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REVIEW

Physical regulation of stem cells differentiation into teno-lineage: current strategies and future direction

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Abstract Tendon injuries are commonly encountered in the clinic, disrupting the patient's normal work/life routine and damaging the career life of athletes. Currently, there is still no effective treatment for tendon injury. Tendon tissue engineering appears to be a promising route for tendon repair and regeneration. However, current strategies utilized in research are still far away from clinical applications due to unsuccessful cellular differentiation to tendon/tenocytes. In this review, we focus on the current physical strategies (mechanical stimulation and extracellular matrix topography) and evaluate their roles in precise and stepwise tendon differentiation. A systematic comprehension of normal tendon development process by structure, gene profile and physical microenvironment analysis is likely suggestive for stepwise tenocyte differentiation.

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Introduction

The physiological function of tendon is to transmit force between muscle and bone, which is mediated by collagen fibers within the tissue ultrastructure. Self-repair of tendon is usually accompanied by the formation of fibro-scar, which is constituted of smaller-sized collagen fibrils (Butler et al. 2004; Liden et al. 2008), and thus the mechanical strength of tendon is difficult to recover. Current therapeutic options for tendon injury include conservative treatments (steroid injection, low-intensity pulsed ultrasound, shockwave and physiotherapy) (Lui et al. 2011) and surgery (direct suture and autograft, allograft and permanent tendon prostheses) (Bagnaninchi et al. 2007; Goh et al. 2003). In the USA alone, about 220,000 tendon reconstructions are performed annually (Maffulli et al. 2003). However, current methods have inherent shortcomings such as a long recovery period, donor site morbidity, immunological rejection, and tendon tissue necrosis (Butler et al. 2004). Therefore, new therapeutic strategies for tendon injury need to be developed.

In recent years, there have been extensive studies on tendon tissue engineering for repairing injured tendon. By utilizing a combination of seed cells, biomaterials and suitable microenvironmental factors, tendon tissue engineering aims to replace the injured tendon through graft implantation. Stem cells have been widely used due to their excellent proliferative capacity within in vitro culture and differentiation potential to tenocytes. However, the utilization of stem cells in tendon tissue engineering also poses uncertainty because stem cells can differentiate into other lineages besides tenocytes. Hence, many studies have attempted to improve the differentiation

efficiency towards the tenocyte lineage by using diverse regulation strategies.

This review focuses on the current physical strategies (mechanical stimulation and extracellular matrix topography) and evaluates their roles in precise and stepwise tendon differentiation.

Current strategies for directing differentiation of stem cells to the tenocyte lineage

Current strategies utilized by investigators include mechanical stimulation, topography of extracellular matrix (ECM), growth and differentiation factors, gene transfection (genetic factors) and co-culture with tendon tissues or cells (Fig. 1). These attempts could be mainly divided into the effect of physical treatments (biomechanics and topography) and biochemical factors. Below we will mainly discuss current physical strategies.

Mechanical stimulation

Previously, induction of cellular differentiation to the tenocyte lineage by mechanical stimulation has been extensively studied (Table 1). It was found that mechanical stimulation, either static (Awad et al. 2000) or dynamic (Abousleiman et al. 2009; Altman et al. 2002; Chen et al. 2008, 2010; Juncosa-Melvin et al. 2006; Kuo and Tuan 2008; Noth et al. 2005; Xu et al. 2011, 2012; Zhang et al. 2008), can induce tendon-specific gene/protein expression, as well as spindle-shaped cell/nuclear morphology and tissue structure similar to normal mature tendon (cell alignment, collagen deposition with parallel orientation, fibril diameter enlargement), together with enhancement of mechanical properties (i.e. failure force, stiffness, modulus). Abousleiman et al. seeded human mesenchymal stem cells (hMSCs) onto human umbilical veins (HUVs) with collagen hydrogel and then placed these into a specifically designed tissue-engineered construct, to which cyclical tension was applied for 2 weeks (Abousleiman et al. 2009). A more than 8-fold increase in cell numbers was observed in these tensioned constructs, together with parallel orientation of collagen fibers and spindle-shaped cell nuclei mimicking the morphology of native tendon tissue. Most importantly, the mechanical stimulation yielded an ultimate tensile strength value and strain values comparable to normal human tendons. These results are promising, indicating the profound regulatory effect of mechanical stimulation on tenocyte lineage differentiation.

Mechanical stimulation has been extensively proposed as a positive regulation factor for tendon differentiation. Nevertheless, there are still several questions that need to be addressed. Firstly, most previous studies focused on differentiation in vitro, usually within a bioreactor. Whether there is enhanced repair with pre-stretched tissue-engineered grafts compared to unstretched grafts or whether natural mechanical stimulation can promote tenocyte lineage differentiation in vivo are still largely unknown. Juncosa-Melvin et al. stretched stem cell–collagen sponge constructs in vitro for 2 weeks, and these constructs were found to induce significant enhancement of rabbit patellar tendon repair, with excellent cellular alignment and mechanical properties (Juncosa-Melvin et al. 2006). In our previous study (Chen et al. 2010), we found that in vivo ectopic mechanical stimulation could enhance the differentiation of human embryonic stem cell derived MSCs (hESC-MSCs) to tenocytes. Furthermore, in a rat Achilles tendon repair model, natural mechanical stimulation caused by tendon rupture also promoted cell differentiation and tendon regeneration (Chen et al. 2010). To verify the effect of mechanical stimulation on tendon differentiation and repair, a more comprehensive and in vivo evaluation needs to be carried out.

