Editorial

The Mkx homeoprotein promotes tenogenesis in stem cells and improves tendon repair

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Tendons are essential structures that transmit mechanical forces generated by skeletal muscles to bones to cause body motion. Acute tendon injury and tendinopathy are common pathologies of the musculoskeletal system. However, a poor understanding of tendon biology has impaired the design of efficient treatments for tendon repair following injury and tendinopathy (1). A recent paper by Liu *et al.*, published in *Stem Cells* in 2014 (2) highlighted an essential role for the Mohawk (Mkx) homeodomain protein in promoting tenogenesis in mouse stem cells and improving tendon repair in a mouse model for tendon injury.

Tendons are mainly constituted of extracellular matrix proteins and scattered tenocytes. Type I collagen is the main component of tendon matrix. It is constituted of α1 and α2 polypeptides encoded by the *Col1a1* and *Col1a2* genes. These peptides assemble in a parallel fashion to form collagen fibrils, fibrils associate to form fibers, that will in turn form fascicles, that finally assemble to form the tendon proper that is surrounded by external sheets (epitenon and paratenon) forming the peritenon. Together with type I collagen, other collagens and extracellular matrix proteins are part of the tendon-specific matrix (1). However, type I collagen is not a specific marker for tendons, as it is expressed in many types of fibroblasts. The lack of specific tendon markers has delayed tendon research for years. The first marker specific for tenocytes, the bHLH transcription factor Scleraxis (Scx), was described in mouse and chick models in 2001 (3). Scx is expressed in tendon (progenitors and differentiated) cells during development and in adult life (3-5). Scx has provided a unique tool to decipher the gene regulatory network controlling tenogenesis during normal and pathological conditions.

In addition to being an excellent tendon marker, Scx has been shown to be involved in tendon formation, to promote tenogenesis in stem cells and to improve tendon repair. *Scx* mutant mice display severe tendon defects (6). *Scx*-deficient tendons display a diminution of *Col1a1* expression and a disorganization of type I collagen fibrils (6). In addition, *Cola14a1* and *Tnmd* gene expression is lost in tendons from E16 in *Scx* mutant mice (6). Tnmd (Tenomodulin) is a type II transmembrane glycoprotein considered as a late tendon differentiation marker downstream of Scx (6,7). Ectopic application of Scx has been shown to activate tenogenesis in human stem cells, based on *Tnmd* expression (8-10), and grafts of Scx-producing stem cells have been shown to improve tendon repair in animal models for tendon injury (10).

In addition to Scx, two other transcription factors have been identified as being involved in tendon formation: the Mkx homeobox protein and the Egr1 zinc finger transcription factor. Although Mkx and Egr1 expression sites are not tendon-specific, Mkx and Egr1 mutant mice display tendon defects, mainly due to alteration of type I collagen production and organization (11-14). Egr1 has been shown to promote tenogenesis in stem cells and improve tendon repair in animal models of tendon injury (12,15). *In vitro* studies showed that when adding BMP12 to growth medium to promote tenocyte differentiation, mesenchymal stem cells (MSCs) isolated from equine umbilical cord blood (16) and bone marrow-derived mesenchymal stem cells (BMMSCs) (9) expressed *Mkx* together with other tendon genes (*Scx*, *Col1a1*, *Dcn*). However, until the publication of the study by Liu *et al.* (2), no data were available concerning a potential role of Mkx in tenogenesis induction in stem cells or during tendon repair

after injury.

Extracellular signals are also essential for tenogenesis. TGFβ is the main signaling pathway involved in tenogenesis during development and repair (1). TGFβ ligands have been shown to activate *Scx* expression in mouse stem cells (17,18). TGFβ ligands are released after tendon injury and the block of TGFβ signaling in the *Smad3* mutant mice impaired tendon healing (19). TGFβ ligands have been largely studied as putative candidates to improve tendon repair (1). Moreover, TGFβ signaling is also sufficient and required for *Scx* expression in tendons during mouse development (17,18). However, there is no clear picture of the regulatory network between extra cellular signals and transcription factors driving tenogenesis.

