The *Mohawk* homeobox gene is a critical regulator of tendon differentiation

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***Mohawk* (*Mkx*) is a member of the Three Amino acid Loop Extension superclass of atypical homeobox genes that is expressed in developing tendons. To investigate the in vivo functions of *Mkx*, we generated *Mkx*^{−/−} mice. These mice had hypoplastic tendons throughout the body. Despite the reduction in tendon mass, the cell number in tail tendon fiber bundles was similar between wild-type and *Mkx*^{−/−} mice. We also observed small collagen fibril diameters and a down-regulation of type I collagen in *Mkx*^{−/−} tendons. These data indicate that *Mkx* plays a critical role in tendon differentiation by regulating type I collagen production in tendon cells.**

Tendons are dense, fibrous connective tissues that connect muscle to bone, transmitting the forces that allow for body movement (1). Tendon damage from overuse or degeneration due to aging is a common clinical problem because damaged tendon tissue heals very slowly and rarely recovers completely (2). The establishment of new therapies, such as regenerative medicine, for injured tendons has been delayed by a limited understanding of tendon biology (1, 3).

Tendons are composed primarily of collagen fibrils that cross-link to each other to form fibers (4). A small number of tendon cells reside between parallel chains of these fibrils and synthesize the specific ECM that contains collagens and proteoglycans (4, 5). The elasticity of tendons is provided by the large amount of collagen, predominantly type I collagen and small amounts of other collagens, including types III, IV, V, and VI (4, 6–9). The proteoglycans found in tendons, including decorin, fibromodulin, biglycan, and lumican, act to lubricate and organize collagen fiber bundles (4, 5). Targeted disruption of these proteoglycans in mice leads to abnormal collagen fibrils in tendons (3, 10–13). Tendon disruptions have also been described in patients with defects in collagen production, such as Ehlers-Danlos Syndrome, in which the type I collagen gene is mutated (14). These studies indicate that the ability of tendon cells to produce ECM is important for tendon formation.

Recently, it was reported that *Scleraxis* (*Scx*), a basic helix–loop–helix (bHLH) transcription factor expressed in the tendon progenitors and cells of all tendon tissues (15, 16), is essential for tendon differentiation. *Scx* knockout mice show severe disruption of force-transmitting tendons, although ligaments, which are tissues connecting bone to bone that closely resemble tendons in their components, and short-range anchoring tendons are not affected (17). It was also reported that *Scx* positively regulates the expression of type I collagen, a main ECM component of tendons (18). However, the type I collagen does not completely disappear from the tendons of *Scx* knockout mice (17), suggesting the presence of other regulatory factors for type I collagen. The tendon differentiation mechanisms remain largely unknown, with *Scx* being the only known transcription factor regulating tendon differentiation.

Mohawk (*Mkx*; also known as *Irx11*) is the sole member of a newly characterized class within the Three Amino acid Loop Extension (TALE) superclass of atypical homeobox genes (19). These transcription factors are essential for a large set of developmental processes, including cell proliferation, differentiation, and posi-

tional specification (20–24). Initial characterization of mouse *Mkx* revealed a dynamic transcription pattern restricted to progenitors of tendon, skeletal muscle, and cartilage, as well as the sex chords of the male gonad and the ureteric bud tip of the metanephrogenic kidney (19, 25, 26). We previously identified *Mkx* as a transcription factor expressed in developing tendons by constructing a whole-mount in situ hybridization database, termed “EMBRYXS” (<http://embrys.jp/embrys/html/MainMenu.html>), which contains expression data of 1,520 transcription factors and cofactors expressed in E9.5, E10.5, and E11.5 mouse embryos (27).

To investigate the in vivo functions of *Mkx*, we generated *Mkx* knockout mice. Here, we show that *Mkx* null mice have hypoplastic tendons throughout their body. Although the size of tendons is drastically reduced in *Mkx* null mice, the cell number in tail tendon fiber bundles shows no significant difference between wild-type and *Mkx* null mice. We also observed abnormal collagen fibrils in *Mkx* null tendons. Furthermore, we show that the expression of type I collagen, which is a major tendon ECM component, is down-regulated in the Achilles tendon of *Mkx* null mice. These data indicate that *Mkx* is a transcription factor controlling tendon differentiation by regulating type I collagen production in tendon cells.

