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Small interfering RNAs in tendon homeostasis

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Abstract

Background: Tenogenesis and tendon homeostasis are guided by genes encoding for the structural molecules of tendon fibres. Small interfering RNAs (siRNAs), acting on gene regulation, can therefore participate in the process of tendon healing.
Sources of data: A systematic search of different databases to October 2020 identified 17 suitable studies.
Areas of agreement: SiRNAs can be useful to study reparative processes of tendons and identify possible therapeutic targets in tendon healing.
Areas of controversy: Many genes and growth factors involved in the processes of tendinopathy and tendon healing can be regulated by siRNAs. It is however unclear which gene silencing determines the expected effect.
Growing points: Gene dysregulation of growth factors and tendon structural proteins can be influenced by siRNA.
Areas timely for developing research: It is not clear whether there is a direct action of the siRNAs that can be used to facilitate the repair processes of tendons.

Key words: tendinopathy, small interfering RNA, short interfering RNA, silencing RNA, RNA interference

Introduction

Tendons are structurally complex tissues that transmit the effects of muscle contraction by transferring the energy of the contraction between muscle and bone, and determining joint motion.[1] The highly specialized mechanical role of tendons makes them a critical structure in the musculoskeletal systems. Tendon injuries and degenerative processes can be debilitating, resulting in pain, functional impotence and high health care costs.[2]

Mature tendons are mainly composed of type I collagen, and of smaller quantities of proteoglycan, glycoproteins and other collagens.[3] The process of tendon formation is named fibrillogenesis, and is determined by the interaction of these molecules.[3] For example, analysing the development of the tendon in chicks, it emerges that collagen type III limits the growth of collagen type I.[3] Different molecules interact both in the proliferation phase and in the self-control of proliferation and maturation.[4–7] The presence of biglycan, a small leucine-rich proteoglycan with two side chains of either chondroitin or dermatan sulfate attached to its core protein, is associated with thicker, more regularly shaped collagen fibrils.[4] Tendon development is also regulated by other molecules, including collagen type V, VII and IX, proteoglycans, and various transcription factor such as scleraxis[8] and transforming growth factor β (TGFβ).[9]

Tendons consist of a central collagen structure with interposition of tenocytes, surrounded by a network of non-collagen molecules, and covered by a thin membrane called epitenon. The epitenon covers the cylindrical fascicles structures oriented longitudinally to the tendon.[10] The fascicles are contained in connective structures similar to the epitenon, called endotenon. The blood supply of a tendon and its nerve supply come largely from the endotenon and epitenon.[3]

Scientific research has focused on a natural process of eukaryotic gene regulation, namely RNA interference (RNAi). This mechanism can be exploited in the fields of genomic research and in the formulation of suitable therapies. Typically, a siRNA (small interfering RNA) is composed of ~20 nucleotides arranged to form a double-stranded RNA molecule.[11]

The RNAi mechanism involves various elements such as detection wire (passenger wire), sense wire (guide wire), enzymes such as Dicer, Argonaute and the central part RISC (RNA-induced silencing complex).[12] The guide wire is a nucleotide sequence recognized by Dicer, which selects it and integrates it into RISC. The guide wire is used to recognize the passenger wire that will be degraded by RISC (Fig. 1).[12]

With the progression of the understanding of the role of siRNA in the physiopathology of several conditions, their possible use in the management of many ailments caused by altered function of gene expression is being increasingly studied. This is the case for endocrinological abnormalities, hepatitis, tumours and pathologies triggered by external agents such as viruses.[12]

SiRNAs selectively target a gene and silence it, inhibiting the expression of the gene that determines the pathology under study. SiRNA technology, though theoretically powerful for the study of functional genomics and the development of therapeutic agents, can be however hindered by the intrinsic instability of siRNAs, as they can be easily digested by nucleases and therefore have a short half-life. Chemical modifications of siRNAs may improve their stability and potency, and the sequence of 4894 chemically modified siRNA is now available.[13]

