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Concise Review: Stem Cell Fate Guided By Bioactive Molecules for Tendon Regeneration

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ABSTRACT

Tendon disorders, which are commonly presented in the clinical setting, disrupt the patients' nor-mal work and life routines, and they damage the careers of athletes. However, there is still no effective treatment for tendon disorders. In the field of tissue engineering, the potential of the therapeutic application of exogenous stem cells to treat tendon pathology has been demonstrated to be promising. With the development of stem cell biology and chemical biology, strategies that use inductive tenogenic factors to program stem cell fate in situ are the most easily and readily translatable to clinical applications. In this review, we focus on bioactive molecules that can poten-tially induce tenogenesis in adult stem cells, and we summarize the various differentiation factors found in comparative studies. Moreover, we discuss the molecular regulatory mechanisms of teno-genesis, and we examine the various challenges in developing standardized protocols for achieving efficient and reproducible tenogenesis. Finally, we discuss and predict future directions for tendon regeneration. STEM CELLS TRANSLATIONAL MEDICINE 2018;7:404–414

SIGNIFICANCE STATEMENT

Tendon disorder represents a major clinical challenge in orthopedic surgery. Currently there is no effective drug system for tendon disease treatments. A better understanding of bioactive molecules for tenogenesis effect on stem cells in vitro and in situ will lead to better treatments for tendon injuries and tendinopathy. Current challenges and future directions are discussed and suggested in order to develop effective chemical cocktails for stepwise inducing tendon regeneration.

INTRODUCTION

Tendon and ligament injuries comprise almost 50% of sports and physical activity-related inju-ries, causing 30–50 million new incidents annually worldwide [1]. Tendon injuries following acute trauma or physical strain constitute a major clini-cal challenge because of the poor intrinsic healing capacity of tendons. Recent advances indicate that the transplantation or grafting of exogenous or endogenous stem cell populations exert regen-erative effects on diseased or injured tendons [2]. Moreover, the precise regulation of the tenogenic differentiation of stem cells is essential for the positive outcome of stem cell-based therapy applied to tendon tissues.

Among the various strategies used for tendon differentiation, mechanical stimulation is consid-ered an important regulator [3, 4]. However, this approach is heavily dependent on equipment, and it is mainly suitable for three-dimensional (3D) constructs [5]. Additionally, because many previ-ous studies utilized custom-made bioreactors, it is difficult to define the inductive conditions [6]. In addition to mechanical stimulation, other physical parameters, such as extracellular matrix (ECM) topography, stiffness, and the influence of decellu-larized matrices on tenogenesis, have shown promise [7–10]. However, the fabrication of nanometer-to-micrometer scale topography usu-ally requires sophisticated instrumentation, and the high inter-batch variability is another concern. Another important approach in treating ligament and skeletal muscle injuries is the use of endoge-nous platelet rich plasma (PRP) [11], which was recently demonstrated to promote the prolifera-tion and myogenic differentiation of myoblasts in vitro when combined with a TGF-β inhibitor [12]. However, controversial conclusions and variable clinical outcomes following PRP treatment for ten-don repair have been reported [13–15]. Addition-ally, variability in the preparation and composition of PRP makes it difficult to compare results across studies [16]. In contrast, bioactive molecules, par-ticularly recombinant growth factors, are readily

available, stable, and dose-controllable. Moreover, they are widely utilized in tissue engineering, and they have demonstrated much potential for translational applications.

With respect to osteogenesis and chondrogenesis, we reached the consensus that specific growth factor formulations exert potent therapeutic effects. However, formulations that are reproducible and can induce tenogenesis consistently remain elusive. Because stem cells can differentiate into specific lineages in vitro, they are potential stable source for regenerative medicine. Therefore, to address the requirements of tenogenic induction protocols, this review will focus on the current progress in the utilization of bioactive molecules in tenogenesis, which may have critical implications for the fields of tendon tissue engineering and future clinical translation.

BIOACTIVE MOLECULES REGULATING TENOGENESIS OF STEM CELLS

Connective Tissue Growth Factor (CTGF/CCN2)

Connective tissue growth factor (CTGF) has been demonstrated to induce the differentiation of human bone marrow stromal cells (BMSC) into fibroblasts, which is evidenced by the marked increase in type I collagen (COL I) and tenascin-C (TN-C) synthesis as well as the elevated expression of typical fibroblastic markers and the reduced capacity to differentiate into non-fibroblastic lineages, including osteocytes, chondrocytes, and adipocytes [17, 18]. In rats, the in vivo control-released delivery of CTGF enabled postnatal connective tissues to undergo fibrogenesis instead of ectopic mineralization [18]. However, it was also reported that CTGF led to mineralization in the human periodontal ligament stem cell line, but the expression of fibroblast-related genes was enhanced when it was combined with TGF-β1 [19]. These results suggested the biphasic effects of CTGF, which could induce either fibroblastic or osteoblastic differentiation, depending on specific micro-environmental conditions. These findings have implications for engineering fibrous tissue because they provide evidence for a reproducible protocol for the fibroblastic differentiation of mesenchymal stem cells (MSCs). The fibroblastic differentiation of stem cells is highly significant in the engineering of tendons.