Another problem that hinders the application of mechanical stimulation on tenocyte lineage differentiation is the exclusion of differentiation towards other lineages, such as bone or

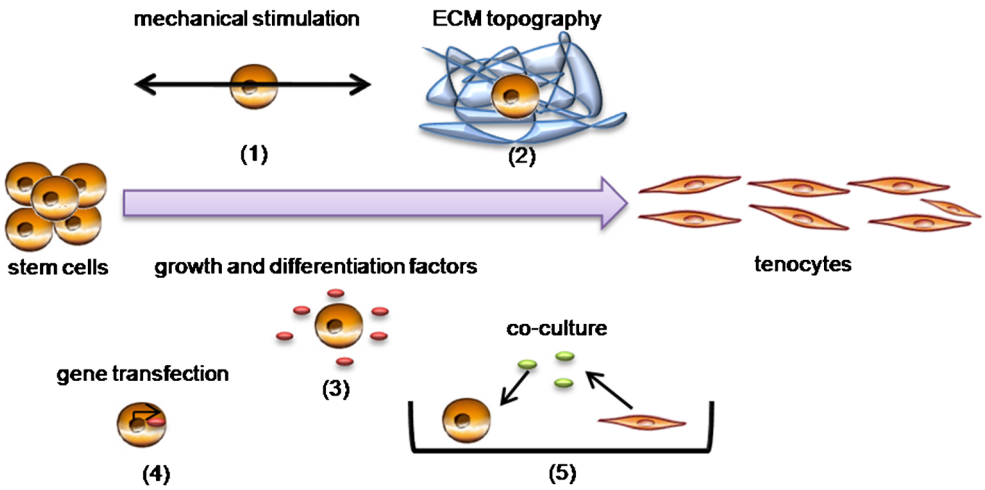

Fig. 1 Current strategies for directing differentiation of stem cells to the tenocyte lineage. (*1*) mechanical stimulation; (*2*) topography of ECM; (*3*) growth and differentiation factors; (*4*) gene transfection; (*5*) co-culture with tendon tissues or cells

Table 1 Mechanical stimulation induces tenogenic differentiation

Stem cells	Study types	Treatments	Findings
Rabbit BMSCs (Awad et al. 2000)	In vitro	Static stretching	Higher seeding densities (4×10^6 or 8×10^6 compared to 2×10^6 cells/mL in collagen gel) caused more well-aligned and elongated cell nuclei.
Rat BMSCs (Song et al. 2010; Zhang et al. 2008) Bovine BMSCs (Altman et al. 2002) Human MSCs (Abousleiman et al. 2009; Altman et al. 2002; Noth et al. 2005)	In vitro, proof of concept	Dynamic stretching	Bioreactors were designed to direct the tenogenic differentiation of stem cells.
Mouse TSCs (Zhang and Wang 2013) Rabbit TSCs (Zhang and Wang 2010a, b) Human MSCs (Chen et al. 2008; Morita et al. 2013)	In vitro, comparative study	Dynamic stretching	High-magnitude stretching (10 % stretching) upregulated the mRNA expression levels of tendon/ligament-related genes (Chen et al. 2008). Cyclic elongation of 10 % induced tendon differentiation of human MSCs, compared with 5 % and 15 % stretching stimulus (Morita et al. 2013). Lower stretch (4 %) of TSCs promoted teno-lineage differentiation, and higher stretch (8 %) increased the expression of both tenocyte-related and non-tenocyte-related genes (Zhang and Wang 2010a, b, 2013).
MouseTSCs (Zhang and Wang 2013) Rabbit MSCs (Juncosa-Melvin et al. 2006) Human ESC-MSCs (Chen et al. 2010)	In vivo study	Dynamic stretching	Mechanical stimulation of stem cell–scaffold constructs significantly improved tendon repair of rabbit (Juncosa-Melvin et al. 2006) and rat (Chen et al. 2010). Intensive treadmill running increased the expression of both tenocyte-related and non-tenocyte-related genes but moderate treadmill running only increased the expression of tenocyte-related genes (Zhang and Wang 2013).
Human MSCs (Kuo and Tuan 2008; Xu et al. 2011, 2012)	In vitro, mechanism study	Dynamic stretching	RhoA/ROCK, cytoskeletal organization, and FAK compose a "signaling network" that drives mechanical stretch-induced tenogenic differentiation of hMSCs (Xu et al. 2011, 2012). It was reported that matrix remodeling and Wnt signaling were involved during tenogenesis of human MSCs in a dynamic, 3D tissue-engineering model (Kuo and Tuan 2008).

cartilage. Although a beneficial role of mechanical stimulation on tenocyte lineage differentiation was demonstrated by many studies, some other studies reported conflicting results. It was shown that tendon stem cells (TSCs) underwent osteogenic differentiation when subjected to repetitive stretching at 4 % or 8 %, 0.5 Hz (Rui et al. 2011). Shi et al. demonstrated that rat TSCs could be induced to the osteogenic lineage after treatment with 2 % elongation uniaxial mechanical tension for 3 days, as shown by increased expression of Runx2 mRNA and protein, Alpl mRNA, collagen type 1 alpha 1 (Col1a1) mRNA, alkaline phosphatase (ALP) activity, and more intense ALP immunocytochemical staining (Shi et al. 2012). An interesting study carried out by Chen et al. (2008) attempted to explore the influence of cyclic mechanical stretching on the differentiation of human MSCs towards the teno- or osteo-lineage. In their study, the typical marker genes of the osteo-lineage were upregulated by low-magnitude stretching (3 % elongation), whereas tendon/ligament-related genes were up-regulated by high-magnitude stretching (10 % elongation) for a prolonged period (Chen et al. 2008). Morita et al. showed that 10 % cyclic elongation is more beneficial for hBMSCs differentiating into tenocytes compared with 5 or 15 % elongation, with upregulated expression of Scleraxis (Scx), Col1, collagen type 3 (Col3) and tenascin (Tnc) (Morita et al. 2013). However, an ex vivo study by Wang et al. (2013a, b) showed that the normal homeostasis of rabbit Achilles tendons could be maintained ex vivo with 6 % cyclic mechanical stimulation, 0.25 Hz for 8 h/day for 6 days. However, lower (3 %) or higher tensile (9 %) strain induced tendon matrix deterioration. And Zhang and Wang found lower stretch (4 %) of TSCs upregulated the expression of Col1 and tenomodulin (Tnmd), and higher stretch (8 %) increased the expression of both tenocyte-related and non-tenocyte related genes (Zhang and Wang 2010a, b, 2013). These results elucidated that there is only a narrow range of mechanical stimulation magnitude which is suitable for teno-lineage differentiation. And tendon homeostasis maintenance may require another different magnitude. However, current reports from a different group

showed discrepancy on the optimal range of magnitude. That is mainly due to the difference of each loading system and loading regimen, such as the time duration of mechanical stretch, tissue fixation method, the stem cell types used, and all other conditions in bioreactor microenvironment (Wang et al. 2013a, b).