Liu and colleagues were the first to address Mkx function in tenogenesis in mouse stem cells and in tendon repair in a mouse model for tendon injury (2). *Mkx* was known to be expressed in progenitor cells of components of the musculoskeletal system, muscle, cartilage, bone and tendon (20,21). The Mkx homeobox protein, closely related to the Iroquois family, has been described as a potent transcriptional repressor and has been shown to block the myogenic conversion of 10T1/2 fibroblasts, to repress *MyoD* transcription in C2C12 cells and to repress *Sox6* transcription in satellite cells (22-24). Mkx also represses the expression of the cartilage marker *Sox9* in human anterior cruciate ligament cells (25). The transcriptional repression of specific muscle and cartilage markers by Mkx in cell cultures leads to the interesting hypothesis that Mkx promotes the tendon lineage by repressing the other lineages in mesodermal progenitors during development. However, *Mkx* mutant mice did not display obvious cartilage, bone or skeletal muscle defects, but showed severe tendon hypoplasia (13,14,26). In *Mkx*-deficient tendons, cell number was not changed but type I collagen production was reduced (transcripts and protein). Tendon defects were first observed in late developmental stages (from E16.5) and *Mkx*-deficient tendons also displayed reduced expression of *Tnmd* and of the extracellular matrix components Decorin (Dcn) and Fibromodulin (Fmd) (13,14).

Liu and colleagues observed that Mkx was present in rat Achilles tendons during postnatal tendon maturation (immuno-staining). Mkx expression was significantly decreased in human samples of tendinopathy tissues compared to normal tendon tissue (assessed by immuno-staining and computational analysis from Gene Expression Omnibus data sets). *Mkx* and *Scx* expression was also higher in human tendon stem/progenitor cells (TSPCs) compared to adipose stem cells and embryonic stem cell-derived mesenchymal stem cells. When *Mkx* expression was silenced by siRNA in mouse TSPCs, the expression of *Scx*, *Tnmd* and *Dcn* was decreased, suggesting a role for Mkx in the maintenance of tendon characteristics in these stem cells (2).

They tested the ability of Mkx to induce tenogenesis in stem cells by overexpressing *Mkx* in C3H10T1/2 cells, a mouse multipotent mesenchymal stem cell (MSC) line. They observed that Mkx decreased the clonacity of C3H10T1/2 cells using the colony-forming unit assay, and impaired the multi-differentiation potential of these MSCs towards adipogenesis and osteogenesis (2). Interestingly, MSCs expressing either *Scx* or *Egr1* transcription factor also displayed a minimal capacity to differentiate into adipocytes and osteocytes (8,10,12). The authors also compared the efficacy of Mkx with that of Scx to promote tenogenesis in MCSs cultured in a multilayered cell sheets adopting a tendon-like structure. Consistent with the Mkx requirement for correct type I collagen production (13,14), the levels of *Col1a1* mRNAs were increased in Mkx-MSCs and the collagen fibrils displayed higher diameters than those of control MSCs (2). The transcription of other tendon-associated collagens, *Col3a1*, *Col5a1*, *Col14a1* and other tendon-associated genes, *Tnmd*, *Dcn*, *Fmod* and *Tnc* was also strongly enhanced in Mkx-producing cells compared to control cells.

Interestingly, Liu *et al*. showed that Mkx induced *Scx* expression in mouse C3H10T1/2 cells and tail TSPCs (2). The *Scx* induction by Mkx in mouse MSCs differs from a concomitant study in which MKX did not activate the expression of *SCX* in human bone marrow MSCs (9). It is possible that ability of Mkx to induce *Scx* and other targets may differ between species and cell types. The ability of Mkx to induce *Scx* in mouse stem cells also differs from the developmental tendon process, where *Scx* is normally expressed in *Mkx* mutant mice (14,26). *Egr1* expression was not increased by Mkx in human bone marrow MSCs (9), and *Mkx* transcription was not increased by Egr1 in mouse C3H10T1/2 cells (12), suggesting independent pathways for the tendon-promoting effects of Mkx and Egr1 transcription factors. Liu and colleagues also observed that the tenogenic effect of Scx was less efficient than that of Mkx, based on smaller collagen fibril diameters and the absence of *Tnmd* activation in mouse Scx-C3H10T1/2 cells compared to Mkx-C3H10T1/2 cells in culture sheet systems that mimick tendon-like structures (2). However, Scx has been shown to activate *Tnmd* expression in human

