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REVIEW ARTICLE

Current concepts on tenogenic differentiation and clinical applications

Yang Liu [a], Chun-Wai Suen [a], Jin-fang Zhang [a], Gang Li [a,b,c,d,*]

[a] *Department of Orthopaedics and Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, China*
[b] *Stem Cells and Regenerative Medicine Laboratory, Lui Che Woo Institute of Innovative Medicine, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, China*
[c] *Key Laboratory for Regenerative Medicine, Ministry of Education, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China*
[d] *The CUHK-ACC Space Medicine Centre on Health Maintenance of Musculoskeletal System, The Chinese University of Hong Kong Shenzhen Research Institute, Shenzhen, China*

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Summary Tendon is a tissue that transmits force from muscle to bone. Chronic or acute tendon injuries are very common, and are always accompanied by pain and a limited range of motion in patients. In clinical settings, management of tendon injuries still remains a big challenge. Cell therapies, such as the application of stem cells for tenogenic differentiation, were suggested to be an ideal strategy for clinical translation. However, there is still a lack of specific methods for tenogenic differentiation due to the limited understanding of tendon biology currently. This review focuses on the summary of current published strategies for tenogenic differentiation, such as the application of growth factors, mechanical stimulation, biomaterials, coculture, or induced pluripotent stem cells. Current clinical applications of stem cells for treatment of tendon injuries and their limitations have also been discussed in this review.

* Corresponding author. Department of Orthopaedics and Traumatology and Li Ka Shing Institute of Health Sciences, Prince of Wales Hospital, The Chinese University of Hong Kong, 30-32 Ngan Shing Street, Shatin, New Territories, Hong Kong, China.
E-mail address: gangli@cuhk.edu.hk (G. Li).

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Introduction

Tendon is a tissue that transmits force from muscle to bone. Tendon injures, such as tendinopathy or acute tendon rupture, are a common type of sports injuries. However, current treatments for tendon injures are unsatisfactory and limited to the nonsteroidal anti-inflammatory drug injection, physical therapy, or surgery [1—3]. Tendon tissue engineering has been suggested to be a promising approach for tendon repair. Since bone marrow stem cells (BMSCs) or tendon-derived stem cells (TDSCs) have outstanding self-renewal and multidifferentiation ability, it is a well-recognized strategy to apply them in tendon tissue engineering [4,5]. Although many genes are reported to be involved in tendon development, they also express in a wide range of other tissues, such as muscle, bone, and cartilage. Owing to the limited understanding of specific tendon makers and molecular interactions between transcription factors and signalling pathways, there is still a lack of a specific method for tenogenic differentiation. Currently, various protocols have been reported to be able to induce tenogenic differentiation. This review focuses on the summary of currently published strategies for tenogenic differentiation, such as the application of growth factors, mechanical stimulation, biomaterials, coculture with another cell source, TDSCs, or BMSCs. An advanced understanding of the current strategies on tenogenic differentiation would be beneficial for tendon tissue engineering and its clinical translation in the future.

Tendon biology

Tendon and associated extracellular matrix markers

Tendon formation relies on the combination of the transcription factors, growth factors, and mechanical stimulation during development [6]. In normal tendon, the primary unit of the tendon is the fibre that made up of collagen fibrils with tendon cells residing inside [6]. The dry mass of human tendons is about 30% of the total tendon mass, with water accounting for 70% [7]. From the dry mass of tendon, collagen type I accounts for 65—80%, and elastin takes up about 2% [7,8]. Collagen provides elasticity to the tendon, which is mainly made up of type I collagen (*Col1*) and a small amount of other collagens, such as types III, IV, V, and VI [9]. The extracellular matrix (ECM) functions as the organizer for collagen fibril assembly [5,10,11], and it is composed of proteoglycan, glycoproteins, and other small molecules. Decorin (Dcn) and biglycan (Bgn) are the common small leucine-rich proteoglycans in tendons that help organize the collagen fibre bundles. Targeted knockout of certain proteoglycan can lead to abnormal collagen fibrils in tendons and impair their mechanical properties [12—14]. Other common proteoglycans are fibromodulin and lumican. It was reported that Tenascin-C (Tn-C), a glycoprotein, is regulated by mechanical loading and is upregulated in patients with tendinopathy [15,16]. Moreover, Tn-C also participates in collagen fibre alignment and orientation [7]. Particularly, Tenomodulin (Tnmd) is a type II transmembrane glycoprotein containing a C-terminal antiangiogenic domain, and it is necessary for tenocyte proliferation and tendon maturation [17,18]. The expression of Tnmd is positively regulated by Scleraxis (Scx) [19]. Mice with loss of Tnmd expression showed impaired tenocyte proliferation, reduced tenocyte density, and increased maximal and greater variation of fibril diameters [18].

Transcription factors of tendon

Currently, Scx, Mohawk (Mkx), and early growth response protein 1 (Egr1) have been identified as the transcription factors for tendon development [9,20,21]. Scx, a basic helix—loop—helix transcription factor, is a relatively specific marker of tendon/ligament lineage and has been reported to be induced at the earliest stage during tendon development [22—24]. Mice with Scx knockdown ($Scx^{-/-}$) have severe disruption of force-transmitting tendons, with limited movement of paws and back muscles, and inability to move the tail [20]. It has also been reported that *Scx* could activate *Col1* together with *Nfatc4* (nuclear factor of activated T cells, cytoplasmic 4) [25]. The matrix in the tendon from $Scx^{-/-}$ mutant mice is also disorganized, with intermixing of tenocytes and endotenon cells [20].

Mkx is a membrane of the three amino acid loop extension superclass of a typical homeobox genes expressed in developing tendons [9,26]. Mice with Mkx knockdown ($Mkx^{-/-}$) showed significantly reduced tendon mass and a small collagen fibril diameter [9]. The expression of *Col1A1* is also decreased in $Mkx^{-/-}$ mice, indicating that *Mkx* plays a role in tenogenic differentiation by regulating the production of collagen type I. Moreover, Liu et al [27] also reported that *Mkx* could dramatically activate *Scx* by binding to the tgfb2 promoter, and Mkx showed lower expression in tendinopathy and it is activated during tendon development.