Because mechanical stimulation may promote the differentiation of other lineages besides tendon, the challenge is to elucidate the possible mechanisms involved when stem cells undergo tenocyte lineage differentiation. The effect of focal adhesion kinase (FAK) on the realignment and mechanotransduction of hMSCs during the process of tenogenic differentiation induced by mechanical stretching has been observed (Xu et al. 2011). Further studies have examined the role of RhoA/ROCK, cytoskeletal organization, and FAK on mechanical stretch-induced tenogenic differentiation. This suggests that these three elements compose a "signaling network", which senses mechanical stretching and drives tenogenic differentiation (Xu et al. 2012). Kuo and Tuan reported the potential involvement of matrix remodeling by matrix metalloproteinases (MMPs) and Wnt signaling during tenogenesis of human MSCs in a dynamic, three-dimensional tissue-engineering model, thus providing insights into the mechanisms of tenogenesis (Kuo and Tuan 2008). However, Shi et al. found mechanical stimulation (2 % elongation) caused osteogenesis via the Wnt5a-RhoA pathway (Shi et al. 2012). It is probably mechanical stimulation activated by the common mechanotransducer RhoA/ROCK. But the cell fate is determined by a different signal pathway network, such as interaction between FAK, RhoA/ROCK and cytoskeletal organization, MMPs and Wnt signaling, or interaction between Wnt5a and RhoA/ROCK.

Topography of ECM

The scaffold fabricated from various biomaterials is one of the key components in tendon tissue engineering, which can modulate cellular adhesion, proliferation, migration, and support cell implantation, as well as bear mechanical stress caused by body movement prior to new tissue formation. The topography of the biomaterials constitutes the microenvironment of stem cells and regulates their differentiation fate (Table 2; Fig. 2a). It has been reported that native tendon sections could promote the differentiation of the seeded MSCs to the tenocyte lineage (Omae et al. 2009; Tong et al. 2012). Omae et al. sectioned tendons in the longitudinal direction and seeded BMSCs onto the decellularized tendon slices (Omae et al. 2009). The composite was incubated in vitro for 2 weeks, and the seeded cells were observed to align between the collagen fibers of the tendon slices. Higher tenomodulin gene expression was observed after culture. Similar results can be obtained by mimicking the topology of natural tendon tissue environment on poly-dimethylsiloxane (PDMS) (Tong et al. 2012).

Other studies have also evaluated whether different material properties could influence tendon differentiation, such as the stiffness of material (Sharma and Snedeker 2012), fiber diameter (Cardwell et al. 2012; Sahoo et al. 2006) and fiber arrangement (Cardwell et al. 2012; Kishore et al. 2012; Yin et al. 2010). When seeded on Col1 substrates, tenogenic differentiation markers were observed only at a moderate rigidity of around 30–50 kPa. However, more rigid substrates with a gradient of 70–90 kPa would robustly induce osteogenic differentiation (Sharma and Snedeker 2012). Our group has found that aligned nanofibers could induce the tenogenic differentiation of human tendon stem/progenitor cells (TSPCs), with the formation of spindle-shaped cells and tendon-like tissues (Yin et al. 2010). Also, higher expression of tendon-specific genes such as Eya2, collagen type 14 (Col14) and Scx were observed with aligned nanofibers, as compared to randomly oriented nanofibers. Cardwell et al. examined the effects of fiber diameter and orientation on tendon differentiation by electrospinning thin mats (Cardwell et al. 2012). C3H10T1/2 cells were cultured on different substrates. The results indicated that fiber diameter affects cellular behavior more significantly than fiber alignment. Larger-diameter fibers (>2 μm) may be more suitable for in vitro tenogenic differentiation.

Similar to mechanical stimulation, the topography of ECM faces the challenge that one particular parameter cannot tell the whole story. For example, aligned fiber arrangement may promote tenogenic differentiation (Yin et al. 2010). However, in some other situations, it also promotes neurogenic differentiation (Wang et al. 2012; Yang et al. 2005). Thus, specifically determining the cell fate of stem cells will be through combining different aspects of topography (ECM components, stiffness, strength, fiber orientation and diameter) to construct a tendon tissue analogue (Fig. 2b).

Biochemical factors

Besides the physical strategies for tendon differentiation, various growth and differentiation factors have been widely reported to regulate stem cell differentiation to the tenocyte lineage by gene transfection or protein treatment. These include GDF-5 (BMP14) (Park et al. 2010; Sassoon et al. 2012), GDF-6 (BMP13) (Helm et al. 2001), GDF-7 (BMP12) (Ni et al. 2011; Violini et al. 2009), bFGF (FGF-2) (Hankemeier et al. 2005), TGFb1/VEGF (Wei et al. 2011), PRP-clot releasate (PRCR) (Zhang and Wang 2010a, b) and insulin (Mazzocca et al. 2011). Also, it has been found that co-culture with native tendon tissue (Lovati et al. 2012) or tenocytes (Barboni et al. 2012; Luo et al. 2009) could enhance tenocyte lineage differentiation.

