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## Review Article

# Tenogenic differentiation of mesenchymal stem cells and noncoding RNA: From bench to bedside

Ying-fei Lu<sup>a,b</sup>, Kai-ming Chan<sup>a,b,c</sup>, Gang Li<sup>a,b,c</sup>, Jin-fang Zhang<sup>a,b,c,\*</sup>

<sup>a</sup> Department of Orthopaedics & Traumatology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong SAR, China

<sup>b</sup> Lui Che Woo Institute of Innovative Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China

<sup>c</sup> Stem Cells and Regenerative Medicine Laboratory, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong SAR, China

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## ABSTRACT

Tendon is a critical unit of musculoskeletal system that connects muscle to bone to control bone movement. More population participate in physical activities, tendon injuries, such as acute tendon rupture and tendinopathy due to overuse, are common causing unbearable pain and disability. However, the process of tendon development and the pathogenesis of tendinopathy are not well defined, limiting the development of clinical therapy for tendon injuries. Studying the tendon differentiation control pathways may help to develop novel therapeutic strategies. This review summarized the novel molecular and cellular events in tendon development and highlighted the clinical application potential of non-coding RNAs and tendon-derived stem cells in gene and cell therapy for tendon injuries, which may bring insights into research and new therapy for tendon disorders.

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## Contents

1. Introduction	1
2. Molecular basis of tendon development and healing	2
2.1. Molecular regulation of tendon differentiation during development	2
2.2. Growth factors in tendon development and formation	2
2.3. Mechanical stimuli induced molecular changes in tendon	2
3. Non-coding RNAs in tendinopathy and tendon development	3
4. Novel strategies of clinical therapy for tendon disorders	3
4.1. Non-coding RNA based gene therapy	3
4.2. Cells used in tendon tissue engineering	3
5. Conclusion	4
Disclosure of conflict of interest	4
Acknowledgment	4
Reference	4

## 1. Introduction

The musculoskeletal system is composed of distinct tissues including bone, muscle, tendon and cartilage. Among them,

tendon is a tough band that connects muscle to bone and transmits the force generated by muscle contraction to the bone [1]. To maintain its tension and bear load in a long-period, the tendon has reduced metabolic rate which also slows down its healing potential after injury [2]. Acute tendon rupture and chronic injuries (or tendinopathy) caused by overuse are quite common in athletes and workers [2,3], but the pathogenesis remain poorly understood. Moreover, tendon healing is often accompanied with formation of fibrotically scarring and adhesion, instead of complete regeneration, which may eventually cause partial loss of tendon function.

\* Corresponding author at: Room 904, 9/F, Li Ka Shing Institute of Health Institute, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China.

E-mail addresses: [gangli@cuhk.edu.hk](mailto:gangli@cuhk.edu.hk) (G. Li), [zhangjf06@cuhk.edu.hk](mailto:zhangjf06@cuhk.edu.hk) (J.-f. Zhang).

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To date, the clinical therapeutic options for tendon injuries were limited to surgical replacement with autografts, allografts, or xenografts [4], with considerable long recovery period. Thus, novel therapeutic strategies are in urgent needed. Studies on the tendon differentiation at molecular level provides insights of the earliest events of tendon development and pathologic changes of tendon.

Tenogenic differentiation involves a series of signaling pathways, transcriptional and epigenetic regulators. Non-coding RNAs (ncRNAs) are a family of functional RNA molecules without being translated into proteins. They serve as important and powerful regulators of various biological activities and play critical roles in a variety of disease progression [5,6]. To date, a few studies have focused on ncRNA functions in tendon development and disorders. In this review, we summarized the molecular events in tendon development and mainly focused on ncRNAs in tendon differentiation and tendinopathy.

