*tnmd*) were reported to mediate tendon differentiation and collagen type I expression [8–11]. Recently, the transcriptional factor *egr1* was demonstrated to promote tenogenic differentiation through activating the expression of tendon markers, such as *scx*, *mkx*, collagen type I alpha 1 (*col1a1*) and collagen type I alpha 2 (*col1a2*) [12–14]. Furthermore, *egr1* also was found to be involved in the tendon development [15]. The function of *egr1* in tendon differentiation and regeneration indicate its potential as a therapeutic target for tendon regeneration.

Abbreviations: **col1a1**, collagen type I alpha 1; **col1a2**, collagen type I alpha 2; **dcn**, decorin; **egr1**, early growth response-1; **fmod**, fibromodulin; **fn1**, fibronectin; **miRNAs**, microRNAs; **mkx**, mohawk homeobox; **scx**, scleraxis bHLH transcription factor; **TDSC**, tendon derived stem cell; **TGF-β**, Transforming growth factor β; **tnc**, tenascin C; **tnmd**, tenomodulin; **UTR**, untranslated region

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MicroRNA (miRNAs) are crucial to exert their regulatory functions in multiple biological activities by binding the 3'-untranslated region (UTR) of the target genes [16]. They have been demonstrated to mediate cell commitment in several types of stem cells such as embryonic stem cells [17], hematopoietic stem cells [18], neural stem cells [19] and mesenchymal stem cells [20]. However, how the miRNA regulates tendon commitment of hTDSC and collagen formation remains largely unknown.

In the present study, hsa-microRNA-124–3p (miR124) was found to be a novel candidate to directly target *egr1*. Further investigation demonstrated that miR124 suppressed the collagen formation of hTDSC while miR124 inhibitor promoted it under tendon linage differentiation. Thus, miR124 may be a potential therapeutic target for tendon injury.

2. Materials and methods

2.1. TDSCs isolation and cell culture

The human TDSCs were isolated from tendon tissue of donors with formal informed consent and this study was approved by Joint Chinese University of Hong Kong-New Territories Ease Cluster Clinical Research Ethics Committee. Cells were collected from tissues of patients undergoing Anterior Cruciate Ligament Reconstruction (ACLR). Followed by being digested by 2 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, the mixture was centrifuged and re-suspended by PBS. After filtering through the falcon cell strainer (70 μm, Thermo fisher scientific, USA), the cells were cultured in Dulbecco's modified Eagle's medium with low glucose (Invitrogen, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin. The cell identifications were performed as previously described [4]. For tendon differentiation, the TDSCs were incubated in the medium with the presence of 10 μg/ml TGF-β1. The medium was changed every 3 days.

2.2. MiRNA prediction and luciferase assays

Three softwares including miRDB (http://mirdb.org/miRDB/), Tarbase (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index), and TargetScan (http://www.targetscan.org/vert_71/) were used to predict the miRNA candidates which could directly target *egr1*. For the luciferase assays, the sequences of 3'-UTR of *egr1* containing the wild type (Wt) or mutated (Mu) binding sites were inserted into the reporter vector pmirGLO. MiR124 mimics or negative control were co-transfected with the reporter plasmids into 293T cells. After incubated for 24 h, the firefly luciferase activities were measured by Luciferase Assay System (Promega, Madison, WI, USA) and normalized to renilla luciferase activity, following the manufacture's protocol.

2.3. Transfection, total RNA isolation and quantitative real-time PCR

The mimics and anti-sense of miR124 were purchased from GenePharma (Shanghai, China). FRET-conjugated oligoes were used to detect the transfection efficiency. Transfection of these oligoes was performed using Lipofectamine 3000 (Invitrogen) as described previously [5]. The total RNA was extracted by using TRIZOL reagent (Invitrogen, USA). For the miRNAs expression assay, the total RNA was reversely transcribed by using NCode™ miRNA First-Strand cDNA Synthesis kit (Invitrogen, CA, USA). To measure the mRNA levels of tendon markers, the total RNA was reversely transcribed by using PrimeScript™ RT Master Mix (TaKaRa, Tokyo, JAPAN). All qRT-PCR samples were performed by using Step-One Plus Real Time PCR system (Applied Biosystems, California, USA) on an ABI 7500 Real Time PCR System. The primers used were shown in Table 1. All reactions were repeated for three times and the relative expression of genes were calculated via $2^{-\Delta\Delta Ct}$ method.

2.4. Western blotting

Transfected cells were collected by M-PER™ Mammalian Protein Extraction Reagent (Thermo scientific, USA). After lysis on ice for 30 min, cell debris were deposited by high-speed centrifugation. Protein lysates were separated by SDS-PAGE (10%) and transferred to PVDF membranes (Biorad, California, USA). The membranes were blocked with 5% skimmed milk for 1 h and incubated with primary antibodies rabbit polyclonal anti-*egr1* (Cell Signaling Technology, Massachusetts, USA) at 4 °C overnight. Then the film was incubated with HRP-labeled corresponding secondary antibodies for 1 h and results were taken using ECL Amersham ECL Prime Western Blotting Detection Reagent (GE healthcare, Massachusetts, USA). The β-actin (Cell Signaling, Massachusetts, USA) was used as the internal control.

2.5. Sirius red staining

The Sirius red staining assay was performed as previously reported [21]. After the medium was removed, the stained cells were washed twice with PBS and fixed in 4% paraformaldehyde (PFA). Then the cells were incubated with Picro-sirius red solution for 1 h at room temperature, and were thoroughly rinsed in acidified water (0.5% acetic acid). To quantify the staining results, after scanning the stained plates, 500 μL of 0.1 M NaOH was used to dissolve the Sirius red, then the absorbance were measured by using microplate reader at 492 nm.

2.6. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed by using the independent *t*-test. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. MiR124 being predicted as a promising candidate for targeting egr1

Three online programs were used to predict miRNA candidates which could directly bind to the 3'-UTR region of *egr1*. Totally 83, 50 and 19 miRNA candidates were screened by Tarbase, miRDB and

Table 1
Sequence of primer used in real time quantitative PCR.

Gene	*Accession no.*		*Sequence (5' to 3')*
gapdh	*NM_002046.5*	*F*	*CCAGGGCTGCTTTTAACTCTGGTAAAGTGG*
		R	*ATTTCCATTGATGACAAGCTTCCCGTTCTC*
col1a1	*NM_000088.3*	*F*	*GTCACCCACCGACCAAGAAACC*
		R	*AAGTCCAGGCTGTCCAGGGATG*
col1a2	*NM_000089.3*	*F*	*AGCAGGTCCTTGGAAACCTT*
		R	*GAAAAGGAGTTGGACTTGGC*
tnc	*NM_002160.3*	*F*	*TTCAGCAGAATTGGGGATTT*
		R	*ACCTAGGTCTCTCGCCCATC*
dcn	*NM_000389.4*	*F*	*TGCAGGTCTAGCAGAGTTGTGT*
		R	*AATGCCATCTTCGAGTGGTC*
fn1	*NM_001306129.1*	*F*	*ACCTCGGTGTTGTAAGGTGG*
		R	*CCATAAAGGGCAACCAAGAG*
egr1	*NM_001964.2*	*F*	*CGAGCTCGTGCCTTTTGTGTGATG*
		R	*CCTCGAGCCCAATCGCAGCTACTT*
miR124-3p	*NR_029668.1*	*F*	*TAAGGCACGCGGTGAATGCC*
		R	*Universal primer*