Table 2 Topography of ECM induces tenogenic differentiation

Stem cells	Treatments	Findings
Dog BMSCs (Omae et al. 2009) Human MSCs (Tong et al. 2012); Human TSPCs (Yin et al. 2013)	Tendon sections	Decellularized multilayer sliced tendon scaffold induced tenogenic differentiation (Omae et al. 2009). Cryostat sections of bovine Achilles tendon induced tenogenic differentiation, which can be reconstructed by PDMS (Tong et al. 2012). Decellularized tendon matrix promoted tenogenesis and inhibited osteogenesis, compared to decellularized bone and dermis matrix (Yin et al. 2013).
Human BMSCs (Sharma and Snedeker 2012)	ECM components (Fn, col1) and stiffness	Tenogenic differentiation markers were observed only on Col substrates with rigidity about 30–50 kPa.
Mouse MSCs (Cardwell et al. 2012) Porcine BMSCs (Sahoo et al. 2006)	Fiber diameter	A novel, biodegradable nano-microfibrous polymer scaffold was developed (Sahoo et al. 2006). Fiber diameter affects cellular behaviour more significantly than fiber alignment (Cardwell et al. 2012).
Human MSCs (Kishore et al. 2012; Tang et al. 2014) Human TSPCs (Yin et al. 2010)	Fiber orientation	Aligned fibers induced stem cell differentiation to tenocytes compared with random fibers (Kishore et al. 2012; Yin et al. 2010). Stem cells differentiated into teno-lineage when seeded on longitudinal tendon sections compared to tendon sections with other angles (Tang et al. 2014).

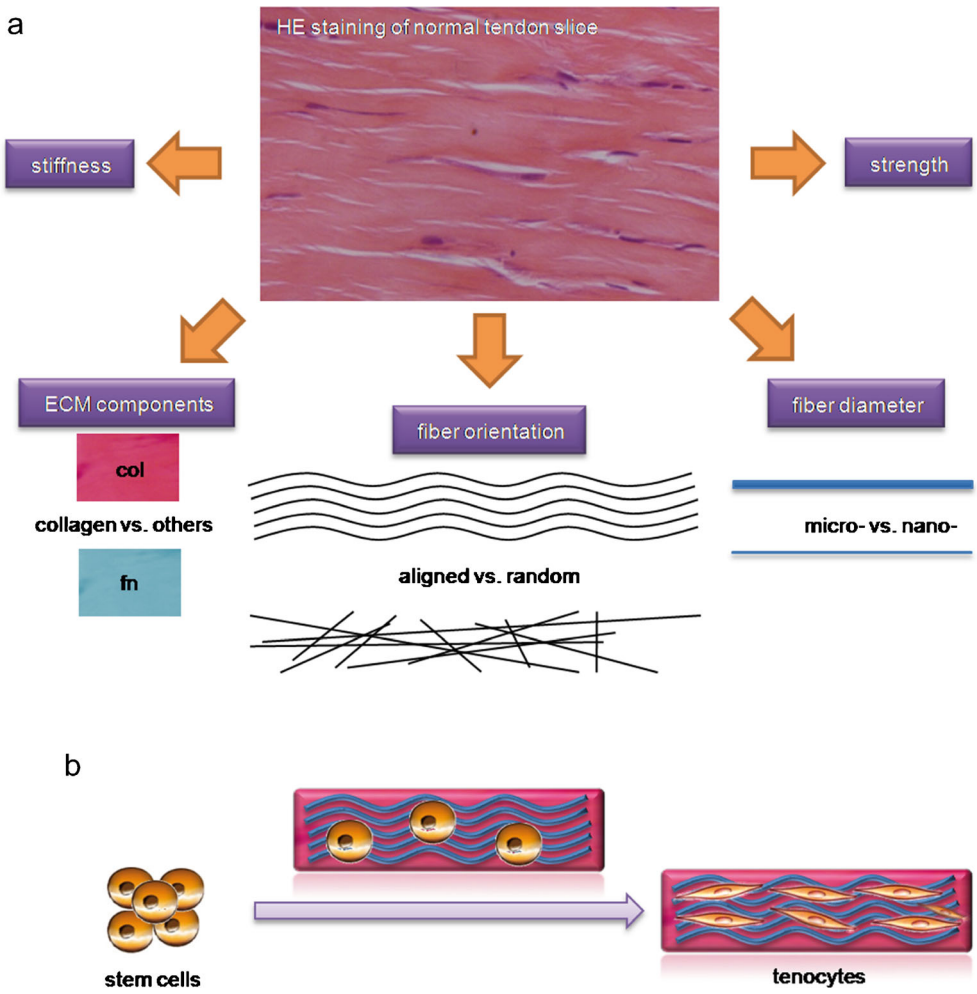

Fig. 2 Effect of ECM topography on tendon differentiation. **a** Some important parameters could be derived from the normal microenvironment of tenocytes, including ECM components, fiber orientation, fiber diameter, stiffness and strength. **b** Future studies can combine different aspects of topography to more precisely and effectively promote tenogenic differentiation of stem cells. *Col* collagen, *Fn* fibronectin

Future direction of differentiating stem cells into teno-lineage

Although various kinds of differentiation strategies have been developed, the ultimate effect is not fully satisfactory. It is still a long way to realizing the ideal of tendon regeneration by fully teno-lineage differentiation of stem cells. It is necessary to tightly control cell differentiation by a combination of genetics, epigenetics and environmental factors. On the one hand, more and more researchers pay attention to combining different strategies to enhance tendon differentiation. On the other hand, we never stop finding new effective differentiation factors.

Combination of physical treatments and biochemical factors

According to our literature review, 66 reports related to teno-lineage differentiation from stem cells had been published by July 2014 (Fig. 3), either by single factor or combination of factors. The number of reports using single factor increased by 86 % from 2005–2009 to 2010–2014 (Fig. 3c), with a peak in 2012 (Fig. 3a). However, the number of reports using two or more factors together increased by 275 % from 2005–2009 to 2010–2014 (Fig. 3c). The number before 2005 is 0. Interestingly, nearly 80 % of them combined physical treatments and biochemical factors.