## 2. Molecular basis of tendon development and healing

### 2.1. Molecular regulation of tendon differentiation during development

Tendon is mainly composed of tenocytes and highly aligned collagen fibers embedded within extracellular matrix (ECM). According to the findings of *in vitro* studies, the progress of tendon development was demonstrated in Fig. 1. Firstly, embryonic stem cells (ESCs) differentiate into mesenchymal stromal cells (MSCs) [7]; then MSCs further developed into tendon progenitors (or known as tendon-derived stem cells, TDSCs) which are stimulated by the transcription factor Scleraxis (SCX), a key regulator of tenocyte differentiation, and its expression in tenocytes is strongly induced by TGF-beta signaling [8]; the tendon progenitors further differentiate into tendon fibroblasts under control of SCX and Mohawk (MKX), the critical regulators of tendinogenic differentiation [9,10]. Finally, the tendon fibroblasts mature into tenocytes through secretion and remodeling of surrounding extracellular matrices (ECM) [11]. During the tendon development progress, a number of genes, such as SCX, MKX, and early growth response (EGR) family member EGR1 and EGR2, are responsible for the cell fate determination and regulation of ECM assembly [12–14].

In contrast to bones and cartilage tissues, lack of specific molecular markers during tendon development has limited the mechanism studies in tendon development. SCX was found to continuously express from early somatic cells to mature tendons during mouse embryonic development and was once regarded as a tendon-specific marker [8,9,12,15]. MKX and EGR1 were also reported to mediate tendon differentiation through activating TGF- $\beta$  pathway [16,17] and regarded as tendinogenic transcriptional factors. Besides these transcription factors mentioned above, genes related to secretion and degradation of tendon matrices, such as collagen type 1 and Tenomodulin (TNMD), were also served as markers to monitor tendon development in the late

stage of embryonic development [18].

### 2.2. Growth factors in tendon development and formation

During tendon development and healing process many growth factors are involved, such as bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), basic fibroblast growth factors (bFGF), transforming growth factor beta (TGF- $\beta$ ), insulin-like factor 1 (IGF-1), platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF), these factors have been reported to regulate cell differentiation, proliferation, chemotaxis and ECM synthesis [19].

The TGF- $\beta$  superfamily, including TGF- $\beta$ , BMPs and GDFs, are the key growth factors for tendon formation. TGF- $\beta$  signaling has critical roles in vertebrate early tendon development. The TGF- $\beta$ /SMAD2/3 pathway is identified as the most important pathway in limb tendon development [20] and disruption of TGF- $\beta$  signaling results in loss of most tendon tissues in mouse embryo [21]. GDFs are also good inducers for tendon formation and regeneration. GDF-5, 6 and 7 (also known as BMP-14, 13 and 12) were shown to modulate tendon matrix synthesis [22–24]. Specially, GDF-5 is found essential in Achilles tendon healing [25]. However, GDFs are also responsible for inducing cartilage and bone formation *in vivo* [26]. More studies on how GDFs regulate cell fate are needed for achieving controlled tendon healing using GDFs.

Other growth factors, such as FGF, IGF-1 and PDGF, are not critical in tendon development, but their expression are increased in tendon healing process, indicating their potential roles in tendon healing. Current studies show that FGF/ERK/MAPK pathway induces tendon differentiation *in vitro* [27], while IGF-1 and PDGF promote cell proliferation, stimulate tenogenic ECM synthesis and enhance tensile strength of tendon in rat models [28–30].

### 2.3. Mechanical stimuli induced molecular changes in tendon

The mechanical stimuli during muscle movements also play indispensable roles in maintaining tendon functions, including tendon development and repair [31–33]. Studies showed that mechanical loading promotes matrix remodeling in MSCs and activates integrin downstream kinases p38 and ERK1/2 [34,35]. A proteomic analysis of tendon tissue showed that the mechanical force altered expression of a large number of proteins in tendon, including extracellular matrix molecules, intra-cellular signaling molecules, cytoskeleton proteins and inflammatory factors [36]. Among these proteins, collagens I and VI, MMP-14, WNT5A and some inflammatory factors, COX, COX2 and PRDX5 may all contribute to the highly compacted and organized tendon tissue structure [36]. *In vitro* studies revealed that the collagen synthesis was stimulated by mechanical loading, which was probably mediated by growth factors TGF- $\beta$ , IGF-1 and IL-6 [37]. During tendon healing process in a rat model, mechanical force stimulates tendon growth in late stage by up-regulating a series of tendon-specific genes, such as SCX and tenomodulin [33], while in the