bone marrow MSCs (9). Again, this discrepancy could be due to the use of different cell lines from different species. Collectively, these results show that in addition to being required for correct tendon formation (13,14,26), Mkx promotes tenogenesis in MSCs from different species (2,9).

To identify direct Mkx target genes during tenogenesis, Liu and colleagues used a chromatin immunoprecipitation sequencing approach to assess Mkx recruitment to regulatory regions in Mkx-C3H10T1/2 cells. No Mkx recruitment to the *Scx* promoter could be detected, but Mkx was recruited to the *Tgfb2* promoter. Furthermore, *Tgfb2* expression was increased in Mkx-C3H10T1/2 cells in 2-dimensional and in cell sheet culture systems, supporting a direct activation of *Tgfb2* transcription by Mkx (2). This result is somewhat surprising, since Mkx has been described as a potent DNA binding transcriptional repressor (23,24). However, one can speculate that Mkx has the ability to bind different partners, which would modify its transcriptional activity. For example, Smad3, a well-known transcriptional activator, was demonstrated to interact physically with Mkx in mouse C3H10T1/2 cells (27). Interestingly, the Egr1 transcription factor is also recruited to the *Tgfb2* promoter region in adult mouse tendons (12). The Egr1 recruitment to the *Tgfb2* promoter in tendons combined with the decrease of *Tgfb2* expression in injured tendons of *Egr1* mutant mice and the increase of *Tgfb2* expression Egr1-C3H10T1/2 cells, indicated a direct activation of *Tgfb2* transcription by Egr1 (12). Mkx and Egr1 are recruited to distinct regulatory regions of the mouse *Tgfb2* gene (2,12). Moreover, TGFβ2 only partially mediates the Egr1 and Mkx effects on tendon gene expression in MSCs, since TGFβ2 is not able to activate *Tnmd* expression, while Egr1 and Mkx are able to do so (2,12). Thus, it appears that *Mkx* and *Egr1* act at the same level in the genetic network controlling tenogenesis, upstream of *Tgfb2*. However, TGFβ pathway inhibition does not fully blocks Mkx and Scx effects on tenogenesis. It will thus be necessary in future work to characterize better the Mxk and Scx TGFβ-independent activities in this process.

Finally, Liu and colleagues tested the ability of Mkx-expressing MSCs to improve tendon healing after Achilles tendon injury *in vivo*. Mkx-expressing MSCs cell sheets were implanted after a complete transverse section of mouse Achilles tendon. Four weeks after implantation of Mkx-expressing cells, the typical structure of tendon was observed at the repaired sites, with a more mature collagen compared to the control tendons. Mkx-C3H10T1/2-grafted tendons had better biomechanical properties than those of GFP-C3H10T1/2-grafted tendons. These data showed that the application of Mkx-expressing MSCs improves tendon repair *in vivo*. This is the first experimental evidence that Mkx-cells promote tendon repair in animal models.

In conclusion, Scx, Mkx and Egr1 are three transcription factors involved in tendon development, and they all display the ability to induce tenogenesis in stem cells. The implantation of stem cells producing any of these transcription factors improves tendon repair in animal models for tendon injury. The genetic interactions between these transcription factors during tenogenesis are not fully understood and could be different between *in vitro* and *in vivo* systems and between species. However, the Mkx and Egr1 transcription factors were demonstrated to act upstream of *Tgfb2* during tenogenesis in mouse mesenchymal stem cells. This is an important step in the elaboration of the gene regulatory network orchestrating tenogenesis, even if other studies will still be needed to elucidate this network fully.

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