The process of tenogenesis and the regulation of tendon homeostasis are guided by the different genes that encode for the structural molecules of the tendon fibres. Some of these genes regulate cell proliferation and regeneration, namely Mohawk (MKX); Scleraxis (Scx); Extracellular signal-regulated kinase (ERK) 2; Sodium-calcium exchanger 1 (NCX1); Early growth response factor 1 (Egr1); Transforming growth factor beta 1(TGF-β1).[9]

Genes are regulated by siRNAs that determine their expression and biological activity. siRNAs participate in the regulation of tendon homeostasis, and their use to intervene in the processes of tendon healing has been experimented.[11]

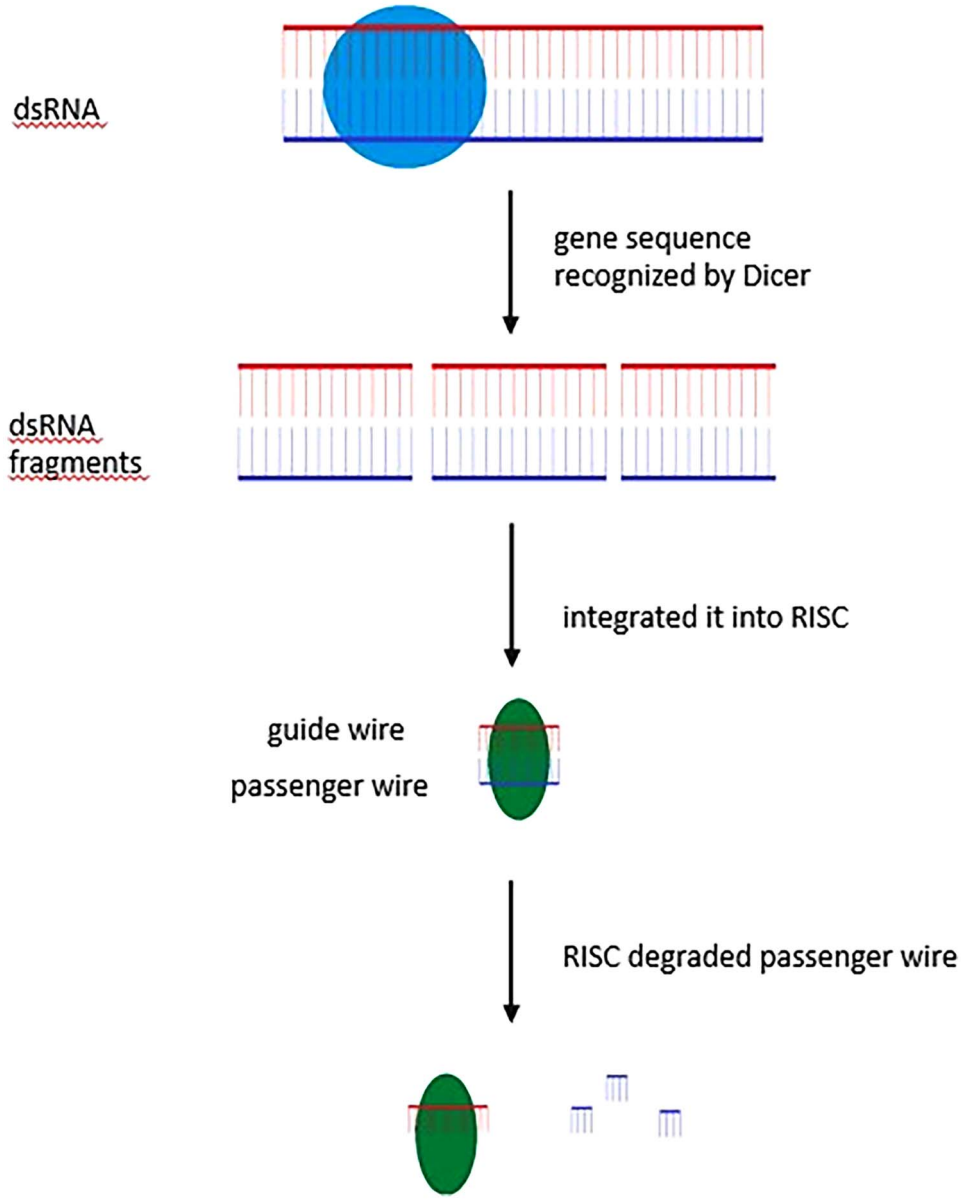

Fig. 1 Mechanism to degrade the messenger wire.