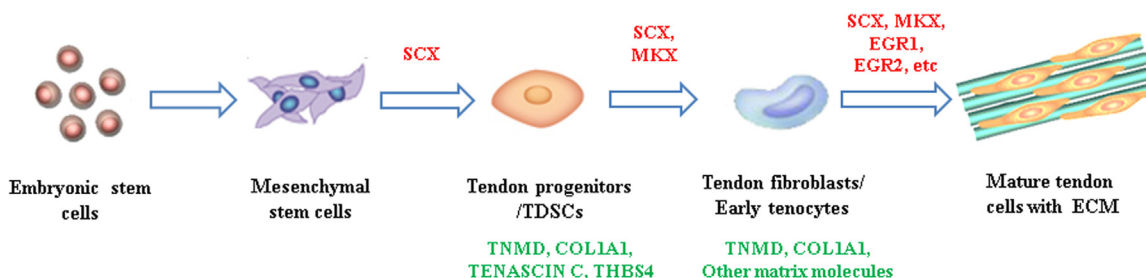


Fig. 1. Hypothetic illustration of tendon development. The transcriptional factors for tendon development are labeled in red, whereas the other molecular markers related to tendon development are labeled in green.

early stage of healing, mechanical loading causes micro-damages to initiate tendon healing [38]. COX-2 is essential in response to micro-damage in loading-stimulated tendon healing [39], and TGF- $\beta$ /Smad2/3 pathway activation contributes to the expression of SCX and maintenance of ECM [40].

Collectively, tendon differentiation is a comprehensive process mediated by many regulators and involving many signaling pathways. To understand the mechanisms of tendon differentiation will help to explore alternative cells and methods for tendon regeneration.

### 3. Non-coding RNAs in tendinopathy and tendon development

Non-coding RNAs play critical roles in both development and differentiation process. The ECM molecules, transcription and growth factors involved in tendon development are all potential targets involved in tendinopathy. Some of the ncRNAs have been proved or predicted to have involvement in tendon development and tendon disorders such as tendinopathy.

Accumulating evidences indicate that miRNAs play important roles in skeleton and muscle development and diseases [41,42], but little is known about their functions in tenogenesis and tendinopathy. A large-scale microarray assay was carried out for screening anomalously expressed genes in tendinopathy. The expression of 318 genes was significantly different in diseased samples compared to normal tendons. MiR-499 was screened out as the most significant miRNA in tendinopathy and may regulate specific genes, such as CUGBP2 and MYB [43]. In another screening study, 35 miRNAs were found in regulating cell proliferation, ECM synthesis, muscle growth and adaption, chondrogenesis and angiogenesis; among them, 12 miRNAs showed changes after mechanical loading and 16 miRNAs were changed after treatment of TGF- $\beta$  [44]. Markedly, miR-338 and miR-381 were both negatively correlated with Scx, indicating that these two miRNAs may regulate tendon development by targeting Scx. In addition, single nucleotide polymorphisms (SNPs), which located within miR-608 gene sequence, could affect the binding efficiency with 3'-UTR of COL5A1 and associate with chronic achilles tendinopathy [45,46], while silence of miR-29a/b could up-regulate collagen type III and TGF- $\beta$ 1, leading to fibrosis in tendinopathy [47,48]. Due to the high abundance of miRNAs in genome and their ability to target hundreds of genes, there should be much more miRNAs involved in tendinopathy and tendon healing than those have been identified.

The long non-coding RNAs (lncRNAs) are also extensively transcribed from mammalian genomes with multiple regulatory functions. Unlike the miRNAs which have simple and clarified regulatory mechanisms, the lncRNAs have much more complex functions that remain largely unknown. To date, few lncRNAs has been reported to be involved in tendon differentiation, but some lncRNAs have been proved to play important roles in musculoskeletal system development, cell differentiation and pathogenesis of diverse diseases through diverse mechanisms. For instance, lncRNA Hotair mediates developmental progress through promoting Histone H3 lysine 27 trimethylation [49]. Disruption of Hotair in mice leads to metacarpal-carpal bones and homeotic transformation of the spine [50]. There are several lncRNAs like Hotair that are involved in chromatin modification, silencing or activating a host of genes potentially including tendon-regulating genes. Linc-MD1 acts as a competing endogenous RNA (ceRNA) to "sponge" miRNAs that regulate muscle-related transcription factors, controlling muscle differentiation in both mouse and human myoblasts [51]. Since a number of miRNAs in tendon differentiation has been identified, ceRNAs that play important roles in tendon could also be predicted according to the base complementarity. Some other lncRNAs, such as MEG3 and H19, may be