Egr1 is a zinc finger transcription factor, and it was reported to be involved in vertebrate tendon formation [28]. Mice with *Egr1* knockdown ($Egr1^{-/-}$) have weaker mechanical properties, and decreased expression of *Scx*, *Col1A1*, and *Col1A2* was observed in adult tendons [21]. Particularly, it was also mentioned that *Egr1* can promote tenogenic differentiation by targeting transforming growth factor (TGF)-β2. As mentioned before, mechanical stimulation is also necessary for tendon development, especially during the late stage of tenogenic differentiation, to promote the maturation of collagen [29,30]. Activation of *Egr1* has been suggested as a possible mechanism during mechanical stimulation, which promotes the maturation of collagen formation [10,30].

Tendon-derived stem cells

Bi et al [5] first identified and characterized tendon stem cells in tendons from human and mouse, followed by Rui et al [31] in isolating and identifying TDSCs from rat tendon. TDSCs showed multipotent and self-renewal capacities, and they have been suggested as an ideal cell source for tendon tissue engineering. Moreover, it is also found that TDSCs have higher *Tnmd*, *Scx*, *Col1*, *Dcn*, *Bgn* expression; osteogenic differentiation; and chondrogenic differentiation abilities when compared with BMSCs [32].

Current strategies on tenogenic differentiation

In the past 2 decades, many studies and reviews have been performed to foster the understandings on tendon development [30,33,34]. It has been recognized that tendon development relies on both biological and biomechanical stimulation [29,30,35]. Embryological studies have revealed that TGF-β, bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and Wnt signalling pathways were involved during the differentiation of skeletal progenitor cells [23,29,35—38]. It was additionally suggested that the divergent differentiation of progenitors are dependent on the temporal coordination of those signals, rather than solely via an individual signalling pathway in an embryonic digit model [23,29]. Apart from the biological factors, mechanical stimulation is also necessary for tendon development, especially during the late stage of collagen maturation [29]. Applications of mechanical stimulation on TDSCs or BMSCs were also reported to promote tenogenic differentiation. Furthermore, discovery of novel biomaterials, using the coculture strategy, or application of induced pluripotent stem cells (iPSCs) was also suggested to be considered for tendon tissue engineering. In this review, we focus on the summary of the currently published strategies for the above subtopics on tenogenic differentiation and their current clinical applications.

Literature search

A comprehensive literature review was performed to obtain experimental studies on the following topics for tenogenic differentiation and clinical applications. We conducted a literature search using the PubMed search engine with the following terminologies relevant to the topic: "tenogenic differentiation", "GDF and tenogenic differentiation", "BMP and tenogenic differentiation", "FGF and tenogenic differentiation", "TGF-β and tenogenic differentiation", "Wnt and tenogenic differentiation", "biomaterial and tenogenic differentiation", "decellularized matrix and tenogenic differentiation", "coculture and tenogenic differentiation", "iPSCs and tenogenic differentiation", "tendon stem cells and tenogenic differentiation", "bone marrow stem cells and tenogenic differentiation", "adipose stem cells and tenogenic differentiation", and "stem cell and tendon". Studies published in the recent 10 years were screened by title first, and then by the abstract, to confirm whether relevant information was provided. Articles shown in the reference list in published systematic reviews not found in the PubMed were also included.

Growth factors

TGF-β ligands

TGF-β signalling plays a key role in tendon formation and has been suggested to be a potent inducer of the tendon transcription factor *Scx* [38]. In embryos with TGF-β signalling disruption (TGF-$\beta2^{-/-}$ or TGF-$\beta3^{-/-}$), loss of tendons and ligaments in limbs, trunk, tail, and head was observed, indicating that TGF-β2 and TGF-β3 might mediate new tendon cells towards tenogenic differentiation. As per *in vitro* studies, the effects of TGF-β on promoting tenogenic differentiation were well reported by enhancing tenogenic gene expression (*Scx*, *Tnmd*, and *Egr1*) and ECM production [38—40]. Recently, a stepwise protocol using TGF-β1 to initiate tenogenic differentiation, followed by a combination of TGF-β/connective tissue growth factor (CTGF) to further maintain the teno lineage, was reported to better promote tenogenic differentiation [40]. As observed in *in vivo* studies, the induced BMSCs could promote neotendon formation, patellar tendon repair, and increased mechanical properties, indicating its potential application in clinical treatment. However, there is also the controversy that TGF-β could promote tissue fibrosis by increasing Col1 production and scar formation during injury healing [41], indicating that the application of TGF-β-treated BMSCs was better than the direct injection of TGF-β ligands into the injury site, to avoid excess collagen formation and scarring during injury healing. By the transcriptome analysis of mouse limb tendon cells during development, Havis et al [35] found that TGF-β signalling was sufficient to drive mesodermal stem cells towards tendon lineage via the intracellular Smad2/3 pathway. It is also reported that mature tendons in $Smad3^{-/-}$ mice showed crimped fibres, lower tenogenic gene expression [42], and impaired tendon healing [43]. However, fewer effects of Smad3 on tenogenic differentiation in BMSCs were reported. Considering the obvious tendon defects in $Smad3^{-/-}$ mice, its effects on BMSCs are warranted to be further studied in the future.

FGF ligands

FGF is a crucial signal for limb outgrowth [44]. Loss of myotomal FGF protein leads to the absence of tendon formation via Myf5 and Myod1 expression [45], and FGF4 was also reported to induce the expression of *Egr1* and *Egr2*, transcription factors expressed in tendons, to help in the maturation of tendon tissues [28]. FGF is also one of the basic components of platelet-rich plasma, which has been reported to promote tendon injury [46]. As observed *in vitro* studies, FGF2 (also known as bFGF) was mostly shown to promote matrix production (e.g., *Col1* and *Tn-C*) [47—50] or *Scx* [51,52] in either BMSCs or other stem cell types. According to *in vivo* studies, FGF2 planted in the fibrin [53,54] or gelation hydrogel [55] was reported to promote rat tendon healing by increasing collagen production and mechanical strength, indicating its potential for future applications. However, Brown and colleagues [39] reported that FGF4 has no effects on the regulation of tendon-related gene expression in tendon progenitor cells, although it was reported to activate the expression of Egr1 and Egr2 *in vivo*. Current results support that FGF2 plays a positive role on tenogenic differentiation, compared with FGF4.

