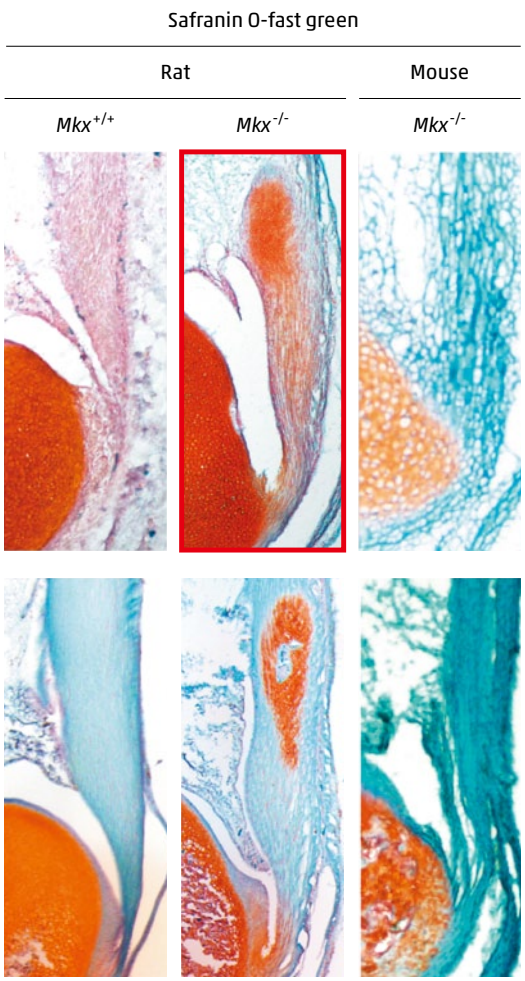
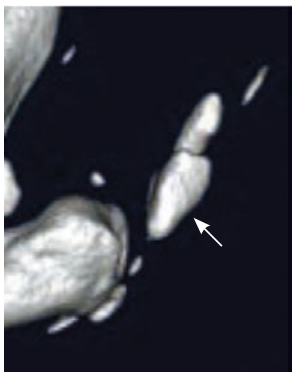
Mohawk transcription factor: A potential target for tissue engineering

Knockout rats represent a powerful animal model

Rats are the experimental animals of choice for several fields of research, including the musculoskeletal system, where smaller animals such as mice impose limitations ranging from an insufficient number of harvestable cells to difficulties evaluating the effects of mechanical load. Rats are also more physiologically similar to humans than mice are. However, it has proven to be technically challenging to derive genetically modified rats because of complications in isolating and maintaining embryonic stem (ES) cells.

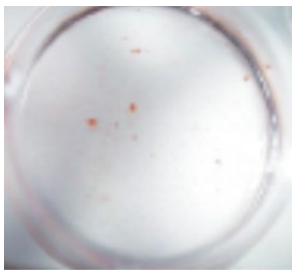
Heterotopic ossification in Achilles tendon of *Mkx*$^{-/-}$ rats



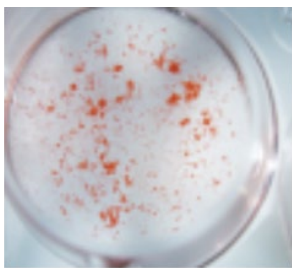


Micro-CT of the Achilles tendons of *Mkx*$^{-/-}$ rat

Mkx$^{+/+}$



Mkx$^{-/-}$



Mkx-deficiency accelerates osteogenic differentiation of tendon-derived cells

The development of genome editing technology for CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) has enabled precision genetic engineering to be achieved without ES cells. The Cas9 nuclease induces double-strand DNA breaks that are repaired by the cellular processes of homologous recombination or non-homologous endjoining. Further target specificity is achieved by the use of guide-RNA to direct nuclease activity.

Research by collaborators, coordinated at TMDU, used CRISPR/Cas9 technology to generate rats with a deletion of the gene encoding the Mohawk transcription factor (*Mkx*), which controls the expression of tendon-related genes.

Mkx plays an important role during tendon development

We derived three lines of F1 rats with targeted *Mkx* knockout, and in all three lines, we observed the distinctive 'wavy tail' phenotype and underdeveloped (hypoplasic) tendons previously seen in *Mkx*$^{-/-}$ mice. However, the tail phenotype was more pronounced in the *Mkx*$^{-/-}$ rats, which also showed the more severe phenotype of early heterotopic ossification of the Achilles tendon (the abnormal growth of bone within soft tissue). In humans, heterotopic ossification can be caused by injury to the soft tissue surrounding bones and joints, such as occurs during musculoskeletal trauma, excessive mechanical stress, or joint replacement, resulting in a painful condition that reduces the range of motion. Similarly, physiological assessment of the ankle joint during gait analysis of *Mkx* knockout rats showed reduced downward flexion of the foot compared with wild-type control *Mkx*$^{+/+}$ rats.