essential in tendon development and differentiation acting as TGF- $\beta$  pathway regulators [52,53]. Recently, a RNA-seq study has profiled lncRNA expression in aging tendon and 34 lncRNAs were found to be up-regulated [54]. This study provided the basis and a good start for further research in lncRNAs in tendon development and disease. The potential importance of lncRNAs in tendon differentiation and tendinopathy deserves more research.

## 4. Novel strategies of clinical therapy for tendon disorders

### 4.1. Non-coding RNA based gene therapy

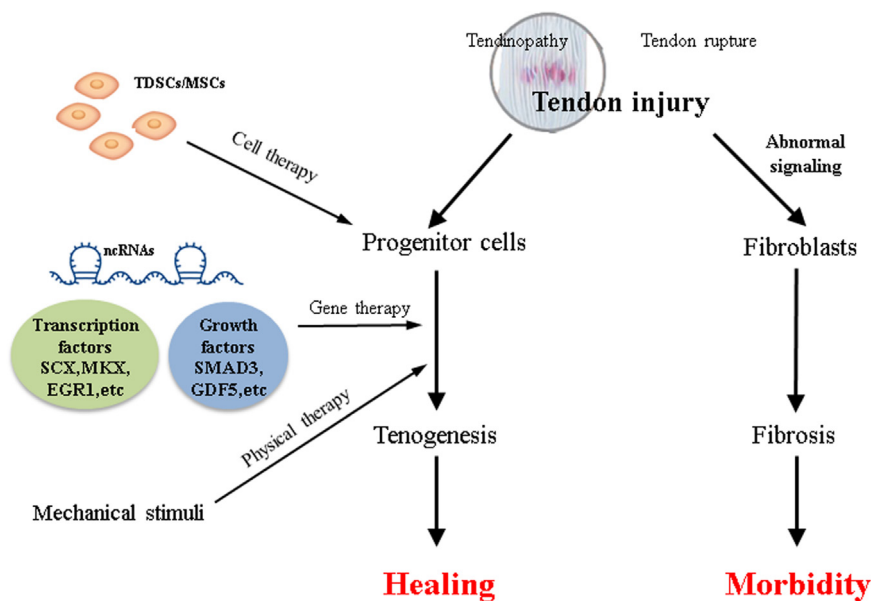
Gene therapy has been used for over twenty years and offered alternative therapeutic potentials for distinct pathologic conditions [55]. The non-coding RNA based gene therapy is still at the initial stage. *In vitro* studies demonstrated that miR-135a augmented proliferation, migration and tenogenic differentiation and inhibited cell senescence of TDSCs [56]. Studies *in vivo* also revealed the effectiveness of miRNA-based gene therapy on tendon repair in which miR-210 injection into the injured tendon accelerated Achilles tendon healing in rat through promoting angiogenesis at the early phase of repair [57]. Synthesized miRNAs targeting TGF- $\beta$  could down-regulate the expression type III collagen and CTGF which were closely correlated to tendon adhesion during wound healing, but did not affect type I collagen in tenocytes both *in vitro* and *in vivo* [58]. However, the tendon strength of group treated with siRNAs targeting TGF- $\beta$  was significantly lower than that of the control group [59], that hinders their clinical use. Tendon adhesion mainly occurs on the superficial layers of the tendons and is the most troublesome postsurgical complication, delivery of miRNAs into the superficial layers, rather than the center of the tendons, might be preferable for prevention of tendon adhesion.