Rat tendon stem/progenitor cells (TSPC) treated with ascorbic acid and CTGF for 2 weeks exhibited the increased expression of tendon-specific markers and the significantly decreased expression of chondrogenic and osteogenic markers [20, 21]. Additionally, engineered scaffold-free tendon tissues formed by TSPC sheets after treatment with CTGF and ascorbic acid, distinctly enhanced tendon healing in a rat patellar tendon window injury model [20]. Similarly, TSPC formed cell sheets after treatment with ascorbic acid and CTGF were evaluated in a rat anterior cruciate ligament reconstruction model, and the improved early graft healing was evidenced by better graft osteo-integration and enhanced integrity of the intra-articular graft recovery [22]. CTGF delivery has also been used selectively to enrich tendon-resident CD146$^+$ stem cells during the early phase of tendon healing in rats. Subsequently, CTGF-pretreated CD146$^+$ progenitor cells were transplanted to exert positive effects on tendon regeneration [23].

FAK/ERK1/2 signaling pathway was reported to regulate the CTGF-induced proliferation and differentiation of CD146$^+$ stem/progenitor cells [23]. However, it was unclear that how CTGF-regulated tenogenic differentiation was linked with TGF-β

signaling pathway. Further investigation of CTGF and its potential crosstalk with TGF-β will likely provide an in-depth understanding of its roles in tendon regeneration.

Hence, CTGF may contribute to the preservation of the structural integrity of tendon tissue, which implies its potential use as a therapeutic agent in tendon regeneration. After treatment with CTGF, the TSPC sheets could be used to promote graft healing in reconstruction, and the engineered scaffold-free tendon tissue could be a novel approach to tendon repair and regeneration. Treatment with 100 ng/ml of recombinant human CTGF and 50 μg/ml ascorbic acid for 4 weeks was demonstrated to have the best effect on monolayer-cultured MSCs [17, 18]. When the TSPCs were treated, the treatment could be adjusted to 25 ng/ml for 2 weeks [20, 21].

Transforming Growth Factor Beta (TGF-β)

The transcriptome analysis of developing mouse limb tendon cells showed that TGF-β was the most significant signaling pathway and exhibited the largest upregulation in differentiated tendon cells compared with TSPCs [24]. In addition, Scx-GFP signaling, which gradually disappeared when TSPCs were cultured in vitro, was reported to reappear upon TGF-β treatment [25, 26]. Moreover, it was demonstrated that the tendon-specific transcription factors Mkx and Egr1 acted partially through the TGF-β signaling pathway [25, 27]. Combined, these results demonstrated the pivotal role of TGF-β signaling in tendon induction.

TGF-β1 enabled equine tenocytes to retain a differentiated state [28]. It also highly upregulated neotendon transcription factors Scleraxis (Scx) and Mohawk (Mkx) expression [29], and significantly impeded the decline of mechanical properties [30]. Treatment with TGF-β2 was able to increase the expression of Col1a1 and Scx genes [24, 25, 27], which were significantly downregulated by specific TGF-β inhibitors [24]. It was also demonstrated that TGF-β2-loaded affigel beads that were grafted into limb buds in an organ culture, robustly induced Scx expression during tendon development [31]. TGF-β3 was reported to promote tendon differentiation of equine embryo-derived stem cells [32]. Moreover, in vivo studies showed that human MSC and bone marrow-derived mononuclear cells had the capacity to generate tendon-like tissues when treated with TGF-β3 [33].

The TGF-β signaling pathway is involved in multiple cellular functions, including cell growth, cell differentiation, and cellular homeostasis. TGF-β1 and the insulin-like growth factor 1 (IGF-1) were reported to enhance the mechanical properties of rabbit patellar tendons at 2 weeks post-surgery [34]. TGF-β was also reported to facilitate differentiation of human keratocytes into myofibroblasts, but TGF-β-mediated improper fibrosis and scar formation limited its use in human application. Recently, single-cell analysis reveals the potential of IGF-1 to inhibit the TGF-β/Smad pathway of fibrosis in human keratocytes in vitro [35]. Additionally, TGF-β signaling was also reported to play essential roles in cartilage formation and maintenance [36]. Thus combined administration of growth factors and guided tenogenesis has gained significant interest in recent years. Recently, it was demonstrated that the combination of tendon-derived ECM extract with TGF-β3 enhanced tenogenic differentiation of human adipose-derived mesenchymal stem cells (ADSCs) [37].

The TGF-β/Smad signaling axis is one of the main TGF-β downstream cascades. It was demonstrated that TGF-β signaling was sufficient and required via Smad2/3 to drive mouse mesodermal stem cells toward the tendon lineage [24]. The embedment of

Smad8/BMP2–engineered MSCs was also reported to result in higher effective stiffness than in the control groups in a full-thickness Achilles tendon defect model at 3 weeks post-surgery [38]. Moreover, although TNF-α inhibited the proliferation and tenogenic differentiation of TSPCs, the expression of tenogenic-related marker genes and the proliferation of TSPCs were significantly increased after simultaneous or sequential treatment with TGF-β1 and TNF-α. [39]. During the processes, the TGF-β and BMP signaling pathways were highly activated as evidenced by highly phosphorylated Smad2/3 and Smad1/5/8 [39]. It was also demonstrated that the addition of TGF-β3 to tenocytes minimized extrinsic scarring, decreased tendon adhesion and promoted tendon healing by significantly downregulating the expression of Smad3 and upregulating the expression of Smad7 [40].