This review analyses the current scientific evidence to provide a critical perspective of the use of siRNA in the regulation of tendon homeostasis.

Methods

The present systematic review was performed following the Preferred Reporting guidelines for systematic reviews and meta-analyses (PRISMA) (Fig. 2).[14]

A systematic search up to October 2020 of articles assessing the role of siRNA in regulation of tendon homeostasis with no restrictions of language was performed.

In the search, we used combinations of the following key terms: tendinopathy, small interfering

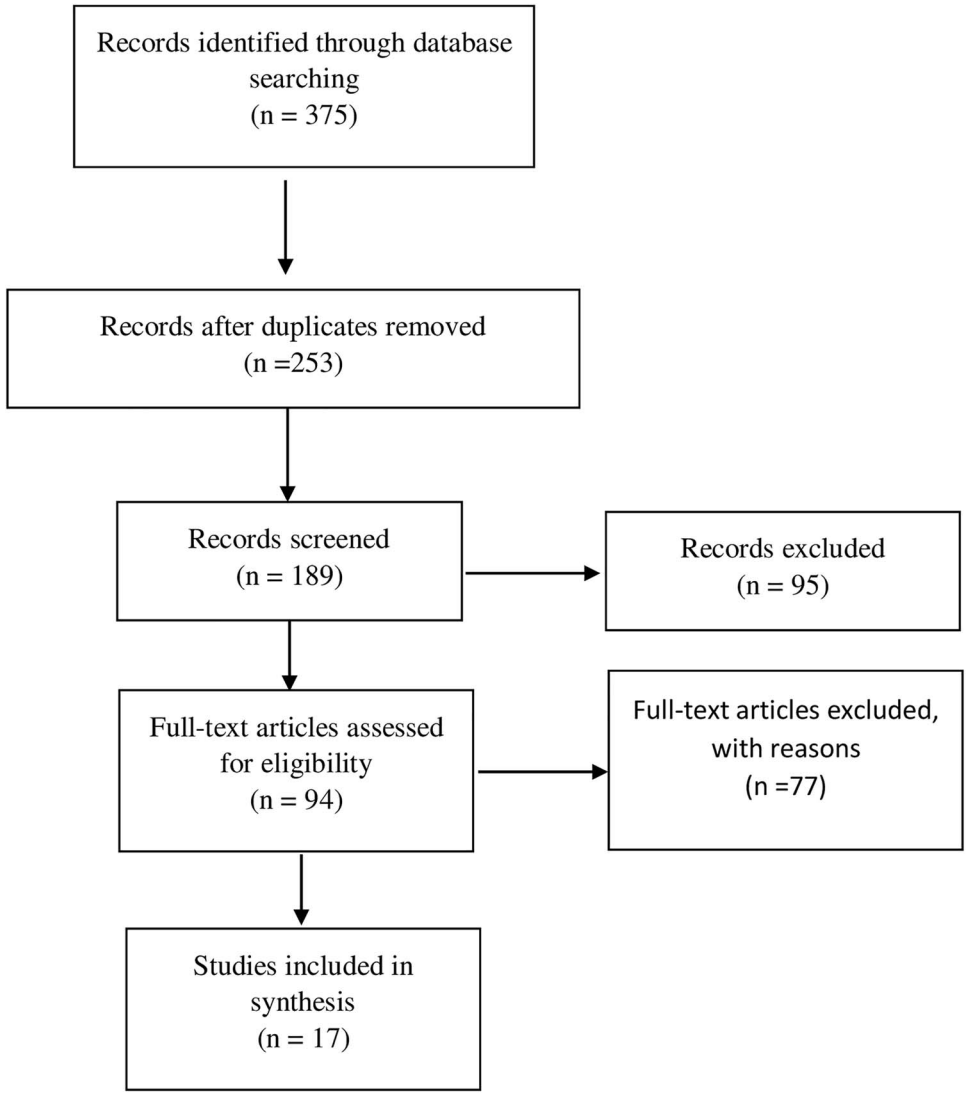

Fig. 2 PRISMA flow diagram.