In most of these attempts (Table 3; Fig. 4), mechanical stimulation (Chen et al. 2009, 2012a, b, c; Farng et al. 2008; Petrigliano et al. 2007) or varying topography of ECM (Ker et al. 2011; Kishore et al. 2012; Sahoo et al. 2010a, b) are combined with growth factors. In the former case, Petrigliano et al. evaluated the collective contributions of bFGF and mechanical strain on BMSC differentiation in a three-dimensional culture system (Petrigliano et al. 2007). After 21 days, cells subjected to both mechanical stimulation and bFGF displayed the highest upregulation of tendon-related genes such as Col1, Col3 and Tnc, as compared to untreated or single-treatment groups. This study suggested that the stimulatory effect of bFGF on tenogenic differentiation of BMSCs could be influenced by mechanical strain. Specific bFGF concentration (500 ng/scaffolds) added for a specific time duration (21 days) was required for a synergistic effect (Petrigliano et al. 2007). In the latter case, bFGF (FGF-2) was incorporated by electrospinning (Sahoo et al. 2010a, b) or with an inkjet-based bioprinter (Ker et al. 2011) and showed a positive synergetic effect in several studies. Sahoo et al. developed a biohybrid fibrous scaffold that comprises both ultrafine PLGA fibers and microfibrous silk (Sahoo et al. 2010a, b). By incorporating bFGF into the ECM-like biomimetic scaffold, they found that mesenchymal progenitor cells (MPCs) were initially stimulated to proliferate and subsequently underwent tenogenic differentiation. Additionally, the mechanical properties of the constructs were enhanced by the combination of ECM and biochemical factors, making the tendon analogue potentially useful for tendon repair and regeneration (Sahoo et al. 2010a, b). Another interesting study by the Ker group used a Spinneret-based Tunable Engineered Parameters (STEP) technique to control the orientation of submicron fibers (Ker et al. 2011). The results showed that the

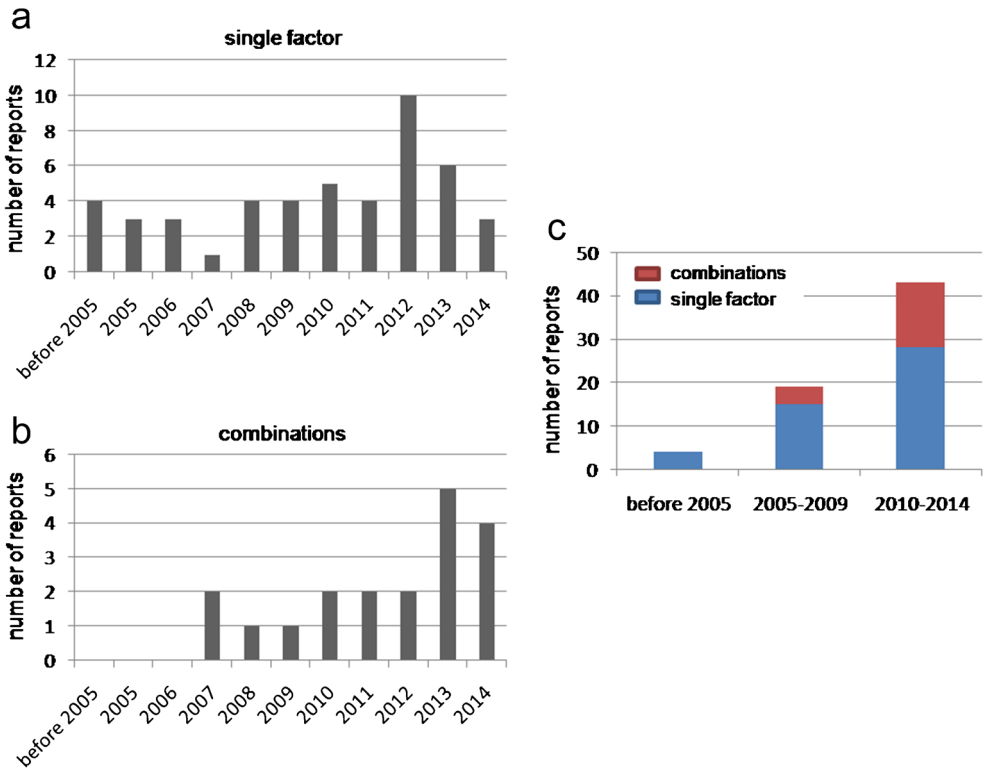

Fig. 3 Reports related to teno-lineage differentiation from stem cells by July 2014. **a** Number of reports by single factor induced teno-lineage differentiation in each year. **b** Number of reports by combination strategies induced teno-lineage differentiation in each year. **c** Comparison of single factor strategy and combination strategy in different time periods

Table 3 Combination of multiple inductive strategies to promote tenogenic differentiation