These results indicated that the local delivery of TGF-β may accelerate the healing process and play a significant role in tendon-to-bone healing. Treatment with 20 ng/ml of TGF-β for 24 hours was demonstrated to be sufficient to induce the tenogenic differentiation of monolayer-cultured MSCs [24, 27]. We can concluded that adult stem cells are able to differentiate into a therapeutically relevant cell type and that the TGF-β driven differentiation of stem cells may provide a model for studying tendon development and better understanding the transcriptional networks that are involved in tendon cell differentiation in different developmental stages.

The Growth Differentiation Factor (GDF) Family

GDF-5 (BMP-14), GDF-6 (BMP-13), and GDF-7 (BMP-12), which belong to the TGF-β superfamily, are essential in tendon development and differentiation [41]. GDF-5 was shown to induce the tenogenesis of rat ADSCs, resulting in an enhanced ECM and tenogenic markers [42, 43]. Similar effects of GDF-5 were reported on human BMSCs [44, 45] and periodontal ligament-derived cells [46]. Additionally, following GDF-5 induction, the obvious downregulation of the non-tenogenic marker genes (*RUNX2* and *SOX9*) was observed, which excluded human BMSCs differentiation into other lineages [44]. GDF-6 was reported to have a positive effect on the tenogenic differentiation of rat BMSCs with the significant upregulation of specific markers (*Scx* and *Tnmd*) [47]. It was demonstrated that treatment of the C3H10T1/2 mouse mesenchymal progenitor cell line with GDF-6 or GDF-7 led to the dose-dependent induction of *Thbs4* expression [48]. Moreover, different mesenchymal stem cell lineages exhibited different tenogenic differentiation capacities in the presence of GDF-7, in which ADSCs exhibited inferior capacity [49]. However, GDF-7 stimulated the expression of tenocyte lineage markers and was used to promote tenogenic differentiation in rat TSPCs [50] and BMSCs [51, 52], as well as in canine and mouse ADSCs [53]. In horse, BMSCs also differentiated into tenocytes after treatment with GDF-7 [54]. The GDF-7-releasing sutures also enhanced Achilles tendon healing and reduced adhesions and scars [55].

GDF-5 also promoted the osteogenic-lineage differentiation of stem cells in vitro, which was demonstrated by histology and biochemical assays for alkaline phosphatase activity as well as the analysis of gene expression [56]. Although the local delivery of GDF-5 by coating sutures in rats showed a promising beneficial effect on tendon repair, which was evidenced by the improved histological and mechanical properties in an early stage, the subsequent appearance of cartilage-like structures demonstrated the chondroinductive capacity of GDF-5 [57, 58]. The effect of GDF-5 in rat ADSCs is similar to that of GDF-7, but it is marginally less effective (based on the expression of *Scx* and *Tnmd*), and the effects with selective (based on downregulation of *Bglap*) [53]. However, neither GDF-7 nor GDF-5 promoted *Col1a1* and *Col3a1* expression in ADSCs [53].

To date, the molecular signaling mechanisms which regulate GDFs-induced tenogenic differentiation remain largely unknown. BMPs are known to signal through the canonical Smad pathway and the non-canonical mitogen-activated protein kinase (MAPK) pathway. Although some research investigated the downstream signaling pathways of GDF-5, GDF-6, and GDF-7, such as changes in Smad-1/5/8 expression or activation level [48, 53], few studies have focused on the underlying molecular mechanism of tenogenesis. Recent research demonstrated that the reorganization of the cytoskeleton plays an important role in tenogenic differentiation induced by GDF-5. The genes that are mainly related to cytoskeleton reorganization signaling were identified as potentially associated with tenogenic differentiation [59].

Hopefully, the adverse side effect might be overcome by modifying the dose, the suture material, or the micro-environment. It was reported that GDF-5 was capable of inducing tendon-like tissues in a synthetic 3D microenvironment in vivo, based on tendon-specific genes and the histological and morphological characteristics of the tissue [45]. The optimal concentration of GDFs 5, 6, 7 varies from 10 to 100 ng/ml, and the treatment time is difficult to determine because of the different cell types. It was shown that low-dose GDF-7 within an absorbable collagen sponge was feasible and likely safe as an adjuvant therapy for rotator cuff repair [60]. The clinical relevance of these results is that accelerated repair might reduce rehabilitation time, leading to an earlier return to work and recreational activities.

Basic Fibroblast Growth Factor (bFGF/FGF-2)

FGF-2 combined with platelet-derived growth factors (PDGF-BB) has been reported to promote cell proliferation and collagen production significantly in canine flexor tendon fibroblasts [61]. Low dosages of FGF-2 triggered a biphasic human BMSC response. Cell proliferation was significantly stimulated in the early stage, and cell differentiation was stimulated in the late stage with upregulated *COL I*, *COL III*, and *FN* [62]. The delivery of FGF-2 through various scaffolds was also shown to induce the tenogenic differentiation of MSCs [63–65]. In the process of healing rat rotator, the FGF-2-treated group demonstrated a significant enhancement of biomechanical strength and histological appearance [66]. Additionally, FGF-2 stimulated the proliferation of the tenogenic stem cells in tendon-to-bone healing, which led to the better recovery of the repaired rotator cuff [66].

FGF signaling has been reported to induce the formation of a tendon progenitor population that expressed *Scx* during somite development [67]. The application of FGF-2 on flexor tendon healing in a canine model resulted in enhanced neovascularization and reduced inflammation in the earliest stages, but it did not result in any positive effect on the mechanical and functional properties of the repaired tendon [68]. Moreover, it was also reported that FGF-4 did not increase *Scx* expression in mouse limbs in both early and late developmental stages and that it showed negative effects on *Scx* and *Col1a1* gene expression in C3H10T1/2 cells [69, 70].

