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MicroRNA 148a-3p promotes Thrombospondin-4 expression and enhances angiogenesis during tendinopathy development by inhibiting Krüppel-like factor 6

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ABSTRACT

Tendinopathy is a common musculoskeletal disorder with characteristic hypervascularity. The mechanism of angiogenesis in tendinopathy remains unclear. The present study aimed to investigate the roles of miR-148a-3p in angiogenesis development of tendinopathy. In this study, we demonstrated that miR-148a-3p expression was increased in tendinopathy tissues and positively correlated with CD34 levels which is a specific marker for angiogenesis. We identified Krüppel-like factor 6 (KLF6) as a direct target gene of miR-148a-3p in tenocytes. Furthermore, reduced levels of KLF6 in tendinopathy tissues was showed using qRT-PCR and immunohistochemical analysis, compared with controls. A negative correlation between the levels of KLF6 mRNA and miR-148a-3p was observed. Then, we verified that miR-148a-3p could regulate Tsp-4 expression by targeting KLF6 in tenocyte and was positively correlated with Tsp-4 levels in tendinopathy tissues. In a coculture system of tenocytes with endothelial cells (ECs), we observed that transfection of Lv-miR-148a-3p markedly upregulated EC angiogenesis. In summary, our data establish a novel molecular mechanism by which miR-148a-3p upregulates Tsp-4 expression in tenocytes to promote EC angiogenesis by targeting KLF6, which could be helpful for the treatment of tendinopathy in the future.

1. Introduction

Tendinopathy is a common musculoskeletal disorder involving the Achilles, patellar, and supraspinatus tendons. Tendinopathy usually affects athletes and individuals who are engaged in repetitive activities [1]. Tendinopathy can result in a significant amount of morbidity and health care expenditure without effective treatment. Histologic studies have shown that consistent changes occur in response to tendinopathy, including degeneration and disorganization of collagen fibers, hypercellularity, and hypervascularity [2,3]. Many current fundamental basic science studies are mainly focused on understanding processes involving tenocyte cellularity, matrix degradation and tendon healing [4,5]. However, only a few studies have been performed to investigate the mechanism of hypervascularity in tendinopathy [6]. Although neovascularization is critical to tendon healing, and some vascularization-enhancing methods including the use of platelet-rich plasma (PRP) are applied in the treatment of tendon injury [7], hypervascularity in tendinopathy does not favor functional recovery of the tissue. Therefore, it is important to discover the mechanism of angiogenesis in the tendinopathy process.

Thrombospondin-4 (Tsp-4) is a member of the thrombospondin protein family, which plays roles in adhesion, migration, apoptosis, proliferation, and extracellular matrix (ECM) remodeling/fibrosis [8,9]. Previous studies have investigated the functional role of Tsp-4 in the nervous system [10], eye [11,12], and heart [13]. Moreover, several recent studies reported that Tsp-4 plays a role in the

Abbreviations: PRP, platelet-rich plasma; Tsp-4, thrombospondin-4; ECM, extracellular matrix; miRs, microRNAs; KLF6, Krüppel-like factor 6.

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acceleration of angiogenesis [14,15]. Tsp-4 is abundant in the tendon [16,17]. Frolova et al. [18] reported that tendon collagen fibrils in Tsp-4 (−/−) mice were larger than those in wild-type mice. Subramanian et al. [19] demonstrated that Tsp-4 could control matrix assembly at myotendinous junctions. However, the role of Tsp-4 in the hypervascularity of tendinopathy remains unclear.

MicroRNAs (miRs) are a group of short, noncoding and single-stranded RNAs, which serve a primary role in the regulation of a variety of cell processes through binding to the 3′-UTR of target genes [20,21]. Previous studies have suggested that miR-148a-3p has an effect in cancer progression, including gastric cancer, hepatocellular carcinoma, and pancreatic cancer [22–24]. Tian et al. reported that miR-148a-3p could affect adipocyte and osteoblast differentiation by targeting Kdm6b [25]. Moreover, the role of miR-148a family on angiogenesis has been reported in several recent studies [26,27]. However, no study has been performed to investigate the role of miR-148a-3p in the development of tendinopathy, especially in the mechanism of angiogenesis which could contribute to the development of tendinopathy.

A previous study reported that Krüppel-like factor 6 (KLF6) regulated Tsp-4 through transcriptional control in cardiomyocytes [28]. Moreover, we found that KLF6 is a potential target of miR-148a-3p using bioinformatics prediction tools (Miranda, miRDB, TargetScan). Therefore, it is of great interest to investigate such a mechanism. In the current study, we demonstrated that miR-148a-3p expression was increased in tendinopathy tissues compared with that in healthy controls. Mechanistically, upregulation of miR-148a-3p increased Tsp-4 expression by inhibiting KLF6 expression, thus promoting angiogenesis in the development of tendinopathy.

2. Materials and methods

This study was approved by the Medical Ethics Committee of Shanghai Tenth People's Hospital affiliated with Tongji University. Written informed consent was obtain from each participant.