Stem cells	Treatments	Findings
Mouse BMSCs (Farng et al. 2008) Rat TSCs (Chen et al. 2012a, b, c) Rabbit BMSCs (Petrigliano et al. 2007) Human ESCs (Chen et al. 2009)	Growth factor + mechanics	HESCs were first induced to ESC-MSCs, then subjected to static stretch to form tissue engineered tendon (Chen et al. 2009). The stimulatory effects of bFGF (Petrigliano et al. 2007) or PRCR (Chen et al. 2012a, b, c) were dose-dependent, and had a synergetic effect with mechanical strain. Additive synergism with mechanical and GDF-5 was not observed (Farng et al. 2008).
Mouse MSCs (Ker et al. 2011) Rat BMSCs (Chai et al. 2013) Rat ASCs (Cheng et al. 2014) Rabbit BMSCs (Sahoo et al. 2010a, b) Equine ESCs (Barsby et al. 2014) Human MSCs (Caliari and Harley 2014; Kishore et al. 2012) Human ASCs (Min et al. 2014);	Growth factor + topography of ECM	The bFGF-releasing scaffolds: sub-micro scaffold (Ker et al. 2011), nano-scaffold (Sahoo et al. 2010a, b), micro- and nano-hybrid scaffolds (Sahoo et al. 2010a, b) promoted tenogenic differentiation. The combination of GDF-6 (20 ng/ml)-treated BMSCs and SIS promoted-tendon differentiation and regeneration (Chai et al. 2013). Aligned collagen-nanoparticle fibers with PDGFs significantly promoted tenogenesis of ASCs compared to random scaffolds with PDGFs (Cheng et al. 2014). ASC tenogenesis were promoted under higher PDGF-BB and lower BMP-2 concentrations (Min et al. 2014). 3D collagen constructs with the addition of TGF-beta3 synergistically improved teno-lineage differentiation of ESCs (Barsby et al. 2014). Aligned collagen scaffolds induced stem cell differentiation to tenocytes compared with random scaffolds. However, an additional effect with BMP-12 on aligned scaffolds was not observed (Kishore et al. 2012). Tenogenesis was enhanced in anisotropic 3D collagen–glycosaminoglycan scaffolds compared to isotropic group. However, this effect was abrogated by supplemented blebbistatin (Caliari and Harley 2014).
Canine ASCs (Schneider et al. 2011)	Growth factor + co-culture	Tenogenesis was induced through a combination of treatment with IGF-1, TGF-beta1 and in high-density co-culture with primary tenocytes.
Human MSCs (Qiu et al. 2014) Human ASCs (Yang et al. 2013) Human iPSC-MSCs (Czaplewski et al. 2014)	Mechanics + topography of ECM	Cyclic tension promoted teno-lineage differentiation of MSCs in collagen scaffold (Qiu et al. 2014). Combination of tendon extracellular matrix and uniaxial tension improved teno-lineage differentiation and inhibited osteogenesis (Yang et al. 2013). Braided fibrous scaffolds with larger angles promoted tendon differentiation under the stimulation of cyclic uniaxial strain (Czaplewski et al. 2014).
Human BMSCs (Lee et al. 2007)	Co-culture + mechanics	Co-culture with anterior cruciate ligament cells and mechanical stress promotes ligament differentiation synergistically.
Equine ASCs (Raabe et al. 2013)	Growth factor + mechanics + oxygen tension	The optimal condition for tenogenic differentiation is found when stem cells received mechanical stimulation with 21 % oxygen tension and GDF-5 or GDF-7 supplementation.

cells underwent myocyte differentiation when the fibers were not patterned with any growth factors. Tenogenic or osteogenic differentiation was promoted when the aligned fibers were printed FGF-2 or BMP2 (Ker et al. 2011). These suggest that the combination of geometric properties of native ECM and biochemical factors is important for directing cellular differentiation and for successful organization of the engineered tissue.

Whereas not all attempts are successful, Farng et al. (2008) cultured scaffolds in a custom bioreactor under static or cyclic strain (10 % strain, 0.33 Hz), which were coated with GDF-5. After 48 h, MSCs treated with mechanical stimulation or GDF-5 alone displayed increased Col1 and Scx expression. However, additional synergism with the mechanical and biological stimuli was not observed. Similarly, the synergistic effect of BMP-12 and collagen orientation on the tenogenic differentiation of human MSCs was not observed as demonstrated by another study (Kishore et al. 2012). It is likely that one treatment was dominant and masked the other treatment on the effect of teno-lineage differentiation. Also, the optimal delivery strategy of growth factors should be evaluated in the combination treatment. A stepwise-treated strategy may be a better choice for synergistic effect, compared with simply treating stem cells with different stimulation at the same time.

Developing a more systemic microenvironment for stem cell tenogenic differentiation by combining physical treatment and biochemical factors requires more fundamental knowledge of tenocyte differentiation and a suitable differentiation model. In addition, epigenetic regulation should be combined into the tenogenic differentiation system, which has been

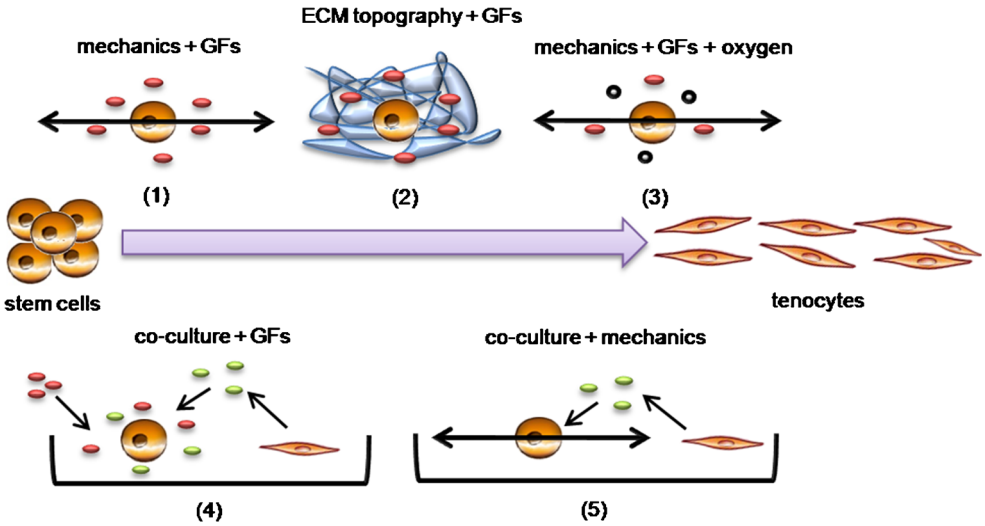

Fig. 4 Current strategies for tendon differentiation by combining two or more inductive factors. (*1*) Combination of mechanics and growth factors; (*2*) combination of ECM topography and growth factors; (*3*) combination of mechanics, growth factors and oxygen tension; (*4*) combination of co-culture and growth factors; (*5*) combination of co-culture and mechanics. *GFs* growth factors

demonstrated to be important for cell fate determination in other tissues (Koh and Rao 2013; Ma et al. 2010). However, to our knowledge, there have not been any reports in the field of tendon differentiation.