FGF signaling pathway played distinct roles in limb tendon development between the chick and mouse models. ERK inhibition was reported to prime mouse stem cells for the tendon lineage sufficiently [24]. More researches are needed to understand

the differences of FGF/ERK pathway in tendon development and regeneration.

In summary, FGF-2 plays a crucial role in cell proliferation and collagen production. And it is usually used at a low concentration of less than 10 ng/ml [62]. Low-dose FGF-2 may be useful for tissue engineering of tendons by promoting stem cell proliferation and increasing mRNA expression of specific ECM proteins and cytoskeletal elements. But its effect on the mechanical and functional properties of the repaired tendon remains elusive.

Others

Adiponectin, an adipocyte-secreted hormone that increases cell sensitivity to insulin and protects against type II diabetes, was found to promote progenitor cell differentiation and proliferation in tendons concurrently. After exposure to recombinant adiponectin in vitro, diabetic-ridden human TSPCs exhibited significantly upregulated tenogenic gene expression with unaltered levels of adipogenic, chondrogenic, and osteogenic gene expression [71].

The intra-tendon delivery of PDGF-BB in a rat Achilles tendinopathy model was reported to increase maximum load-to-failure and stiffness with respect to the control and the steroid treatment [72, 73]. However, there were no significant differences in histology, and a long-term evaluation was not conducted. Although PDGF-BB delivery was demonstrated to promote early healing in a rat rotator-cuff repair model, there was an absence of either a better structure or stronger mechanical properties in later stages of healing [74].

Because the expression of MAPK or MAP kinase and MAP kinase phosphatases was downregulated in developing tendon cells, a reduction in MAPK activity during tendon differentiation was implicated. The PD18 inhibitor blocked the ERK/MAPK pathway and induced the activation of *Col1a1*, *Col1a2*, *Scx*, and *Thbs2* expression in mouse E9.5 limb explants and MSCs [24]. However, another MAPK signaling inhibitor U0126 did not show any positive effect on tenomodulin (*Tnmd*) expression of MSCs [75].

The loss of mesenchymal Wls, which is necessary for the secretion of multifarious Wnts, prevents the differentiation of distal mesenchyme. The regulatory role of Wnts in undifferentiated distal limb mesenchyme cells was investigated [76]. Wnts were demonstrated to activate the *Scx* promoter and to be crucial for the induction of distal tendon/ligament [76]. Hence, the Wnt protein is a potent inducer of *Scx* expression and tendon differentiation. Another study of Wnt signaling involved the treatment of BMSCs with the glycogen synthase kinase-3 (GSK-3) inhibitor-BIO. In 3D BIO-containing collagen gel, BIO induced the expression of *Tnmd*, *Col14a1*, Decorin (*Dcn*), and fibromodulin (*Fmod*), but not in monolayer BMSCs [75]. The inhibition of GSK-3 with siRNA corresponding to GSK-3 also resulted in the upregulation of *Tnmd*, which suggested that Wnt/β-catenin signaling mediated the expression of *Tnmd*.

COMPARATIVE STUDIES OF BIOACTIVE MOLECULES

Although many studies have used growth factor supplements to induce tenogenesis with varying degrees of success, their efficacy is difficult to evaluate because of the different cell types, varying durations, and different readout parameters. Hence, it is necessary to compare the bioactive molecules directly in order to evaluate and determine which is the most potent in inducing tenogenic differentiation.

Recently, the tenogenic potential of FGF-4, TGF-β3, TGF-β2, and PD18 was evaluated in MSCs. It indicated that TGF-β signaling was more effective than the other two growth factors in inducing tenogenic differentiation [24]. Additionally, TGF-β2/3 signaling suppressed the cartilage marker *Sox9* significantly. However, neither differences in the effects of TGF-β2 versus TGF-β3 nor synergetic effects were observed [24]. Similarly, another study showed negligible effects of FGF-4 on the differentiation of TSPCs that were isolated from the axial and limb in a series of developmental stages [69]. This study also analyzed the gene response profiles of TSPCs to TGF-β2, FGF-4, mechanical loading, and their combinations. Based on *Scx* expression, TGF-β2 was demonstrated to induce tenogenesis of TSPCs in all stages, whereas mechanical loading affected the late-stage TSPCs. When the growth factors were applied in combination, TGF-β2 continued to be tenogenic, whereas FGF-4 appeared anti-tenogenic [69]. In contrast, it was revealed recently that FGF-4 significantly increased *Scx* expression, whereas PD18 decreased *Scx* expression in undifferentiated cells of chick limb. However, TGF-β2 positively regulated *Scx* expression as previously described. This result demonstrated that the function of TGF-β in tendon development was similar in chick and mouse limbs, whereas FGF-4 and TGF-β2 showed independent effects [77]. Another study on the tenogenic differentiation capacity of human ADSCs and amniotic fluid stem cells (AFSC) evaluated the growth factors EGF, bFGF, PDGF-BB, and TGF-β1 [78]. Based on the results of *Col I*, *Col III*, *Dcn*, *Tn-C*, and *Scx* expression, it was shown that TGF-β1 induced *Scx* expression 10 times more than other growth factors did on day 7; EGF induced the greatest amount of *Tn-C* expression on day 7 and *Col III* expression on day 14; TGF-β1 and PDGF-BB induced *Col I* expression 10 times more than other growth factors did on day 21. The expression of genetic markers showed different patterns in human AFSCs and ADSCs, which could indicate that the tenogenic lineage commitment induced by biochemical signals might be affected by the origin of the stem cells [78].