RNA, short interfering RNA, silencing RNA and RNA interference, with no limit of year of publication.

Editorials, single clinical case reports, abstracts, technical notes, conference presentations, narrative reviews, expert opinions and systematic review articles were excluded.

Two investigators independently conducted the systematic search, through October 2020, using full-text archives of Embase, Google Scholar, Scopus and PubMed. The titles and abstracts were examined separately by the two investigators to remove duplicates and evaluate the eligible studies according to the inclusion criteria. The full text of each article was examined by both investigators if either of them perceived ambiguity, and the bibliographies of the articles included were reviewed by hand to identify further related articles. Where present, discrepancies were resolved through discussion with the senior investigator.

In total, 17 studies met the inclusion criteria and were included in the analysis. The PRISMA flowchart shows the details of the search (Fig. 2).

Results

The studies included in the review are summarized in Table 1.

Healing at the tendon-to-bone junction is slow and often incomplete.[32] Chen *et al.* tried to accelerate this process implanting tendon-derived stem cells (TDSCs) with silenced transforming growth interacting factor 1 (TGIF1) gene in a supraspinatus tendon tear model in rats. They showed that siRNA cells transfected and treated with TDSCs express higher levels of chondrogenic proteins.[19]

The genes that participate in chondrogenesis and fibrinogenesis were studied. Comparing by western blot the group where TGIF1 had been silenced and the control group, there were significant differences in protein concentration and transcription factors, including aggrecan, collagen II and Y-box transcription factor in the sex-determining region 9 (Sox9). When TGIF1 was silenced, the concentration of the typical fibrogenesis markers (scleraxis, tenomodulin and collagen I) was greatly decreased.[19]

To further study the function of Pin1 in the regulation of the process of senescence of tendon stem/progenitor cells (TSPC), Chen *et al.* used Pin1 siRNAs to inhibit the early stages of TPC-1 cells.[20] TSPC transfected with siPin1 showed a considerable increase in the percentage of senescence-associated β-galactosidase positive cells, suggesting that Pin1knockdown accelerates the senescence of TSPC. Furthermore, telomerase activity and the expression of protein 16 (p16INK4A) were determined in cells transfected by siRNA, showing that the Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1 (Pin1) deletion was associated with increased expression of p16INK4A and decreased telomerase activity. Knockdown of Pin1 seems to induce senescence of TSPC.[20]

The fibrogenic and proinflammatory activity of fibroblasts is suppressed by siRNA targets Transcription factor protein 65 (p65).[23] Fibroblast proliferation, apoptosis, inflammation and tendon adhesion could be modulated by the subunit P65 of the NF-κB complex.[23] p65-siRNA inhibits proliferation, promotes apoptosis and reduction of the production of ECM in tendon cells, which may not be favourable for tendon healing processes.[23]

In the Achilles tendon of rats, siRNA-mediated knockdown of the adenosine monophosphate kinase subunit a1 (AMPKa1) reduced the inhibitory effect of hepatocyte growth factor (HGF) on TGF-β1-induced myofibroblastic differentiation in the tendon fibroblasts.[17]

To determine the ability of mouse and human tenocyte to migrate, Jamil *et al.* used a gene array approach in a modified *in vitro* scratch test.[24] Normally, Angiopoietin-like 4 (ANGPTL4) exerts a positive effect on tenocyte proliferation and the cell cycle progression, as well as adhesion and migration. Treatment with siRNA ANGPTL4 resulted in a reduction in the migration rate of tenocytes.[24] The phenomenon of cell migration is fundamental for multiple crucial pathological and physiological processes such as wound healing, remodelling, cell growth and differentiation, tissue adaptation and inflammation.[33,34]