BMP (growth differentiation factor) ligands

Growth differentiation factors (GDFs) are a subfamily of the BMPs that are indispensable in skeletal tissue development, such as joint formation, due to their associations with osteogenic or chondrogenic differentiation [37].

GDF5 (also named as BMP14), GDF6 (also named as BMP13), and GDF7 (also named as BMP12) were first reported to promote ectopic neotendon/ligament formation in rats by Wolfman et al [56]. Tendons in GDF5$^{-/-}$ mice showed weaker mechanical properties, reduced collagen production, and smaller collagen fibrils, and Achilles tendon injury also exhibits a 1–2-week delay in injury healing [36,57,58]. Similarly, defects in joint, ligament, and cartilage formation were observed in mice with GDF6 deficiency [59]. Tail tendons in GDF6-deficient mice showed a lower collagen content, which further contributed to the impaired mechanical property [60]. In mice with GDF7 deficiency, the Achilles tendon in GDF7$^{-/-}$ mice showed a smaller fibril diameter when compared with the wild-type controls, while there was no significant difference in the expression of tendon proteoglycans (*Dcn*, *fibromodulin*, *lumican*, and *Bgn*). There may be compensating effects in GDF family members during tendon formation, as GDF5 and GDF6 showed a two-fold increase in tendons of GDF7$^{-/-}$ mice [61].

In *in vitro* studies, the role of GDFs in tenogenic differentiation was well summarized by Lui et al [34] and is not shown in detail in Table 1 except for new studies. The mechanisms of GDFs on promoting tenogenic differentiation may be caused by activating cytoskeleton reorganization signalling (stress fibre formation) [62] or activating the Smad1/5/8 signalling pathway [63]. Considering the osteogenic or chondrogenic differentiation effects of the BMP family, *Noggin* was suggested to play an essential role in switching from osteogenic or chondrogenic to tenogenic differentiation of stem cells during the development [33].

As observed in *in vivo* studies, suture coating with GDF5 was reported to promote collagen synthesis and cell migration in tendon fibroblast [64], and promote injury healing by increasing collagen formation and maximal loading in a rat flexor tendon laceration [65]. In a clinical randomized control trial, implantation of recombinant human bone morphogenic protein 12 (rhBMP12) (GDF7)/absorbable collagen sponge was shown to promote rotator cuff injuries in 14 of 16 patients at 1-year follow-up, indicating its safety and potential to promote tendon injury repair in the future [66]. However, there is a lack of conclusion on the effects of GDF6 in tendon injury healing currently. Jelinsky et al [67] showed that injection of GDF6 or GDF7 on Day 1 after tendon injury could promote injury healing, but no related histology or mechanical testing data were shown. While Gulotta et al [68] reported that injection of GDF6-overexpressing MSCs (mesenchymal stem cells) could not promote injury healing in a rat supraspinatus tendon repair model, compared with the MSC-only group. Collectively, GDF5 or GDF7 may have potential therapeutic effects on tendon injury. For clinical translation, suture coating with GDFs or combining GDFs with biomaterials could be a potential consideration for tendon injuries.

Wnt ligands

Wnt signalling is essential and plays multiple roles during vertebrate limb development [69]. As a classic signalling pathway on promoting osteogenic differentiation, recent studies also indicated that it plays a role in tendon/ligament formation during embryogenesis [33,35]. In an *in vitro* study, Miyabara et al [70] reported that the activation of Wnt/β-catenin signalling could induce *Tnmd* expression in BMSCs via glycogen synthase kinase-3. It was also reported that *Wnt4* and *Wnt5a* were highly involved in the dynamic loading of tenogenic differentiation [29,71]. However, there are fewer studies showing that ectopic Wnts could promote tenogenic differentiation directly, indicating that it may be one of the signalling pathways involved in tendon development, instead of the pathway that could activate tendon development directly.

Others

Recently, Havis et al [35] reported that MAPK (Mitogen-activated protein kinase), calcium, Wnt, and Hedgehog signalling were greatly involved in mouse limb tendon development using transcriptomic analysis, in addition to the TGF-β signalling pathway. Our group also found that inhibition of the ERK (Extracellular signal–regulated kinases) signalling pathway could promote tenogenic differentiation via activation of *Scx*, *Col1*, *Dcn*, and *Tnmd* (unpublished data). Our results are consistent with those of Havis et al [35] that inhibition of ERK MAPK signalling can activate Scx in mouse limb mesodermal progenitors and mesenchymal stem cells. Moreover, the Hedgehog signalling plays an important role during tendon development via its downstream effector *Gli1* [72]. Carbone et al [73] also reported that Hedgehog signalling is mechanosensitive and active during tendon–bone healing in a rat anterior cruciate ligament reconstruction model.

Connective tissue growth factor

CTGF (also named as CCN2) is important in biological processes such as skeletal development and differentiation [74]. According to *in vitro* studies, CTGF could upregulate *Col1* and *Tn-C* expression in human BMSCs, and accelerate the formation of fibrosis-like tissue from connective tissue without ectopic mineralization [75]. Particularly, CTGF showed better effects in tendon cells by activating *Scx*, *Tnmd*, and other ECM marker expression, compared with that in BMSCs [76,77]. It is also reported that the engineered scaffold-free tendon tissue, produced via CTGF and ascorbic acid-treated TDSCs, showed significantly higher *Tnmd*, *Scx*, *Col1*, *Dcn*, and *Thbs4* expression, and augmented tendon repair in both patellar tendon defect [78,79] and anterior cruciate ligament reconstruction models [80]. The basal expression level of *Scx* in BMSCs was significantly lower than that in TDSCs [32]. The limited effects of CTGF on *Scx* expression in BMSCs indicate that it cannot activate *Scx* directly. To solve the problem, a stepwise differentiation strategy was suggested by Yin et al [40]. They first stimulated BMSCs with TGF-β1 to activate the tendon-specific marker expression (e.g., *Scx*), and then followed by combination with CTGF. The results indicated that the expression of *Scx* was further activated (as well as that of *Tnmd*), compared with TGF-β1 only [40]. It is

Table 1 Factors regulating tenogenic differentiation.