Our recent study investigated the treatment of rat BMSCs with TGF-β1, BMP-12, CTGF, and their combinations, which showed that TGF-β1 treatment led to the greatest upregulation of tenogenic genes. It was observed that TGF-β1 combined with CTGF elevated Tnmd mRNA and protein expression in the late stages, so a stepwise differentiation strategy was developed [79]. It was also reported that *Scx*-GFP expression levels decreased over time in primary tenocytes after culture in vitro [26]. In addition to the induction of lineage-specific differentiation, the maintenance of a tenocyte lineage phenotype should receive increased attention and consideration in the research. It was demonstrated that treatment with TGF-β and GDF-8 resulted in the maintenance of *Scx*-GFP expression in adult tenocytes. [26].

However, the varying patterns of gene expression induced by growth factors may be too complicated to provide any valuable use in future applications. Because the immunofluorescence results for the detection of tendon ECM proteins exhibited no significant difference between each culture condition, it was difficult to reach a conclusion without quantitative data. Although the general conclusion was that both stem cell types could be biochemically induced to the tenogenic lineage, the most suitable growth factors and the proper duration for stimulation are still unclear. Hence, further research is needed to provide comprehensive evidence for the tenogenic differentiation efficacy of a range of bioactive molecules in order to reach a consensus on future therapeutic applications.

Elucidation of Molecular Regulatory Mechanisms of Tenogenesis

The formation of highly organized tendon tissue structures relies on the precise temporal-spatial regulation of signaling pathways. The knowledge about the mechanisms of the signaling pathways that regulate the tenogenic master gene *Scx* is limited. It has been demonstrated that TGF-β/Smad2/3 signaling pathways play an important part in this activity. Upregulated *Scx* by canonical TGF-β2 signaling was attenuated by MAPK activity, which indicated that the convergence of MAPK signaling to this pathway had a negative effect [80]. Moreover, the bioinformatics analysis of tendon transcriptome in diverse developmental stages showed that the two most crucial signaling pathways for tenogenesis were TGF-β and MAPK [24]. TGF-β signaling was demonstrated to be either pro-tenogenic or pro-chondrogenic depending on the cell type and culture conditions [31, 81–83].

The roles of TGF-β signaling in tendon homeostasis and tendon development have been elucidated, while the roles of Wnt/β-catenin signaling in tendon remain mostly elusive. Recently, a novel regulatory mechanism was found in equine BMSCs, suggesting that Wnt/β-catenin signaling mediated the expression of Tnmd, whereas Scx and Mkx were not affected [75]. During this process, tendon-related ECM components were also upregulated, which showed that the activation of Wnt/β-catenin signaling induced the tenogenic differentiation of BMSCs. However, it was also demonstrated that the activation of Wnt/β-catenin signaling in TSPCs suppressed the expression of Scx, Mkx, and Tnmd and reduced the amounts of Smad2 and Smad3, which are intracellular mediators in TGF-β signaling [84]. Combined, these results indicated that Wnt/β-catenin signaling might have completely different effects on different type of cells, which warrants further elucidation.

Identification of Developmental Stage-Specific Tendon Markers

The deeper understanding of tendon biology in terms of development and morphogenesis would advance the establishment of an optimal inductive condition for tenogenesis in vitro [85]. The identification and characterization of TSPCs in tendon tissues would constitute a milestone step in understanding tendon physiology, thus facilitating progress in this field [86]. Currently, there is a dire need for specific and definitive markers to identify TSPCs. At present, TSPCs have almost all the same markers that are present in MSCs, such as the positive expression of CD73, CD90, and CD105, as well as the negative expression of CD31, CD34, and CD45. Although TSPCs express higher levels of the commonly utilized tenogenic-related markers, such as *Scx*, *Tnmd*, and *Col I*, these genes could not distinguish TSPCs from tenocytes. Because of the lack of definitive developmental stage-specific markers, it is difficult to evaluate the effects of tenogenic inducers.

Advances in high-throughput screening technologies have brought about the revolution of big data. Combined with bioinformatics analysis, these technologies provide powerful tools for identifying developmental mechanisms and potential markers for each developmental stage. Several public released datasets are available on the Gene Expression Omnibus (GEO) database. These microarrays and RNA sequencing (RNA-seq) datasets thus provide a valuable resource for tendon research. The extensive analysis of regulated genes during limb development will provide scientists with the in-depth understanding of the expression dynamics that govern limb morphogenesis. However, the currently available whole-genome microarray and RNA-seq datasets mainly comprise the initial stages of limb development. However, postnatal development stages, including the critical maturation transition from fetal tendon to adult tendon tissues, should be taken into consideration. Because adult tissue regeneration is generally considered to recapitulate developmental processes, insights gained from research in developmental biology have been considered to be important for teno-lineage differentiation in vitro [87]. A recent study successfully developed a protocol that recapitulated the various differentiation stages of developmental skeletal myogenesis, which represents an advance in the field by improving the efficiency and timing of the generation of striated, contractile fibers from mouse and human pluripotent stem cells [85]. Additionally, the stage-specific markers established in developmental biology will provide useful information for evaluating the various tenogenesis stages in vitro. The comparison of RNA-seq in the gene expression profiles of *Scx*-GFP positive cells and *Scx*-GFP negative cells from E13.5 forelimb samples identified the novel transcription factor *Foxf2* as a specific marker of differentiated limb tendon [88]. The comparison of genes that were expressed in adult rat and human tendon tissue with those in other musculoskeletal tissues showed that *Thbs4* and *Tnmd* exhibited the most tendon-specific expression [89]. Based on these findings, definitive markers could serve as useful reporters of tenogenesis evaluation in vitro and in vivo.