2.1. Patients and tissue samples

Tissue samples were collected from patients who underwent surgical resection at the Department of Orthopedics of Shanghai Tenth People's Hospital affiliated with Tongji University between January 2015 and June 2017. In cases with tendinopathy (supraspinatus tendinitis with partial tendon injury), enrollment was limited to patients who were diagnosed by MRI before operation, and tendinopathy was confirmed by arthroscopic surgery. All patients (40.4 ± 10.3 years) had a positive Jobe test during physical examination and underwent conservative treatment for more than 3 months. In control participants (36.3 ± 11.5 years), enrollment was limited to those who were diagnosed with shoulder instability by physical examination and MRI, which was also confirmed by arthroscopic surgery. Other shoulder disorders were not observed in all participants. All tissue samples were stored at −80 °C for further use.

2.2. Cell culture and transfections

Tendon was digested by dispase (1 U/ml; STEMCELL Technologies, USA) and collagenase type II (2 mg/ml; Worthington Biochemical Corporation, USA) for 48 h. Isolated tenocytes were grown in α-MEM consisting of 10% FBS (Cambrex, USA), 50 μg/mL kanamycin (Sigma-Aldrich, USA) and type I collagen (Koken, Tokyo, Japan). Tenocytes were transfected with a lentivirus encoding pre-miR-148a-3p (Lv-miR-148a-3p) or control mIR (Lv-miRctrl) in 6-well plates and was cultured according to the manufacturer's instructions.

Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Manassas, USA). The cells were cultured at 37 °C in EC basal medium 2 (EBM2) containing low serum and a specific EC growth supplement.

2.3. Target gene prediction and dual-luciferase reporter assay

Target gene prediction was carried out using bioinformatics prediction tools (Miranda, miRDB, TargetScan). The wild-type (wt) and mutant-type (mut) 3′UTR of KLF6 mRNA were inserted into dual-luciferase reporter vectors (Promega Corporation, USA). 293 T cells were cotransfected with Lv-miR-148a-3p or LV-miRctrl and with wt or mut KLF6 3′UTR. A Dual Luciferase Reporter Assay System (Promega Corporation, USA) was used to detect the luciferase activity.

2.4. Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted from human tenocytes using an RNAiso Plus reagent (Takara Bio, Japan). First-strand cDNA of the total RNA and small RNA was synthesized with a PrimeScript RT reagent kit (Takara Bio, Japan). SYBR Premix Ex Taq II (Takara Bio, Japan) on a StepOne instrument (Life Technologies, USA) was used to analyze mRNA expression. miRcute miRNA qPCR Detection kit (Tiangen Biotech, China) was used to determine miR expression. Relative mRNA expression was normalized to GAPDH and relative miR-148a-3p expression was normalized to U6. The primers sequence was shown as follows: miR-148a-3, Forward, 5′-GCTAGCCTCCGAAGCAAACAATGAAA-3′, Reverse, 5′-AAGCTTCGTCTACAAGGACTAACCGAAA-3’; KLF6, Forward, 5′-TATCTTCAGGATGAGCCCTGCTAC-3′, Reverse, 5′-AGACTTCACCAATGGGATCAGAGG-3’; Tsp-4: Forward, 5′-TGGAAGGACTCCAGGAATGT-3′, Reverse, 5′-TCATAAAAGCGCACCCTGA-3’; GAPDH, Forward 5′-CCCCGGTTTCTATAAATTGAGC-3′, Reverse, 5′-CACCTTCCCCATGGTGTCT-3’; U6, Forward, 5′- CTCGCTTCGGCAGCACA-3′, Reverse, 5′- AACGCTTCACGAATTTGCGT-3’; All expressions were measured using the $2^{-\Delta\Delta CT}$ method.

2.5. Western blot

Protein was extracted from tissues and cells using RIPA buffer containing 1% protease inhibitors (Beyotime, China). A BCA Protein Assay Kit (Thermo Fisher Scientific, USA) was used to quantify the protein content. Primary antibodies, including anti-KLF6 (Abcam, UK) and anti-Tsp-4 (Santa Cruz, USA) and anti-β-actin (Abcam, UK), were utilized according to the respective manufacturers' instructions. Primary antibodies against β-actin were used as a control.

2.6. Immunohistochemical staining

To localize KLF6, Tsp-4, and CD34 expression, sections were stained with human polyclonal Abs against KLF6 (Abcam, UK), Tsp-4 (Santa Cruz, USA), and CD34 (Abcam, UK) according to the manufacturers' instructions. All steps were performed as previously described [29]. The expression levels of KLF6 and Tsp-4 were analyzed by optical microscopy. Positive staining of CD34 showing brown spot was considered as microvessels, which was identified within low power and was counted within high power. The median of microvessel amount for controls was used as control, as described previously [30].

2.7. Coculture of tenocytes and ECs

A coculture system was established using a Transwell chamber (Corning Inc., USA). Tenocytes (1×10^4) were seeded on 6-well

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