Silencing experiments evaluated the expression of collagen type III, which is involved in the process of tendon healing after injury.[30]

Scx is a transcription factor required for the development of tendons during embryogenesis and to induce tenogenesis in mechanically stimulated stem cells. Its function in adult tenocytes is however not well-defined. Nichols *et al.*[25] studied the role of Scx in mediating the mechanoresponse in adult equine tenocytes. They subjected equine tenocytes to cyclic mechanical strain and exposed them to siRNA targeting Scx or a control siRNA before performing RNA-sequence analysis. Vimentin, a fibroblast marker was highly expressed. Among the proteins of the extracellular tendon matrix, the collagen Iα2 and IIIα1 were highly expressed and not influenced by Scx knockdown.[25] In Scx knockdown tenocytes, some of the main gene networks downregulated were those involved in focal adhesions and extracellular matrix-receptor interaction. On the other hand,

Table 1 Studies included

Study	siRNA target gene*	Function on tendon	Type of study
Xue *et al.* (2009)[15]	EPCR	EPCR stimulate proliferation of tenocyte and healing	*In vitro*
Sakamoto *et al.* (2009)[16]	NCX1	NCX1 is a protein found in tendon fibroblasts imputed to tissue repair	*In vitro*
Cui *et al.* (2013)[17]	AMPKa1 subunit	The balance with growth factors such as HGF and TGF-β1 regulates extracellular matrix deposition	*In vitro*
Ruan *et al.* (2013)[18]	ERK 2	ERK 2 participates in the recruitment and proliferation of fibroblasts in the formation of post-surgical tendon adhesions	*In vivo*
Chen *et al.* (2015)[19]	TGIF1	TGIF1 participates in the regeneration of the insertion site between tendon and bone	*In vitro*
Chen *et al.* (2015)[20]	Pin1	Pin1 knockdown induces TSPCs(tendon stem/progenitor cells) senescence	*In vitro*
Li *et al.* (2015)[21]	TGIF1	TGIF1 is a very important transcription factor in tendon repair	*In vivo*
Wu *et al.* (2017)[22]	MKX, Egr1	MKX and Egr1 have the function of regulating the development and tendon repair processes.	*In vitro*
Chen *et al.* (2017)[23]	RELA	Influences fibroblasts proliferation, apoptosis, inflammatory markers, and tendon adhesion	*In vitro*
Jamil *et al.* (2017)[24]	ANGPTL4	ANGPTL4 is stimulated by exercise and improves tendon healing	*In vitro*
Nichols *et al.* (2018)[25]	Scx	Scx is a transcription factor frequently used as a tendon marker, Scx has a pro-tenogenic effects	*In vitro*
Xu *et al.* (2018)[26]	FOXP1	FOXP1 participates in various biological processes, cell cycle, proliferation, differentiation and aging of TSPCs	*In vitro*
Yao *et al.* (2019)[27]	IRE1, ATF-6	ATF-6 and IRE1 are implicated in tendon adhesion processes and apoptosis	*In vitro*
Zhao *et al.* (2019)[28]	Pitx1	Pitx1 determines the structure and tendon morphology	*In vitro*
Wang *et al.* (2020)[29]	TGF-β1, Smad 2, Smad3	TGF-β1, Smad 2 and Smad3 are implicated in the signalling pathways leading to tendon healing	*In vivo*
Liao *et al.* (2020)[30]	Collagen type III	collagen type III is normally present in tendon repair processes	*In vitro*
Jackson *et al.* (2020)[31]	Flii	Flii is a cytoskeletal protein involved in adhesion of tendon cells	*In vitro*