Moreover, recent molecular studies found that individual cells exhibited substantial differences in gene expression, even in a seemingly homogenous population. The use of imaging multi-color flow cytometry has been demonstrated to help researchers to visualize and quantify the reactions of individual cultured cells to bioactives or other physiological impulses and to evaluate the potency of these bioactives in stimulating muscle commitment [90]. The cutting-edge technology used in single-cell quantitative gene analysis combined with high-throughput arrays has allowed the analysis of gene expression profiles to investigate the heterogeneity of cell populations [91–93]. This high-content microfluidic real-time platform is useful in comparative studies on the regulation of developmental processes in single cells [94, 95]. This method overcomes the limitations of heterogeneous cell populations and small sample quantities, and it is potentially useful for analyzing the differential regulation of gene expression in normal versus diseased tissues and organs. The nestin+ tendon stem cells subpopulation was identified by single-cell gene analysis [96]. Technical improvements have enabled the RNA sequencing of single cells, and an increasing number of studies have utilized the power of single-cell analysis in reconstructing cellular lineages in several tissues and revealing sequential stem cell states [97–99]. Hence, high-throughput single-cell qRT-PCR and single-cell RNA sequencing have important implications for the characterization of previously unidentified subpopulations and for the further analysis of the mechanisms of stem cell self-renewal and differentiation in tendon biology [100].

Development of Small Molecule Cocktails for Stepwise Tenogenic Induction

Induced pluripotent stem cells (iPSC) is currently a "hot" topic in stem cell research. These stem cells are obtained by reprogramming differentiated somatic cells through the recombinant overexpression of specific transcription factors. Such advances and breakthroughs in cellular reprogramming indicate the reversibility of cell lineage fate [101, 102]. However, genetic manipulation and the use of viral vectors in reprogramming have evoked concern

Table 1. Biochemical strategies of tenogenic differentiation

Supplementation	Target cell	Optimal concentration	Treatment time	Outcome	References
CTGF with ascorbic acid	Human BMSC	100 ng/ml	4 weeks	COL I and TN-C contents↑	Lee CH, et al. Conf Proc IEEE Eng Med Biol Soc 2006
	Human BMSC	100 ng/ml	4 weeks	*COL I, COL III, TN-C, FN, VIM, MMP-1* (4–10 folds↑); COL I and TN-C contents↑	Lee CH, et al. J Clin Invest 2010
	Rat TSPC	25 ng/ml	2 weeks	*Tnmd, Scx, Thbs4* (1–2 folds↑); *Col1a1, Eln, Dcn, Bgn* (1–3 folds↑)	Ni M, et al. Bio-materials 2013
	Rat TSPC	25 ng/ml	2 weeks	*Col1a1, Tnmd* ↑; chondrogenic (*Acan, Col2a1*) and osteogenic (*Alpl*) markers↓	Lui PP, et al. Cytotherpy 2016
	Rat CD146⁺ tendon cell	100 ng/ml	1 week	*Col1a1, Col3a1, Tn-C, Vim, Tnmd* and *Scx* (3–25 folds↑)	Lee CH, et al. J Clin Invest 2015
TGF-β1	Mouse flexor tendon tenocyte	10 or 100 ng/ml	24, 48 hours	*Mmp-16, Dcn*↓; *Bgn, Col V, Col XII, Serpine1, Scx*, and *Mkx* (5–10 folds↑)	Farhat YM, et al. PLoS One 2012
TGF-β2	Mouse MSC	20 ng/ml	1, 24 hours	*Scx* (6–8 folds↑) and *Col1a1* (3–4 folds↑)	Guerquin MJ, et al. J Clin Invest 2013
	Mouse TSPC and MSC	/	12, 24, 48 hours	*Scx* (6–8 folds↑), *Col1a1* (3–4 folds↑), *Col5a1* (2–3 folds↑) at different time points	Liu H, et al. Stem Cells 2015
TGF-β2/3	Mouse MSC (C3H10T1/2)	20 ng/ml	24 hours	*Scx* (6–8 folds↑), *Col1a1*(3–4 folds↑), *Sox9* (4–6 folds↓)	Havis E, et al. Development 2014
TGF-β3	Human bone marrow-derived mononuclear cells	20 ng/ml	7 days	Synthesis of collagen fibrils and appearance of fibripositors	Kapacee Z, et al. Matrix Biol 2010
	Equine embryo-derived stem cells	20ng/ml	72 hours	Scx, Tn-C, Col1a1, and cartilage oligomeric matrix protein (Comp) ↑	Barsby T, et al. Tissue Eng Part A 2013
GDF-5 (BMP-14)	Rat ADSC	100 ng/ml	3, 6, 9, and 12 days	ECM (*Col I, Dcn, Acan*) and tenogenic marker gene (*Scx, Tnmd,* and *Tn-C*) expression↑ Tnmd, Tn-C, Smad-8, and Mmp-13 protein expression↑	Park A, et al. Tissue Eng Part A 2010
	Human BMSC	100 ng/ml	4 days	*COL I, SCX, and TN-C* expression (2.31 ± 0.27, 2.30 ± 1.81 and 3.55 ± 0.27 folds↑)	Tan SL, et al. Cells Tissues Organs 2012
	P4–6 periodontal ligament-derived cells	10 ng/ml	3 weeks	SCX (2 folds↑), TNMD markedly, ACAN and COL *III* (4–8 folds↑)	Xia D, et al. Growth Factors 2013
	Human BMSC	/	3 days	*COL1A1, COL3A1, DCN, SCX,* and *TN-C*↑	Govoni M, et al. Tissue Eng Part A 2017
GDF-6 (BMP-13)	Mouse BMSC	100 nM	3 days	*Col I* (3 folds↑), *Thbs4* (10 folds↑)	Berasi SP, et al. Growth Factors 2011
	Rat BMSC	20 ng/ml	2 weeks	*Tnmd* (5.61 folds↑) and *Scx* (4.18 folds↑), Tnmd protein expression↑	Chai W, et al. Chin Med J (Engl) 2013
GDF-7 (BMP-12)	Horse BMSC	50 ng/ml	14–21 days	*Tnmd* and *Dcn* expression	Violini S, et al. BMC Cell Biol 2009
	Mouse BMSC	100 nM	3 days	*Col I* (3 folds↑), *Thbs4* (12 folds↑)	Berasi SP, et al. Growth Factors 2011
	Rat BMSC	10 ng/ml	12 hours	*Scx, Tnmd, Tn-C, Col I* (2–4 folds↑)	Lee JY, et al. PLoS One 2011