New differentiation factors from tendon development study

It is challenging to discover new crucial genes or treatments for tenogenic differentiation, but the physiological development process of tendons may help to overcome this problem. In fact, many of the growth factors or important genes mentioned above were initially identified in the biological development of tendon or with gene knock-out mice models. For example, Scx is expressed in early tendon progenitor cells and is continuously expressed throughout tendon development (Brent et al. 2003; Schweitzer et al. 2001). GDF-5 knock-out mice displayed thinner patellar tendon (Mikic 2004), and knock-out of GDF-6 in mice influenced matrix remodeling and collagen deposition in tail tendon (Mikic et al. 2009). Systematic study of the tendon development process from embryonic to postnatal to totally mature, may uncover new important genes or factors regulating tenogenic differentiation and help elucidate the inherent mechanism from ESCs → MSCs → TSCs → TPCs → differentiated tenocytes. Current putative tendon specific genes or growth factors regulating tenogenic differentiation were mostly discovered during the embryonic development stage of tendon (Brent et al. 2003; Shukunami et al. 2006; Storm and Kingsley 1996). Some studies have also focused on the postnatal tendon development process in mice (Ansorge et al. 2011; Ezura et al. 2000; Liu et al. 2012; Zhang et al. 2006), but the objectives of these studies were only to observe the structural alteration or to evaluate the function of pre-discovered genes. The structural formation of tendon has not so far been clearly elucidated by an inherent molecular basis. Our unpublished data indicated that it is quite useful to screen new differentiation factors from postnatal tendon development. We compared the mature process (histology staining, TEM scanning and polarized light evaluation) of post-natal Sprague–Dawley rats at P0, P1, P2.5, P4, P7, P14, P28 and P56. Using microarray and siRNA technology, we have identified c-fos as a new tendon early-stage differentiation factor (unpublished data). Other potential crucial genes are under evaluation, which may account for the tendon differentiation and maturation.

Future research directions might rely on systematic comprehension of the tendon development process by combining structural and functional analysis. With such progress, we may acquire really effective strategies to manipulate stem cells differentiation to tenocytes.

Stepwise tenogenic differentiation

Due to the various possible combinations and interactions, it is difficult to find the correct combinations to effectively regulate teno-lineage differentiation. However, some principal rules can be obtained according to normal tendon development. The most important one is to adopt the concept of stepwise differentiation to combine different effective factors in the right order. Current stem cell types utilized for tenogenic differentiation include ESCs, MSCs and TSCs. ESCs, being at the nascent primary stage of development, have the potential to differentiate into all cell types within the body. MSCs, the most widely utilized stem cells in current research, are able to differentiate into all mesenchymal lineages. TSCs, or TSPCs, the specialized stem cells existing in normal tendon tissue, also display differentiation potential to several mesenchymal lineages, and might be a more promising cell source than MSCs for tendon regeneration (Tan et al. 2012). From ESCs → MSCs → TSCs → TPCs → differentiated tenocytes, the stemness of cells is gradually lost, with the cell types

becoming more defined towards the tenocyte lineage (Fig. 5). Thus, it is reasonable to speculate that different stem cell types need different stimulation for effective tenogenic differentiation. Moreover, a stepwise differentiation strategy is necessary for precise control of ESCs or MSCs differentiating into mature tenocytes, to avoid the risk of teratoma formation or other lineage differentiation in current attempts.

Stepwise differentiation in other lineage

The necessity of stepwise differentiation has been confirmed in research on other tissues. For example, directly differentiating hESCs into muscle progenitors has not been successful. However, the generation of skeletal myoblasts could be achieved from ESCs through mesodermal transition (Barberi et al. 2007; Darabi et al. 2008, 2012; Goudenege et al. 2012). In the first step, Goudenege et al. (2012) cultured hESCs in a myogenic medium to achieve mesenchymal differentiation. In the second step, they overexpressed myogenic factor MyoD to achieve the ultimate conversion. This kind of stepwise differentiation is effective and avoids the formation of teratomas. Oldershaw et al. (2010) reported a three-step protocol for differentiation of hESCs into chondrocytes. Human ESCs are differentiated through primitive streak–mesendoderm and mesoderm intermediates to chondrocytes with chemical combinations. These chemicals are based on the development knowledge of pathways active in sequence. Besides the muscle and cartilage tissue, stepwise differentiation from hESCs into other tissues/cells have been reported, such as liver (Hay et al. 2008), heart (Laflamme et al. 2007), insulin-secreting beta cells (D'Amour et al. 2006), neurons (Yan et al. 2005) and oligodendrocytes (Nistor et al. 2005).