Results

Generation of *Mkx* Mutant Mice. We inactivated the *Mkx* gene by homologous recombination in ES cells using a targeting vector to replace the *Mkx* gene from the start of translation to the end of exon 2 with the Venus gene and PGK-neomycin-resistance (PGK-neo) cassette (Fig. 1A). We predicted that the Venus gene, an improved GFP gene (28), would be expressed in *Mkx* positive cells in heterozygous mice generated with the targeting construct. We identified two correctly targeted ES cell clones by Southern blot analysis (Fig. S1A and B). These two clones were microinjected into 8-cell stage embryos and germ-line transmission in the resulting chimeric mice was confirmed by Southern blotting and genomic PCR (Fig. 1B and Fig. S1C). *Mkx*^{+/-} embryos showed robust Venus expression that recapitulated *Mkx* expression faithfully at E13.5 forelimb and tail (Fig. 1C). Also, these Venus knockin heterozygous mice showed tendon-specific Venus expression in the tail of E16.5 embryos (Fig. 1D). In addition to embryos, we investigated Venus expression in adult mice. Because *Mkx* expression in tendons of adult mice had not been determined, we analyzed

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Fig. 3. Tendon mass is decreased in *Mkx* null mice. (A) H&E staining of the patellar (a–d) and tail (e–h) tendons in 3-month-old wild-type and *Mkx* null mice. Yellow arrowheads indicate a fiber bundle in the tail tendon. [Scale bars: (a and c) 1 mm; (b and d) 50 μm; (e and g) 200 μm; (f and h) 50 μm.] (B) Patellar tendon thickness of 7-day-old wild-type and *Mkx* null mice. Error bars, SEM ($n = 8$). (C) Cell density of tail or Achilles tendons (Left) and cell number in a tail tendon fiber bundle (Right) in 3-month-old wild-type and *Mkx* null mice. Error bars, SEM (Left, $n = 8 \sim 10$; Right, $n = 14$). ns, no significance. (D) Azan staining of E18.5 embryonic tails in wild-type and *Mkx* null mice. Yellow arrowheads indicate a fiber bundle in the tail tendon. [Scale bars: (Left) 500 μm; (Right) 200 μm.] (E) Transmission electron microscopic view of collagen fibrils in the Achilles tendon of wild-type and *Mkx* null mice. (Scale bars, 200 nm.)

tendons in *Mkx* null embryos was not affected, but we observed a low density of aniline blue staining (Fig. 3D; yellow arrowheads), which detects collagen fibers. To analyze the collagen fibrils of *Mkx* null tendons in detail, we performed an ultrastructural analysis of Achilles tendons using electron microscopy. Collagen fibril diameters in the *Mkx* null mice were uniformly smaller than those of wild-type mice (Fig. 3E). These data suggest a reduction of collagens in *Mkx* null tendons and a critical role for *Mkx* in tendon differentiation in vivo.

***Mkx* Null Tendon Cells Reduced Type I Collagen Production.** Despite tendon mass reduction in *Mkx* null mice, tendon cell number showed no significant changes between wild-type and *Mkx* null mice (Fig. 3C). This suggests that *Mkx* null tendon cells have a reduced ability to synthesize ECM or enhance ECM catabolic activity. In addition, histological analysis by azan staining of E18.5 *Mkx* null tail tendons (Fig. 3D) and ultrastructural analysis of Achilles tendons (Fig. 3E) suggested a reduction of collagens in *Mkx* null tendons. To investigate the production of collagens by *Mkx* null tendon cells, we first measured the amount of soluble collagens in *Mkx* null tendons by Sircol Soluble Collagen Assay. This experiment indicated that total soluble collagens were decreased in Achilles and tail tendons of *Mkx* null mice compared with those from wild-type mice (Fig. 4A). Furthermore, we found a reduction in the protein level of type I collagen, which is a main component of tendon ECM, in Achilles