Table 1. *Continued*

Supplementation	Target cell	Optimal concentration	Treatment time	Outcome	References
	Rat BMSC	50 ng/ml	2 weeks	*Scx* (2.56 folds↑), *Tnmd* (3.07 folds↑), *Tn-C* (1.88 folds↑), Tnmd protein expression↑	Ni M, et al. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2011
	Canine/Mouse ADSC	100 or 1,000 ng/ml	3, 7, 14 days	Scx, Tnmd gene, and protein expression↑	Shen H, et al. PLoS One 2013
	Rat TSPC	50 ng/ml	14 days	Scx, Tnmd, Col I, and Tn-C gene, and protein expression↑	Liu J, et al. Cell Physiol Biochem 2015
FGF-2	Human BMSC	3 ng/ml	14 days	*COL I, COL III, FN* expression (1–2 folds↑)	Hankemeier S, et al. Tissue Eng 2005
FGF-2 or PDGF-BB	Canine flexor tendon fibroblast	10 ng/ml	24 hours	Cell proliferation and collagen production↑	Thomopoulos S, et al. J Hand Surg Am 2005
PD18 (a mitogen-activated protein kinase inhibitor)	Mouse MSC	3.3 μM	24 hours	*Scx* and *Col1a1* (∼2 folds↑)	Havis E, et al. Development 2014
Wnt 3a	Mouse MSC	/	48 hours	*Scx* promoter, luciferase assay	Zhu X, et al. Development Biology 2012
BIO (a glycogen synthase kinase-3 inhibitor)	Equine BMSC	10 μM	7 days	*Tnmd* (∼3 folds↑), *Col14a1, Dcn,* and *Fmod* gene expression↑	Miyabara S, et al. J Equine Sci 2014
BMP2 + Smad8	Mouse MSC	/	4, 7, 10, 14 days	Morphological characteristics and gene expression profile of tendon cells both in vitro and in vivo	Hoffmann A, et al. J Clin Invest 2006
Recombinant human adiponectin	Diabetic ridden human TSPC	10 μg/ml	48 hours	*COL I, III, TNMD,* and *SCX* expression (2.0 ± 0.3, 1.73 ± 0.25, 1.63 ± 0.21 and 1.74 ± 0.24 folds↑)	Rothan HA, et al. Int J Med Sci 2013

Abbreviations: /, not determined; ADSC, adipose-derived mesenchymal stem cells; BMSC, bone marrow stromal cells; COL I, type I collagen; CTGF, connective tissue growth factor; FGF, fibroblast growth factor; GDF, growth differentiation factor; MSC, mesenchymal stem cells; PDGF-BB, platelet-derived growth factors; TGF-β, transforming growth factor beta; TN-C, tenascin-C; TSPC, tendon stem/progenitor cells.

about the safety of iPSCs in therapeutic applications. Recently, an increasing number of studies have identified small molecules that can substitute reprogramming factors as well as enhance reprogramming efficiency, which could overcome the limitations of traditional reprogramming protocols and achieve cellular reprogramming efficiently and safely [103, 104]. Recent reports showed that neural progenitor cells and neurons were induced from fibroblasts by using the appropriate chemical cocktails [105]. Moreover, small molecule-based direct lineage-specific conversion from mouse or human fibroblasts to various cell lineages has been achieved [106, 107], thus demonstrating the feasibility of a pure small molecule-based approach to reprogramming. These studies have led to new possibilities for reprogramming cell lineage fate by using chemical agents. To date, only a few of these small molecules have demonstrated tenogenic potential. Recently, the retinoic acid receptor agonist was found to maintain tendon stem cell characteristics [108]. The strategy for inducing tendon differentiation from pluripotent or multipotent stem cells could be designed based on developmental processes in tendons. In order to recapitulate the stage-specific regulatory signaling during tendon morphogenesis, stepwise approaches should be taken into consideration. In the future, we anticipate that chemical screening will be employed to identify small molecule cocktails for teno-lineage-specific differentiation, which will provide a step toward therapeutic applications.