Factor	Study types	Species[a]	Cell model	Injury model	Effects	Study level[b]
TGF-β signalling pathway						
TGF-β2; TGF-β3 [33]	*In vivo*	Mice	*TGF-β2−/−*; *TGF-β3−/−* mice	TGF-β2−/−; TGF-β3−/−	Loss of tendons during development	3
TGF-β2 [34]	*In vitro*	Mice	TPC	/	↑*Scx*	2
TGF-β1 + CTGF [40]	*In vitro* & *in vivo*	Human	BMSCs	Nude mice & rat patellar tendon	↑*Scx*, *Tnmd*, & ECM; ↑mature collagen formation and mechanical properties of new formed tendon	3
TGF-β1 antibody [41]	*In vitro* & *in vivo*	Rabbit	Tenocytes	Inject into transected flexor digitorum tendon	↑Collagen formation; limited range of motion	2
Smad3 [42,43]	*In vivo*	Mice	*Smad3−/−* mice	Flexor digitorum longus tendon repair	Tendons showed crimped fibres and ↓*Col1* and *Tn-C*; ↓mechanical properties and ↓collagen formation	3
FGF signalling pathway						
FGF2 [47]	*In vitro*	Human	BMSC		↑*Col1*, *Col3*, fibronectin at 3 ng/mL	1
FGF2 [51]	*In vitro*	Mice	C3H10T1/2/inkjet-based bioprinter		↑*Pea3*, *Erm*, *Scx*	2
FGF2 [52]	*In vitro*	/	Patterned with submicron polystyrene fibres		↑*Scx* expression	2
FGF2/FGF5 [48]	*In vitro*	Foals	Umbilical cord blood stem cells/ASCs		↑*Tn-C* expression in matrix gel	1
FGF4 [39]	*In vitro*	Mice	TPCs		No effect despite regulation of other genes	2
FGF2 [54]	*In vivo*	Rat	In fibrin sealant (100 mg/kg)	Rotator cuff tendon defects	↑Collagen maturation; mechanical properties	2
FGF2 [55]	*In vivo*	Rat	Gelatin hydrogel containing 5 μg of FGF2	Supraspinatus tendon to insertion sites	↑*Scx*, *Tnmd*; ↑mechanical strength; collagen fibres with an aligned orientation	3
bFGF (FGF2) [49]	*In vivo*	Chicken	/	FDP tendons, injected vectors carrying bFGF transgenes	↑Col1 production and cell proliferation; mechanical strength; ↑fibre arrangement	2
bFGF (FGF2) [50]	*In vivo*	Rat	Slow release by an osmotic pump	Chronic supraspinatus tendon lesion	↑Col1 production	1
BMP signalling pathway						
GDF5 coating suture [64]	*In vitro*	Rat	Fibroblast	/	↑Cell migration; cell proliferation; collagen synthesis	1

GDF5 coating suture [65]	*In vivo*	Rabbit	/	Zone II flexor tendon lacerations	↑Collagen formation; increase maximal loading	1
GDF6 [68]	*In vivo*	Rat	BMSC with overexpression of GDF6 or BMSC seeded in fibrin sealant	Implant in supraspinatus tendon—bone interface	No significant difference on histology and mechanical properties	2
rhBMP12 or rhBMP13 [67]	*In vivo* injection	Rat	/	Achilles tendon transection model	↑Rat and quality of tendon repair	1
rhBMP12 [66]	*In vivo*	Human	rhBMP2 in absorbable collagen sponge	Implanted in rotator cuff repair	1 y follow-up; 14/16 patients with complete healing	2
GDF7 [63]	*In vitro*	Canine	ADSCs	/	↑*Scx*, *Tnmd*; ↓*Ocn* expression; ↑Smad1/5/8 signalling pathway	2
Wnt signalling pathway						
GSK-3 inhibitor [70]	*In vitro*	Equine	BMSC in collagen gel	/	↑*Tnmd*, *Dcn*, and *Fmod*	2
Wnt5a, Wnt4 [29]	*I vitro*	Human	MSC-seeded collagen gel under mechanical stimulation	/	↑*Wnt4* & *Wnt5a*	2
Wnt5a [71]	*In vitro*	Human	Tenocytes seeded in polyglycolic acid long fibres under mechanical stimulation	/	↑*Wnt5a*	2
Other signalling pathway						
Indian Hedgehog [73]	*In vivo*	Rat		ACL reconstruction using a flexor tendon graft with pretension	Indian Hedgehog signalling was active at the healing tendon—bone interface	1
ERK/MAPK [35]	*In vitro* & *in vivo*	Mice	C3H10T1/2	E9.5 mouse limb explants	Inhibition of the ERK/MAPK could ↑*Scx* in mouse limb mesodermal progenitors	2
CTGF						
CTGF + Vc [78,79]	*In vitro* & *in vivo*	Rat	TDSC	Tendon-like cell sheet implanted in rat patellar tendon defect model	↑*Scx* and ECM gene expression; ↑collagen formation and mechanical properties	2
CTGF [76]	*In vitro*	Rat	Overexpression of CTGF in TDSCs	/	↑*Scx*, *Tnmd*, *Tn-C*, *Col1*	2
CTGF [75]	*In vitro* & *in vivo*	Human	BMSC	Rat calvarial defect model (with CTGF seeded in PLGA microspheres)	↑*Col1*, *Tn-C*; promote fibrosis tissue formation *in vivo*	2
CTGF [77]	*In vitro* & *in vivo*	Rat	Tendon cells	CTGF seeded in fibrin glue and implanted in patellar tendon defect	↑*Col1a1*, *Col3a1*, *Tnc*, *Vim*, *Tnmd*, *Scx*; ↑dense alignment of collagen fibres and maximal loading	3
Mechanical stimulation						
Mechanical stretching [81]	*In vitro* & *in vivo*	Mice	Adult tenocyte cell line	*ScxGFP* transgenic mice	Gradual and temporary loss of tensile loading causes reversible loss of Scx expression	3