Stepwise differentiation in teno-lineage

Our group adopted this stepwise differentiation approach when utilizing ESCs for tendon regeneration in a previous study (Chen et al. 2009), and further explored the regenerative effect of implanting these newly-formed ESC-MSCs within a rat Achilles tendon repair model (Chen et al. 2010). No teratoma was found in any of our samples. Also, the stepwise differentiation from ESCs to ESC-MSCs enhanced tenogenic differentiation (Chen et al. 2010). However, there is a still long way to go, from MSCs to TSCs/TSPCs to tenocytes. Alberton et al. (2012) converted human BMSCs into tenogenic progenitor cells (Chen et al. 2012a, b, c) by ectopic expression of Scx, and observed the upregulation of Col1, Tnmd and several tendon-related genes. When induced towards three different mesenchymal lineages, hMSC-Scx cells failed to differentiate into chondrocytes or osteoblasts, indicating a more restricted differentiation potential. Our group believes that mechanical stress plays an important role in tenocyte maturation and tendon formation. In a recent study (Chen et al. 2012a, b, c), scleraxis was overexpressed in hESC-MSCs to initiate tenocyte lineage differentiation, which means the transition from hESC-MSCs to TSPCs. Mechanical stimulation was introduced to promote tenocyte commitment. The synergistic effect between force and scleraxis augmented the tenocyte lineage differentiation and ectopic tendon regeneration. A recent report by Barsby et al. showed that ESCs differentiated into tendon-lineage when seeded within 3D collagen constructs (Barsby et al. 2014). The 3D microenvironment exerted the mechanical stimulation on the stem cells and caused their differentiation. And this effect could be synergistically enhanced by the addition of TGF-beta3.

These indicate that physical treatment such as mechanical stimulation and ECM may contribute more to the differentiation stage from ESCs to mature tenocytes (Fig. 5). However,

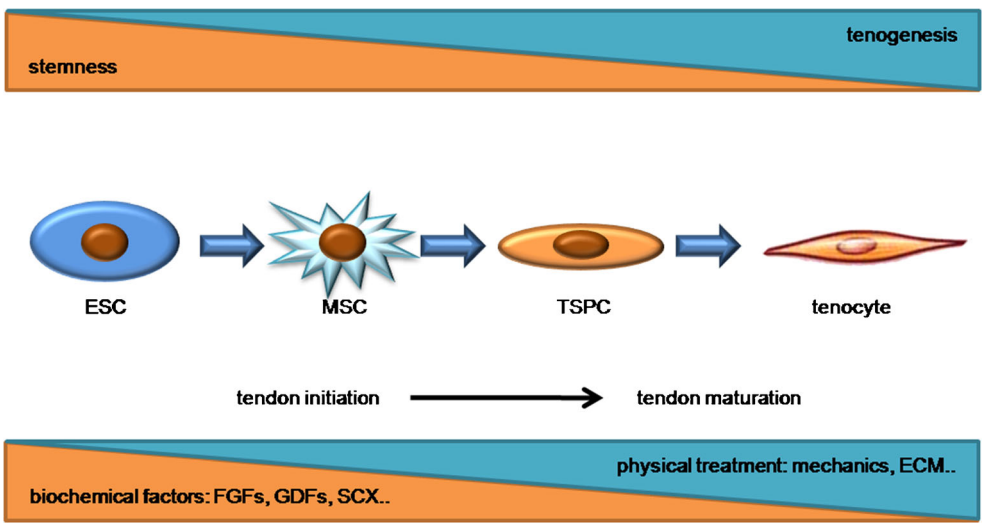

Fig. 5 Tenocyte development and differentiation. **a** The stemness of cells is gradually lost from ESC to tenocyte. **b** From tendon initiation to tendon maturation, biochemical factors may play a dominant role in the first step, while the physical treatments (mechanics and ECM) may be critical to the maturation

current studies on the stepwise tenogenic differentiation are still far from sufficient to uncover the transition events between the various cell types (especially from MSCs to tenocytes), and to find more useful strategies to constitute a systemic induction model.

The potential of physical factors in teno-lineage stepwise differentiation

The importance of physical microenvironment on stepwise tendon differentiation should be taken into account (Fig. 5). The main role of tendon is force transmission. Mechanical loading is essential in tendon formation and maintenance. Mechanics deprivation could result in tendon degeneration (collagen fibers disorienting and nuclear morphology becoming round) (Hannafin et al. 1995). ECM topography regulates the cell fate of TSPCs. Genetic disruption of ECM components, e.g., Bgn and Fmod, caused cell fate from tenogenesis to osteogenesis, and tendon becoming thinner and more translucent (Bi et al. 2007). As summarized in Tables 1 and 2, it has been widely observed that physical treatment such as mechanical stimulation and ECM can influence tenocyte lineage differentiation. Normal tendon development is accompanied by collagen fiber deposition and maturation, which accounts for the topography of ECM and mechanical microenvironment (Ansorge et al. 2011; Ezura et al. 2000; Liu et al. 2012; Zhang et al. 2006). Thus, it is reasonable to speculate that the physical microenvironment exerts an influence as profound as biochemical factors on development of tendon. Some studies we have mentioned above have already shown that physical treatments are important for stepwise teno-lineage differentiation (Barsby et al. 2014; Chen et al. 2012a, b, c). However, because of the practical difficulties of evaluating tissue ECM and mechanics during in vivo development, our understanding of the process remains elusive. Despite tendon-like constructs having been developed in vitro to study the mechanical properties of newly synthesized collagen matrix (Kalson et al. 2010), technical problems of studying the physical microenvironment in vivo need to be solved for further understanding their role in tendon development and differentiation.

Conclusions

Tendon differentiation and repair is enhanced by current physical strategies and the combinations of physical treatments and biochemical factors. However, these tenocyte-like cells derived from stem cells are still quite different from normal tenocytes. To develop an efficient tenogenic differentiation system for stem cells, several key issues need to be further addressed.

Firstly, a more detailed and controllable differentiation system of single inducing factors needs to be established. In order to exclude differentiation towards other lineages within different microenvironments, the stretching magnitude and time course of mechanical stimulation, and the stiffness range and material types of ECM topography, need to be precisely compared and controlled. In addition, future studies should evaluate the effect of epigenetics in tenogenic differentiation.

Secondly, the stepwise differentiation strategies need to be adopted when combining different treatments. The natural stepwise development process of tendon is a good model. A systematic comprehension of tendon development process by structure, genetic/epigenetic profile and physical microenvironment analysis may pave the way for highly effective tendon differentiation from stem cells and ultimately successful tendon tissue regeneration.

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