*Endothelial cell protein C receptor (EPCR); Sodium-calcium exchanger 1 (NCX1); Adenosine monophosphate kinase (AMPK) - AMPKa1 subunit; kinase (ERK) 2; Transforming growth interacting factor 1 (TGIF1); Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1(Pin1); Mohawk (MKX); Early growth response factor 1 (Egr1); Transcription factor p65 (RELA gene); Angiopoietin-like 4 (ANGPTL4); Scleraxis (Scx); Extracellular signal-regulated; Forkhead box P1 (FOXP1); Inositol requiring kinase 1 (IRE1); Activated transcription factor 6 (ATF-6); Paired-like homeodomain 1 (Pitx1); Trasforming growth factor beta 1(TGF-β1); Mothers against decapentaplegic homolog 2-3 (SMAD2, SMAD3); Flightless I (Flii).

tenocytes exposed to Scx siRNA exhibited longer vinculin-containing focal adhesions, and a decreased capability to migrate on soft surfaces.

Sakamoto *et al.* employed two siRNA for NCX1 to specifically knock down NCX1 expression, and studied tenocyte migration and contraction in a wound healing scratch model. The siRNAs used significantly decreased the expression levels of NCX1 mRNA. NCX1 is involved in the migration and contraction of tendon fibroblasts: when NCX1

is suppressed, the motility and contractility of tenocytes are negatively affected.[16]

Wu *et al.* used siRNA to reduce the expression of Mkx or Egr1. Egr1 was inhibited and the expressed genes were evaluated, comparing them with a control group. The expression levels of all target genes, including TGF-β1, Bgn, Col Iα1 and Col Iα2, were reduced. Furthermore, the suppression of Egr1 downregulated the transcription factors Scx and Mkx.[22]

Forkhead box P1 (FOXP1) is implicated in the regulation of the ageing processes of tendon stem progenitor cells (TSPC). Xu *et al.* used FOXP1-siRNA to knock down FOXP1 in TSPCs from young animals. B-galactosidase staining showed that FOXP1-siRNA increased the percentage of b-galactosidase positive cells. Moreover, FOXP1 deletion also increased the expression of p16INK4A in young TSPCs.[26]

In the study of Xue *et al.*, when tenocytes were pre-treated with endothelial cell protein C receptor (EPCR) siRNA, the effect of Adenomatous Polyposis Coli (APC) on proliferation, MMP-2 and type 1 collagen synthesis and mitogen-activated protein kinases was blocked. APC promotes the growth, MMP-2 activity, type I collagen deposition and migration of tenocytes.[15]

In an *in vivo* rat tendon injury model, Yao *et al.* showed that the activated transcription factor 6 (ATF-6) is involved in the inhibition of fibrosis by hydroxycamptothecin (HCPT). They used siRNA targeting activating ATF-6 to pre-treat fibroblasts and then incubated them with TGFβ1 and/or HCPT. The effect of inhibition of fibrosis of HCPT disappears after knockdown of ATF-6.[27]

Flightless I (Flii) protein is involved in cell adhesion processes. In a study on a murine digital flexor tendon model, after knockdown of Flii by siRNA *in vitro*, inhibition of this protein reduces cell adhesion, reducing type 1 collagen expression and increasing TGF-β1 levels.[31]

Of the studies analysed, three were *in vivo* investigations[18,21,29] the results of which confirmed the findings of the *in vitro* studies.[15–17,19,20,22–28,30,31]

Li *et al.* analyzed derived mesenchymal stem cells from the bone marrow (BMSCS) using western blot. Non-transduct biological samples with TGIF1 siRNA showed lower levels of chondrogenic proteins (Aggrecan, Sox9 and type II collagen) than BMSC-treated samples translated with TGIF1 siRNA.[21]

In a chicken flexor tendon repair model, tendon adhesions in the siRNA ERK group were reduced compared with the control group. There were however no significant differences in the rate of failure following tendon repair in the two groups.[18]

The study of Wang *et al.* was carried out on rats: some fibroblast cells were treated against TGF-β1 and Smad 2 and Smad 3 with specific siRNA. The siRNA inhibited the expression of the mRNA of the target genes in fibroblast cells. Clinically, the rats, after the transfer of the siRNAs, developed reduced physical mobility and a limp, or dragged the limbs during movement. Inflammation occurred in 20% of rats.[29]