(*continued on next page*)

Table 1 (*continued*)

Factor	Study types	Species[a]	Cell model	Injury model	Effects	Study level[b]
Mechanical stretching [82]	*In vitro* & *in vivo*	Mice	TDSC with low/high mechanical stretching	Mice underwent MTR (4%)/ITR (8%) treadmill running	↑*Col1*, *Tnmd*; with no effects on nontendon marker gene expression in LTR treatment	2
Uniaxial stretching [86]	*In vitro*	Rat	TDSCs	/	↑*Runx2*, *Dlx5*, *Alp*, and *Col1A1* at 1 Hz, 8%	2
Mechanical stretching [39]	*In vitro*	Mice	TPCs	/	↑Tenogenic differentiation in late-stage cells	2
Mechanical stretching [83]	*In vitro*	Rat	TDSCs in a P(LLA-CL)/Col scaffold	/	↑*Col1*, *Tn-C*, *Tnmd*, *Scx* at 0.5 Hz, 4%	2
Mechanical stretching [29]	*In vitro*	Human	BMSC-seeded collagen gel	/	Help maintain the Scx and matrix expression	2
Mechanical stretching [88]	*In vitro*	Human	BMSC encapsulated in poly(ethylene glycol)-based hydrogel material	/	↑*Col1I*, *Col3*, and Tn-C at 10%, 1 Hz	1
Mechanical stretching [89]	*In vitro*	Mice	Tenocytes or MDC seeded on PGA	/	↑Mature collagen structure, thicker collagen fibrils, mechanical properties, ↑Tnmd in MDC-seeded group	3
Mechanical stretching [85]	*In vitro*	Human	TPCs	/	↑ECM markers and *Mmp13* and *Mmp14* at 8%	2
Mechanical stretching [84]	*In vitro*	Mice	C3H10T1/2 pretreated with BMP12	/	↑*Scx* expression at 5%, 0.5 Hz	2
Biomaterial						
Scaffold						
Poly (l-lactic acid) nanofibres [91]	*In vitro* & *in vivo*	Human	TPCs seeded in aligned scaffold	Implanted subcutaneously into the dorsal surface of nude mice	↑*Scx*, ↓*Runx2*, and *ALP* expression in aligned group; more collagen production *in vivo* in aligned group	3
Ultrafine PLGA fibres [93]	*In vitro*	Rabbit	BMSC seeded in bioactive bFGF-releasing ultrafine PLGA fibres	/	↑*Col1*, *Fbn*, and *Bgn*; mechanical properties	2
Electrochemically aligned collagen [94]	*In vitro*	Human	BMSCs seeded in ELAC		↑*SCX*, *TNMD*; ↓*OCN* expression in ELAC threads	2
Fibrin or collagen hydrogels [95]	*In vitro*	Mice	TPCs seeded in collagen or fibrin hydrogels		↑*Scx*, *Mkx*, *Col1*, *Tnmd*, *Tn-C*, and *Fmod*, and collagen alignment in fibrin group	2
Collagen-GAG	*In vitro*	Equine	Tenocytes seeded in scaffolds		↑*Tn-C* and *Scx* and ↓*MMP1* and	2

scaffolds [96]			with different densities of aligned tracks of ellipsoidal pores		*MMP13* in scaffold with highest density of ellipsoidal pores	
Collagen-polydioxanone sheath [97]	*In vivo*	Rabbit	/	Achilles tendon defect	↓Peritendinous adhesion and ↑diameter, density, and alignment of the collagen fibrils	2
RGD-coupled alginate microspheres [98]	*In vitro* & *in vivo*	Human	TGF-β3-loaded RGD-coupled alginate microspheres encapsulating PDLSCs		↑*Scx*, *Dcn*, *Tnmd*, and *Bgn in vitro*; ↑ectopic neotendon regeneration *in vivo* in PDLSC-seeded group	3
Magnetic nanoparticles [99]	*In vitro* & *in vivo*	Human	ADSC culture on sophisticated magnetic polymer scaffolds	Subcutaneous implantation	↑*Tn-C*, *Col1*; good biocompatibility and integration within the surrounding tissues *in vivo*	1
Decellularized matrix						
Patellar tendons [102]	*In vitro* & *in vivo*	Rabbit	Human TDSC seeded	Implantation in nude rat	Preserve the stemness of TDSCs; ↑*Tnmd*, *Col1*, *Col3*; promote neotendon formation	2
Tendons [101]	*In vitro* & *in vivo*	Porcine	Human TPCs seeded	Achilles tendon reconstruction	↑*Scx*;↓ *Runx2*; mature structure with larger collagen fibrils and stronger mechanical properties	3
Achilles tendon [103]	*In vitro*	Bovine	Human ADSC culture in collagen scaffold under mechanical stimulation		↑*Scx*, *Tnmd*; ↓*Runx2*, *ALP*, *OCN*; ↑mechanical properties	3
Superficial digital flexor tendon [104]	*In vitro*	Equine	Equine BMSC seeded in and under moderate mechanical stimulation	/	↑*Scx* and *Tn-C* after 24 h mechanical stimulation	2
Superficial digital flexor tendon [105]	*In vitro*	Calves	Human ADSCs with TGF-β3 treatment seeded	/	↑*Scx*, *Tn-C*	2
Coculture						
BMSC & TDSC [106]	*In vitro* & *in vivo*	Rat	Ratio at 1:1	Patellar tendon defect	↑*Scx*, *Tnmd*, *Tn-C*, *Dcn*, *Col1*; ↑mature collagen formation and ↑mechanical properties in repaired tendon	3
BMSC & TDSC [107]	*In vitro*	Canine	Raito at 1:1	/	↑*Dcn*, *Tnmd*, *Scx*, and *Col1*/*Col3*	2
BMSC % autologous ACL cells [108]	*In vitro*	Pigs	Ratio at 1:1	/	↑*Tn-C*, *Col1*, *Col1*/*Col3*	1
ADSC & TDSC [109]	*In vitro*	Human	Ratio at 3:1	/	↑*Scx*, *Tn-C*	2
Induced pluripotent stem cell						
iPSC-NCSCs [110]	*In vivo*	Human	iPSC-NCSCs suspended in fibrin gel	Scaffold transplanted into patellar tendon defect	↑COL1 production	2