CONCLUSION

In this review, we described the biochemical strategies of tenogenic differentiation (Table 1) and the direct comparative studies on them (Table 2). The delivery method, timing, and duration of the administration of the bioactive factors should be considered in order to achieve the best healing results and eliminate adverse side effects. Furthermore, the efficiency and efficacy of soluble factor treatment was shown by the confirmatory results of pairwise studies. We also discussed potential molecular regulatory mechanisms. The data obtained in these studies demonstrated that TGF-β/Smad, MAPK/ERK, Wnt/β-catenin, and so on establish a complex signaling network that directs tenogenic differentiation.

Table 2. Direct comparative studies

Comparison	Target cell	Outcome	References
FGF-4, PD18 versus TGF-β2/3	Mouse MSC	TGF-β signaling was sufficient to drive mouse mesodermal stem cells toward the tendon lineage ex vivo and in vitro Examination of the tendon markers *Scx* (6–8 folds↑), *Col1a1* (3–4 folds↑) and the cartilage marker *Sox9* (3–4 folds↓) ERK inhibition by PD18 activated *Scx* and *Col1a1* (∼2 folds↑), and had no effect on *Sox9* expression FGF does not have a tenogenic effect	Havis E, et al. Development 2014
Mechanical forces, FGF-4 versus TGF-β2	Mouse TSPC	Based on *Scx* expression, TGF-β2 was tenogenic for TSPCs at all stages, while loading was for late-stage cells only, and FGF-4 had no effect	Brown JP, et al. J Biomech 2014
Mechanical forces, FGF-4 versus TGF-β2	Mouse MSC and TSPC	TGF-β2 alone and combined with loading were tenogenic based on increased *Scx* levels in both MSCs and TSPCs. Loading alone had minimal effect. FGF-4 downregulated tendon marker levels in MSCs but not in TSPCs	Brown JP, et al. Stem Cell Res Ther 2015
BMP-2 versus TGF-β1 versus GDF-5	Human MSC	TGF-β1 and GDF-5 show similar *COL I* (3–5 folds↑), *COL II* (1–2 folds↑), and *SOX9* (1–2 folds↑) expression BMP-2: *COL I* (2–3 folds↓), *COL II* (100 folds↑), *SOX9* (1–2 fold↑)	Murphy MK, et al. Stem Cells 2015
EGF, bFGF, PDGF-BB, and TGF-β1	Human ASC	TGF-β1 induced *SCX* expression 10 folds more than other growth factors on day 7 EGF induced *TN-C* (10 folds↑) and *COL III* (3–5 folds↑) expression on day 14 TGF-β1 and PDGF-BB induced *COL I* expression 10 folds more than other growth factors on day 21	Gonçalves AI, et al. PLoS One 2013
TGF-β1, −2 and −3; GDF-5, −7 and −8; FGF-4; IGF-1; PDGF; EGF and VEGF	*Scx*-GFP tenocyte	TGF-β1, −2 and −3 were the most potent and induced similar levels of Scx-GFP expression. GDF-8 was ∼4 folds less potent than the TGF-βs Any other cytokines/growth factors examined had no effect	Maeda T, et al. Curr Biol 2012
IGF-1 versus TGF-β3	Human tenocyte	Collagen synthesis and expression of Scx and Tnmd TGF-β3 is more effective than IGF-1	Qiu Y, et al. Cells Tissues Organs 2013
TGF-β1, BMP-12, CTGF, and their combinations	Rat BMSC	TGF-β1 alone significantly and efficiently induced Scx expression and collagen production. TGF-β1 combined with CTGF elevated *Tnmd* mRNA and protein expression on day 7	Yin Z, et al. Stem Cells Translational Medicine 2016
Mechanical forces, FGF, TGF-β versus PD18	Chick undifferentiated limb cell	Both FGF-4 and TGF-β2 promote tendon commitment and act downstream of mechanical forces PD18 decreased *Scx* expression	Havis E, et al. Development 2016

Abbreviations: bFGF, basic fibroblast growth factor; CTGF, connective tissue growth factor; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor 1; MSC, mesenchymal stem cells; PDGF-BB, platelet-derived growth factors; TGF-β, transforming growth factor beta; TSPC, tendon stem/progenitor cells.

Because of its potential relevance in medicine, we highlight the importance of the combined influence of the TGF-β and MAPK pathways in tenogenic differentiation. We hope that our review will give readers a better understanding of the regulation of bioactive molecules in tendon regeneration. We also hope that this review will guide the development of optimal small molecule cocktails for the stepwise induction of tenogenic differentiation. Based on the findings of this review, the role of bioactive molecules in guiding stem cell fate in tendon regeneration warrants further exploration.

Author Contributions

Z.Y., G.L., and K.M.C.: conception and design; Y.J.Z., Z.Y., and X.C.: manuscript writing and editing, final approval of manuscript; H.W.O. and X.C.: critical discussion for manuscript; B.C.H.: final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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