Fig. 4. Type I collagen productivity is decreased in *Mkx* null tendon cells. (A) Soluble collagen measurement in whole soluble protein of Achilles or tail tendons of 8-week-old wild-type or *Mkx* null mice. Error bars, SEM ($n = 3$). (B) Western blot analysis of type I collagen (Col1) and β -actin (actin) in Achilles or tail tendons of 8-week-old wild-type or *Mkx* null mice. (C) Real-time PCR analysis for *Col1a1* and *Col1a2* in Achilles tendons of 8-week-old wild-type or *Mkx* null mice. Error bars, SEM ($n = 3$). (D) Proposed tendon differentiation network.

and tail tendons of *Mkx* knockout mice by Western blotting (Fig. 4B). We also found that the mRNA levels of *Col1a1* and *Col1a2*, which encode the type I collagen, were decreased in Achilles tendons of *Mkx* null mice through quantitative real-time PCR (Fig. 4C). However, the expression of the transcription factor *Scx*, which is a positive regulator of type I collagen, was not reduced and rather increased in these tendons (Fig. S6), indicating that the decrease of collagen I is not due to the down-regulation of *Scx*. We also investigated the expression of other tendon component genes such as *elastin* (*Eln*), *fibrillin1* (*Fbn1*), *Col3a1*, *Col6a1*, *TenascinC* (*Tnc*), and proteoglycans in mutant Achilles tendon by real time PCR analysis. This experiment indicated that decorin (*Dcn*), which is a proteoglycan regulating collagen fiber formation, was down-regulated (Fig. S6). These data reveal that *Mkx* plays an important role in regulating the expression of type I collagen and its associate molecules in tendon cells.

Discussion

We investigated the in vivo functions of *Mkx*, the homeobox gene expressed in developing tendons, by analyzing the tendons of *Mkx* knockout mice. We examined two major tendons, Achilles and tail tendon, whose developmental origins are known to be different. Achilles tendon in the limbs is induced in mesenchyme directly and the tail tendon in the trunk is derived from syndetome (15, 29). As a result, cell density (Fig. 3C), collagen quantity (Fig. 4A) and type I collagen expressions (Fig. 4B) were reduced in both Achilles tendon and tail tendon of *Mkx* null mice. This indicates that *Mkx* may play a role in various tendons, regardless of the developmental origins, although such factors are unknown.

The bHLH transcription factor *Scx* is also known to be a positive regulator of tendon differentiation via its role in promoting type I collagen expression (17, 18). However, phenotypes of both null mutants are different. Although *Scx* null mutants exhibit a loss of segments or complete tendons (17), *Mkx* null mice have

reduced tendon mass without a decrease in the number of tendon cells. These data suggest that *Scx* is essential for the initiation of tendon differentiation, whereas *Mkx* plays a critical role in tendon maturation (Fig. 4D). Regardless of the high expression of *Scx* in *Mkx* null mice (Fig. S6), type I collagen gene expression is decreased (Fig. 4C), indicating the existence of other positive regulators of type I collagen that should be regulated by *Mkx*. Recent work indicates that *Mkx* functions as a transcriptional repressor by recruiting the Sin3A/histone deacetylase co-repressor complex (30), therefore *Mkx* may act as a repressor for negative regulatory factors of type I collagen.

It has been reported that the *Tgfb2*, *Tgfb3* double knockout mice or *Tgfb2* knockout mice show loss of most tendons, and TGF- β led to an early induction of a tendon master gene *Scx* expression (31). This indicates that TGF- β -*Scx* pathway regulates the initial differentiation of tendon. Here, we show that *Mkx* plays a critical role at the tendon maturation stage. It would be of interest to examine whether TGF- β may also be critical for late tendon differentiation via *Mkx* regulation.

To get more insight into the function of *Mkx* on the collagen network development, we examined a set of other tendon component genes' expressions and observed that decorin was also decreased in *Mkx* null mice tendons (Fig. S6). Reduced decorin, known to be a regulator of collagen assembly, expression may provide an explanation for the collagen fibril size reduction phenotype in *Mkx* null mice. Although we focused on tendon development, *Mkx* may also play a role in tendon homeostasis in adults. In this regard, it would be interesting to examine the potential function of *Mkx* in aging tendons.

These findings will serve as a basis for understanding molecular mechanisms of tendon differentiation and may provide a therapeutic target for tendon injuries and tendon related diseases, such as Ehlers-Danlos Syndrome.