(*continued on next page*)

Table 1 (*continued*)

Factor	Study types	Species[a]	Cell model	Injury model	Effects	Study level[b]
iPSC-derived MSCs [92]	*In vitro* & *in vivo*	Human	Seeded onto aligned ultrafine fibres	*In situ* rat Achilles tendon repair	Cells shown elongated; ↑*Scx* and *COL1A*; promote the maturation of repaired tendon	3

1. Papers studying the mRNA or protein expression of ECM proteins such as Col1, Col3, Tn-c; fibronectin that are related but not very specific for tendons (e.g., Scleraxis, Mkx, Egr1, Tenomodulin).
2. Papers studying the expression of more specific molecular markers of tendon such as Scleraxis, Mkx, Egr1, Tenomodulin, or histological formation of tendon-like tissue, or microstructure of tendon fibrils.
3. Papers reporting the expression of more specific molecular markers and histological formation of tendon-like tissue or microstructure of tendon fibrils.

ACL = anterior cruciate ligament; ADSC = adipose-derived stem cells; ASC = adipose-derived stem cells; Bgn = biglycan; BMP = bone morphogenetic protein; BMSC = bone marrow stem cell; Col1 = type I collagen; CTGF = connective tissue growth factor; ECM = extracellular matrix; ELAC = electrochemically aligned collagen; ERK = Extracellular signal−regulated kinases; FDP = flexor digitorum profundus; FGF = fibroblast growth factor; GAG = glycosaminoglycans; GDF = growth differentiation factor; GSK-3 = glycogen synthase kinase-3; iPSC-NCSC = induced pluripotent stem cell-derived neural crest stem cell; ITR = intensive treadmill running; LTR = long terminal repeat; MAPK = Mitogen-activated protein kinase; MDC = muscle-derived cells; MSC = mensenchymal stem cells; MTR = moderate treadmill running; PDLSC = periodontal ligament stem cell; PGA = polyglycolic acid; PLGA = poly-d-l-lactic-co-glycolic acid; P(LLA-CL)/Col = poly(L-lactide-co-ε-caprolactone)/collagen; RGD = arginine−glycine−aspartic acid tripeptide; rhBMP = recombinant human bone morphogenic protein; Scx = Scleraxis; TDSC = tendon-derived stem cell; TGF-β = transforming growth factor β; Tn-C = Tenascin-C; Tnmd = Tenomodulin; TPC = tendon progenitor cells.

[a] Indicating the species of cell model or origins for decellularized matrix.

[b] Modified according to Lui et al [34].

suggested that CTGF could act as one essential assisted factor during tenogenic differentiation, especially in ECM production.

Dynamic mechanical stimulation

Apart from the biological factors, differentiation of embryonic tendon progenitor cell is also regulated by mechanical stimulation *in vivo* [33]. Formation of immature tendon tissue is possible in the absence of biomechanical stimulation, but advanced differentiation of tendinous tissue requires dynamic stimulation [33,39]. Mechanical stimulation could regulate the release of TGF-β from ECM, which maintains the *Scx* expression through the TGF-β/Smad2/3 signalling pathway [81]. Gradual or temporary loss of mechanical stimulation would cause a loss of *Scx* expression, indicating that it can help maintain the *Scx* expression level [29,81]. The moderate intensity (such as 4% elongation, 0.5−1 Hz) was mostly shown to promote tenogenic differentiation by promoting matrix production [82], and *Scx* expression when seeded in collagen scaffold [83] or pretreated with BMP12 [84]. Intensive mechanical loading (8% elongation) would help promote further ECM formation [85], and may also promote osteogenic differentiation [82,86,87]. This may also have a relationship with the production of calcified tissues in patients with tendinopathy. Considering the tendon tissue engineering, the mechanical stimulation applied on stem cell-seeded collagen scaffold [29] or hydrogel [88] was also reported to promote tenogenic differentiation by producing mature collagen with thicker fibrils and strong mechanical properties [89].

Collectively, mechanical stimulation plays an essential role during tenogenic differentiation, especially in the late stage, to induce collagen maturation. The intensity of loading is important for the maintenance of tendon property; moderate intensity could help promote ECM and *Scx* expression, while intensive intensity would lead to osteogenic differentiation, as reported in some studies. For clinical applications, the mechanical loading applied on the stem cell-seeded biomaterials may have the potential to produce tendon-like tissue in the future.

Biomaterials of scaffold and decellularized matrix

Scaffold

The application of novel biomimetic scaffold materials (biological or synthetic origin) is a good strategy for tendon tissue engineering, especially for patients with large tendon defects [90]. The printed modified polymicrofibres or nanofibres or collagen-based hydrogels are the common scaffolds for tendon tissue engineering. It indicated that aligned nanofibres could provide a better microenvironment for stem cells to attach compared with random nanofibres, as evidenced by the cells showing a more elongated shape with increased *Scx* and ECM marker expression [91,92]. As per *in vivo* studies, implantation of the scaffold could help promote the maturation of repaired

tendon with more collagen formation and increased mechanical properties, compared with cells only. Particularly the nanofibre scaffold that can slow down the release of the growth factor, such as FGF2, would have advanced effects on tenogenic differentiation [93]. By contrast, the collagen-based hydrogel is also a good scaffold for tissue engineering, by increasing the *Scx* and ECM expression when seeded with BMSCs or tendon stem cells [94—97]. Oryan et al [97] reported that collagen-polydioxanone sheath implants could increase new tendon formation with increased diameter, density, and alignment of the collagen fibrils in a rabbit tendon defect model. A codelivery system, TGF-β3-loaded arginine—glycine—aspartic acid tripeptide-coupled alginate microsphere encapsulating the tendon stem cells, could also promote neotendon formation [98]. Novel material, such as magnetic nanoparticles, is also reported to promote *Tn-C* and *Col1* expression in ASCs (Adipose-Derived Stem Cells) [99].