Molecular analysis of the *Mkx*$^{-/-}$ rats revealed the reduced expression of tendon-

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related genes compared with the controls, which was accompanied by a corresponding reduced tensile strength of patellar tendons. Also, we observed elevated expression of genes associated with osteogenesis and chondrogenesis (bone and cartilage growth, respectively), which is indicative of heterotopic ossification. This was confirmed by micro-computed tomography. Meanwhile, transmission electron microscopy revealed smaller collagen fibril diameters in the tail tendons of *Mkx*$^{-/-}$ rats than in wild-type rats.

Mechanical stress prevents tendon ossification

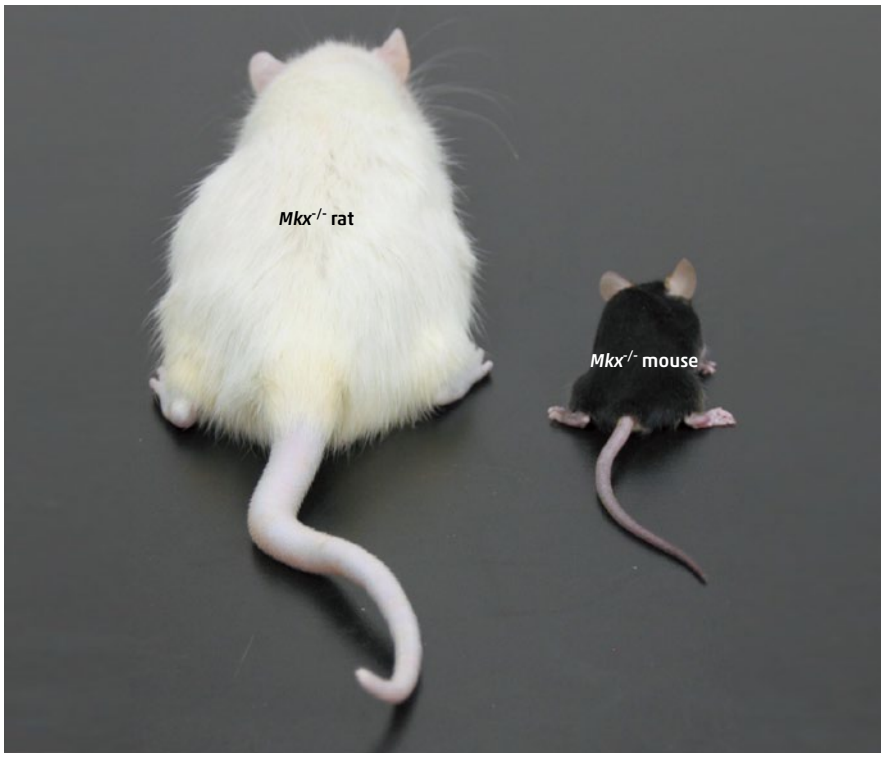
Abnormal tendon ossification in neonatal rats involves the development of cartilage within the tendon at birth, then the replacement of cartilage with bone. We found that in the absence of *Mkx,* tendon stem cells and progenitor cells that should differentiate into tendon cells instead become cartilage cells. Thus, tendon-derived *Mkx*$^{-/-}$ cells showed a tendency to undergo osteogenic and chondrogenic differentiation because absence of *Mkx* represses the tendon-related genes, such as extracellular matrix genes, and promotes the genes associated with chondrogenesis and osteogenesis. Conversely, *Mkx* overexpression reduced the differentiation of *Mkx*$^{-/-}$ cells into bone cells, cartilage cells, or adipocytes.

A lack of tendon cells derived from *Mkx*$^{-/-}$ mice has hampered previous attempts to perform a genome-wide search of *Mkx* targets. However, we obtained a sufficient volume of tendon cells from *Mkx*$^{-/-}$ rats to show that putative *Mkx* targets include both tendon-related genes (such as *Fmod*) and collagen genes, and also the *Sox* family of genes, which are associated with chondrogenic differentiation. *Fmod*-deficient mice have previously been shown to have heterotopic ossification of both the Achilles tendon and the knee joint.

Furthermore, because mechanical stress influences tendon development and promotes mesenchymal stem cells to differentiate into tendon cells, we investigated the effect of the loss of *Mkx* on the cellular response to mechanical stress. We stimulated *Mkx*$^{-/-}$ tendon-derived cells by mechanical stretching and observed increased expression of chondrogenic genes and enhanced differentiation into bone or cartilage cells. We surmise that the larger size of rats compared to mice causes greater mechanical stimulation of the tendon, resulting in increased chondrogenic differentiation and a more severe phenotype.

These findings suggest that *Mkx* controls the differentiation of tendon cells while simultaneously preventing their development into bone or cartilage. The introduction of *Mkx,* therefore, has potential as a novel repair mechanism for tendon damage or tissue engineering.

A white rat and black mouse with the characteristic wavy phenotypes in the tail associated with disruption of *Mkx*



Gene targeting of the transcription factor Mohawk in rats causes heterotopic ossification of Achilles tendon via failed tenogenesis
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