Discussion

In human medicine, most tendon lesions occur at the insertion of the tendon into bone, and, though presenting acutely, in the vast majority of cases the histological changes typical of a long-standing failed healing response are well estatblished.[35–40]

One study investigated the process of healing after detachment and reintegration of the patellar tendon to the patella in a sheep model.[41] At optical microscopy, it appears that the tendon joins perfectly to the bone. At electron microscopy, however, profound alterations to the structure of the extracellular matrix, hypercellularity and no fibrocartilage were noted. Therefore, surgical re-insertion does not fully reconstitute the native enthesis, and the bone-tendon junction is likely more susceptible to subsequent injuries.[20,42]

The present systematic review evaluated the effects of the most important factors and molecules useful for the regeneration and healing of tendons through the use of siRNA. TGFβ seems to modulate the normal healing process of the tendon at its

insertion.[17] The Smads inhibitor and the Smad co-repressor influence the Smad signalling pathway produced by TGIF1. TGIF1 is related to the inflammatory response: silencing of TGIF1 results in a reduced inflammatory response and faster healing.[21]

Fibroblast proliferation, cell adhesion and inflammatory stimulation are enhanced by Transcription factor p65 (RELA gene).[23] During the process of maturation of tenocytes of the axial and appendicular skeletal muscle system, Scx is overexpressed: this overexpression is maintained even when the tendon is mature. Scx is a selective marker of growth and differentiation of tendon cells.[25] Its role appears to be related to tenocyte fibrillogenesis.[43] Scx is intimately related to Sox9.[44] Some mesenchymal cells, in the early stages of cartilage formation, come directly from precursors expressing Sox9.[45] Some progenitors of tendon cartilage in murine embryos come directly from precursors expressing Scx and Sox9.[45]

The process of tendon healing does not always progress as planned, but the mechanisms of failure are not well defined yet.[38,46,47] During the process of differentiation, the chondrogenic and tenogenic genes determine the transformation towards the formation of ligaments or tendons starting from connective tissue cells.[48]

TGFβ is a development factor for both the chondrogenic and fibrogenic lines. Pryce *et al.*[9] showed that the expression of TGFβ in the differentiation of muscles and cartilage is fundamental both for the formation of tendons starting from tendon progenitors, and to maintain homeostasis of the tendon itself. At the tendon lesion point, the structure is more fragile and tends to break down after a second trauma. Better healing at the bone-tendon interface takes place if cells capable of undergoing chondrogenesis are added to that area.[36,43]

In a chicken flexor tendon repair model, Ruan *et al.* used a siRNA targeting ERK 2 delivered by a lentiviral system to prevent tendon adhesion formation. ERK 2 is fundamental in the process of proliferation of fibroblasts and in the formation of collagen-mediated tendon adhesions formation after flexor tendon surgery.[18]

Most of the published work focuses on the enthesis (bone-tendon interface) whereas in the body of the tendon the situation may be different, as the damage in this region is often connected to associated pathologies.[2,39,40,49]

Conclusion

This systematic review summarizes the current published evidence on the role of siRNAs on homeostasis and tendon repair processes. siRNAs can be useful to identify molecules that participate in various tendon repair processes. Using siRNAs, some authors evaluated the effects of some transcription factors and their biological effects in this field. Identification, activation and gene silencing can be a useful mechanism and a pharmacological target on which to work to produce new specific drugs suitable to repair tendon damage. SiRNAs can be useful to identify target genes for such future drugs.

Tendon healing proceeds through inflammation and the recruitment of cytokines and leukocytes. These processes occur through specific proteins, some of which are the subject of recent studies. An important goal would be to be able to regulate the genes that encode these responses through siRNA, acting on the silencing of antagonistic proteins, to induce faster and better healing following tendon injuries.

Conflict of interest statement

The authors have no potential conflicts of interest.

Data availability

Data available on request.

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