Decellularized matrix

In recent years, the decellularized matrix has become a popular alternative for tendon tissue engineering. With the advantage of its biological origin, it can provide the microenvironment with rich ECM proteins for stem cell niches [100].

Currently, the most popular use of the decellularized matrix is to seed stem cells (such as tendon stem cells, adipose stem cells, Etc.) onto it, to provide the microenvironment for stem cell attachment. Comparing with the decellularized matrix from other origins (e.g., bone and cartilage), the tendon-derived decellularized matrix showed better effects on tenogenic differentiation and also inhibited osteogenetic differentiation [101]. According to *in vivo* studies, the stem cell-seeded decellularized matrix implants could form tendon-like tissue, with mature collagen fibrils and increased mechanical properties [101,102]. Particularly combining the stem cell-seeded decellularized matrix with mechanical stimulation [103,104] or TGF-β treatment [105] was also reported to promote *Scx* and tendon ECM marker expression, compared with the stem cell-seeded decellularized matrix alone.

In summary, scaffolds such as nanofibres or collagen-based hydrogel and decellularized matrix are potential alternatives for tendon tissue engineering, as they can support the microenvironment for stem cell niches. Nanofibres with alignment or the decellularized matrix derived from tendon showed better effects compared with others. Furthermore, growth factors or mechanical stimulation can also be applied when using the scaffold for tendon regeneration.

Coculture

Considering the limited cell source of TDSCs, some studies also indicated the positive effects of coculturing TDSCs with BMSCs, adipose-derived stem cells, or muscle-derived cells on tenogenic differentiation. Wu et al [106] demonstrated the advantage of coculture of TDSCs with BMSCs at 1:1 ratio with significantly upregulated tenogenic gene marker expression (*Tnmd*, *Scx*, *Tn-C*, and *Dcn*) and collagen matrix production, and also enhanced tendon injury healing. Schneider et al [107] and Canseco et al [108] also showed similar results. Moreover, the coculture of TDSCs with ASCs at 1:3 ratio was also reported to promote expression of tenogenic genes, such as *Tn-C* and *Scx* [109]. Current studies suggested that BMSCs and TDSCs at 1:1 ratio may be a better cell source for tendon tissue engineering.

Induced pluripotent stem cells

As iPSCs have the unique properties of self-renewal and differentiation to many types of cell lineage, these also are an ideal cell source for tissue engineering. Studies found that human iPSC-derived neural crest stem cells could help in tendon repair by transplanting the fibrin gel with cells seeded in a rat patellar tendon defect model [110]. The application of iPSC-induced MSC on aligned ultrafine fibres also showed increased *Scx* and *Col1* expression, and produced mature collagen during tendon repair [92]. For clinical translation, iPSCs are suggested to be a promising cell source, as these are relatively easy to isolate from patients. However, there are still some limitations associated with iPSC application. It is of concern that the basal reprogramming factors may cause other diseases as well, for example, c-Myc plays an important role in the formation of most human cancers [111]. Currently, we are still at the beginning to prove the concept of application of iPSCs in tendon tissue engineering; there is still a long way to go to translate iPSCs into clinical applications.

Clinical applications of stem cells for tendon injuries

Effects of clinical treatments on tendon injuries, such as tendinopathy or tendon rupture, are limited. Normal healing of tendon injuries is prolonged, due to the limited self-regenerative ability and poor vascularity of tendon tissues [30]. The application of cell therapies (e.g., autologous tenocytes, fibroblast, or autologous BMSCs) for the treatment of tendon injuries has been conducted in clinical trials in recent years and summarized by Ho et al [112]. Although the authors indicated that tendon healing was promoted using cell therapy, the studies are limited by nonrandomized and short-term clinical follow-up and thus unable to indicate its long-term effects for injury healing.

Recently, a 4.5-year clinical follow-up study with application of autologous tenocyte injection was reported to promote healing in patients with chronic resistant lateral epicondylitis [113,114].Briefly, the authors isolated autologous tenocytes from patellar tendon needle biopsy and expanded cells *in vitro*. Then the autologous tenocytes were injected into the site with tendinopathy under ultrasound guidance. During the 4.5-year clinical follow-up, the results showed increased function recovery in lateral epicondylitis patients, with decreased pain and an increased range of motion at arm, shoulder, and hand [114]. No complications were observed in any patient, indicating that autologous tenocyte injection is relatively safe. Meanwhile, a case report using the autologous tenocyte injection for the

treatment of a 20-year-old elite gymnast with a rotator cuff tendon injury was also reported by the same group [115]. The partial-thickness tear was healed by magnetic resonance imaging observation in 1-year follow-up. The patient reported substantial improvement of clinical symptoms and was able to return to national-level competition. Moreover, Lee et al [116] reported that injection of allogeneic adipose-derived stem cells mixed with fibrin glue can also improve the performance of patients with lateral epicondylosis through 52 weeks of follow-up. The application of bone marrow containing both plasma with rich growth factors and BMSCs was also reported to improve function recovery in patients with tennis elbow in a 12-week clinical follow-up [117]. However, the limitations of the study are a small number of participants and absence of a control group.

The overall results indicated the therapeutic value of stem cell (TDSCs or BMSCs) injection for tendinopathy, which is relatively safe (Table 2). There are no reported safety issues with using the autologous cell therapy currently. However, some problems still need to be considered for its future clinical applications. Most current clinical trials are limited by a small number of participants, lack of control group, or short-term follow-up. Well-designed, nonrandomized, long-term studies with enough

Table 2 Clinical studies of stem cell application.