To date, several non-coding RNAs has been identified in regulating tendon healing and tendinopathy. Using gene therapy strategies to introduce ncRNA genes or their inhibitors into tendon tissues may be novel options for clinical management of tendinopathy and the possible novel therapeutic strategies were summarized in Fig. 2.

### 4.2. Cells used in tendon tissue engineering

Tendon tissue engineering is to suspend tendon forming cells in structural materials to produce functional tendon tissues. The specific characteristics of tendon make the choice of cells and scaffolds different from other tissues. Fibroblasts from tendon, tendon derived mesenchymal stem cells, and ESCs are the main types of cells used for tendon engineering. ESCs bring risk of teratoma formation [60] as well as ethical controversy to be applied in clinical treatment. Tendon repaired by fibroblasts and tendon derived mesenchymal stem cells does not necessarily lead to complete regeneration, but may be accompanied with ectopic bone or cartilage formation [61].

MSCs of different source have different properties; bone marrow- and periosteum-derived MSCs have higher potential for osteogenesis, whereas adipose tissue- and synovium-derived MSCs are superior for adipogenesis [62]. Bone- and adipose tissue-derived MSCs formed ECMs with lower quantity and quality than chondrocytes during chondrogenic differentiation [63]. Consequently, the tendon-derived MSCs provided a novel cell source for treating tendon injury with reduced risk of ectopic bone and cartilage formation. The TDSCs (tendon derived stem cells) have reported in mouse, rat, rabbit and human tendon [64–67]. TDSCs exhibit pluripotent stem cell-like property with the ability to regenerate tendon-like tissues *in vivo* [64] and could enhance



**Fig. 2.** Healing process after tendon injury and potential novel therapeutics. Strategies include stem cell-based cell therapy, ncRNA-based gene therapy and physical therapy are developed for improving tendon repair and reducing morbidity and pain for patients of tendon disorders.

tendon repair in rat models [68]. Moreover, comparing to other stem cells, TDSCs could form neo-tendons without scaffold [69]. Most studies on tendon tissue engineering are scaffold-based. However, concerns of scaffolds such as affecting cell proliferation, differentiation, risk of infections, immunological responses, low bioactivity and biocompatibility [70–72]. Thus TDSCs may be an ideal choice of stem cells for tendon repair and regeneration. Considering the need of multiple operations in clinical therapy for tendinopathy, the source of autologous TDSCs would be sufficient for personalized treatment.

Combination of gene therapy with cell therapy and tissue engineering would significantly improve the tendon repair comparing to the use of single technique. Transfection with genes related to tendon regeneration, such as GDFs, into cells prior to injection at the wound site, may help to achieve complete tendon regeneration by MSCs and TDSCs. For example, biologically active variant of Smad8 (that promotes BMP-2 expression) in murine MSC cell line C3H10T1/2 could form tenocytes and neo-tendon tissue *in vivo*, whereas MSCs alone could not promote complete tendon regeneration [73]. On the other hand, the MSCs or TDSCs could be regarded as gene delivery vehicles with lower risks than those of viral and non-viral carriers.

## 5. Conclusion

The first choice for tendon injury management is still conservative therapy for pain release purposes. For patients who failed conservative therapies, surgical replacement may be the next choice. However, tendon has poor self-repair capability, and it is difficult to obtain complete recovery. Hence, novel therapeutics are needed for improving tendon healing. At present tendon regeneration still lacks of optimal cell preparation strategies; Tendon-specific differentiation factors, biomarkers and reliable tenogenic induction conditions for tendon regeneration need further carefully investigation.

The non-coding genes take up 98% of total human genome, which highlights their importance in development and diseases. Increasing number of ncRNAs have been identified with functions, which makes ncRNA-based therapeutics appealing, for example anti-miR-122 oligonucleotide is used currently in Phase II clinical

trial for HCV treatment [74]. Identification of ncRNA involved in tendon differentiation and repair may put the missing pieces of puzzle back to the molecular network of tendon biology. Understanding the biological functions and abnormalities of these ncRNAs in tendon development and tendinopathy may also provide valuable information and therapeutic targets for tendon-specific biomarkers and novel therapeutics.

## Disclosure of conflict of interest

None.

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