Cell source	Injury type	Patient no.	Type of treatment & study	Outcome measure	Effects	Level of evidence[a]
Autologous tenocyte [113,114]	Chronic lateral epicondylitis	15	Ultrasound-guided injection	VAS score, QuickDASH, UEFS, grip strength, MRI scanning	Improved clinical function and MRI tendinopathy scores in 4.5-y follow-up; no complications observed at the patellar tendon biopsy site for any patient	4
Autologous tenocyte [115]	Rotator cuff	1 (case report)	Ultrasound-guided injection	VAS, QuickDASH, Oxford shoulder score, MRI	Reduced pain; partial-thickness tear healed on MRI; back to national-level competition	4
Allogeneic ADSCs [116]	Chronic lateral epicondylitis	12	Mixed with fibrin glue injection	VAS, modified Mayo clinic performance index for elbow, ultrasound images of tendon defect	Improved elbow performance and pain; defect decrease observed under ultrasound	4
Bone marrow aspirate[b] [117]	Tennis elbow	30	Direct injection	PRTEE	Decreased PRTEE in 1-y follow-up	4

ADSC = adipose-derived stem cells; MRI = magnetic resonance imaging; PRTEE = patient-rated tennis elbow evaluation; QuickDASH = quick disabilities of the arm, shoulder and hand; UEFS = Upper Extremity Functional Scale; VAS = visual analogue scale.
[a] According to the Centre for Evidence Based Medicine (http://www.cebm.net).
[b] Containing plasma rich in growth factors and mesenchymal stem cells.

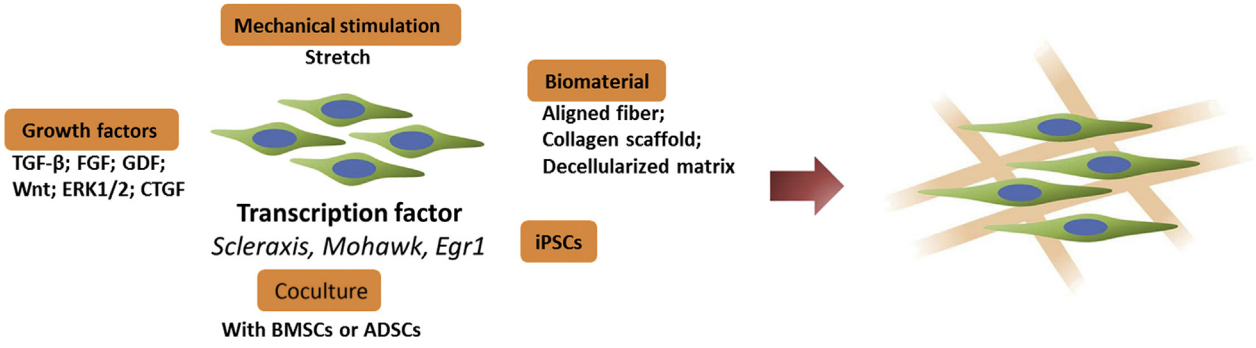

Figure 1 Summary of the current understanding and concepts of tenogenic differentiation. ADSC = adipose-derived stem cell; BMSC = bone marrow stem cell; CTGF = connective tissue growth factor; FGF = fibroblast growth factor; GDF = growth differentiation factor; ERK: Extracellular signal—regulated kinases; iPSC = induced pluripotent stem cell; TGF-β = transforming growth factor β.

participants (both treatment and control) are needed to confirm its healing effects. Moreover, as stem cells such as TDSCs or BMSCs were all reported to show multipotent differentiation abilities [31,32], as well as tumorigenesis [118], more long-term well-designed randomized controlled trials are still needed to confirm its safety with respect to nonectopic bone formation or tumour formation. Apart from that, application of rhBMP12 has been shown to be relatively safe and effective in patients with rotator cuff in clinical trials [66]. Injection of BMP12-pretreated TDSCs or BMSCs may have advanced effects on repaired tendon healing [66], as it may avoid the potential of nontenogenic differentiation of injected stem cells. This is warranted to be considered for future applications.

Conclusion

The understanding of tenogenic differentiation has greatly improved and broadened in recent 10 years. As summarized in this review, current experimental data support the strategies of applying growth factors (especially TGF-β, GDF5/7, and CTGF), moderate mechanical stimulation, biomaterials (fibres with alignment or tendon-derived decellularized matrix), coculture of BMSCs and TDSCs at 1:1 ratio showed promising effects on promoting tenogenic differentiation of stem cells (TDSCs or BMSCs) (Figure 1). However, a lack of consensus on tendon-specific markers led to challenges regarding assessing the efficacy of strategies for tenogenic differentiation; the discovery of specific markers will undoubtedly help identify a novel therapeutic approach. On the contrary, the development of tendon relies on both biological and biomechanical stimulation [6,30]; applications of the growth factor or biomaterials in combination with mechanical stimulation will be the future directions for tendon tissue engineering.

However, there is still a large gap between experimental research and clinical applications. For tendon injury healing, current clinical trials are limited to the usage of stem cells (TDSCs or BMSCs) only for the treatment of tendinopathy, and most of the current strategies for tenogenic differentiation are still in the preclinical stage, because of the high standard of safety requirements for clinical trials in contrast to the experimental research work [112]. To translate the current findings to clinical applications, we need to pay attention to their safety (e.g., stem cell or growth factor treatment for tendon adhesion [41], potential nonectopic bone formation [119], or tumour formation [118]), and long-term follow-up with enough participants (both treatment and control) in nonrandomized studies. Experimental work has found that pretreated BMSCs or TDSCs with growth factors (such as BMP12 and CTGF) promotes tenogenic differentiation and inhibits osteogenic differentiation, whereas application of biomaterials seeded with BMSCs or TDSCs facilitates their adhesion and survial. To translate these findings from bench to clinical settings, using these new strategies in patients with tendon injuries is warranted.

Conflicts of interest

The authors indicate no potential conflicts of interest.

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