Materials and Methods

Generation of *Mkx* Mutant Mice. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at the National Institute for Child Health and Development. A vector was constructed to replace the endogenous *Mkx* locus with the Venus gene and PGK-neo cassette by homologous recombination in ES cells (Fig. 1A). 5' and 3' sequences flanking the endogenous *Mkx* locus were amplified by PCR from a C57BL/6 genomic BAC clone (BACPAC Resource Center). These homology arms were cloned into a vector incorporating both a neomycin resistance cassette for positive selection and a diphtheria toxin (DT-A) gene for negative selection. The targeting vector was linearized and electroporated into TT2F ES cells. Recombinant ES clones were isolated after culture in medium containing G418 antibiotic and screened for proper integration by Southern blotting with the 5' probe, 3' probe and neo cassette sequence (Fig. S1 A and B). Two clones exhibited proper integration, which was validated through genomic sequencing, and were chosen for microinjection into eight-cell stage embryos. The resulting chimeric offspring were crossed to C57BL/6 mice and germ-line transmission was confirmed by Southern blotting (Fig. S1C) and PCR (Fig. 1B). The floxed PGK-neo cassette was removed by crossing with Meox-Cre transgenic mice (purchased from The Jackson Laboratory) (32) and Cre-mediated neo excision was analyzed by genomic PCR. PCR primer sequences for genotyping are shown in Table S2.

Whole-Mount Visualization of Fluorescent Signals and in Situ Hybridization. For whole-mount visualization of fluorescent signals, embryos were observed directly and tissues were skinned before observation. Whole-mount in situ hybridization for *Mkx* was performed as described previously (33). The details of *Mkx* probe synthesis for whole-mount in situ hybridization can be obtained on the "EMBRYOS" web site (<http://embryos.jp/embryos/html/MainMenu.html>).

Histological Analysis and Immunohistochemistry. *Mkx* null mice and wild-type littermates were obtained from an intercross of *Mkx*^{+/−} maintained on a C57BL/6 background. For histological analysis, patellar and tail tendons were harvested from embryos or adult mice and fixed with 4% paraformaldehyde in PBS at 4 °C overnight. Tissues were dehydrated, embedded in paraffin, and sectioned, and each section was stained with H&E or azocarmine-aniline blue (azan). For immunohistochemistry, tails from E16.5 embryos were dissected

and fixed with 4% paraformaldehyde in PBS at 4 °C for 2 h. The tissues were embedded in O.C.T. compound (Sakura Finetek) and frozen rapidly in liquid nitrogen. Specimens were sectioned at 10 μ m. Cryosections were air-dried and blocked with Blocking One (Nacalai Tesque) for 1 h. The sections were then incubated with anti-myosin heavy chain antibody (MF20; DSHB) at 4 °C overnight, rinsed, and incubated for 1 h with Alexa 594 (Molecular Probes). These experiments were performed with at least three independent samples to confirm reproducibility.

Tensile Testing. To evaluate the mechanical properties of the Achilles tendon, we used the entire tendon unit (from the myotendinous junction to the calcaneal tuberosity). A uniaxial materials testing system (Autograph AGS-G; Shimadzu Corp. Ltd.) was used to determine tensile properties with a 500 N load cell, as described previously (34) with some modifications. To facilitate gripping during testing, the proximal end of the Achilles tendon and foot of the mouse were fixed in custom-made clamps and the specimens were pulled at a constant strain rate of 0.5 mm/sec. All samples broke within the gauge length. The initial length and cross-sectional area of each specimen were measured using digital calipers and by microscopy of H&E stained sections, respectively. Force data were collected in Trapezium (Shimadzu Corp. Ltd.) software at a frequency of 50 Hz. For each specimen, a stress-strain curve was created from the load-displacement curve and Young's modulus was calculated from each stress-strain curve using the cross-sectional area.

Counting Cell Numbers of Tendon. The paraffin-sections or cryosections of tail or Achilles tendons from 3-month-old *Mkx* null or wild-type mice were prepared and stained with H&E or DAPI as described in Histological Analysis and Immunohistochemistry. The hematoxylin or DAPI stained nuclei were counted in each 50 μ m $\times$ 50 μ m area or tail tendon fiber bundle. At least eight areas or bundles were counted in three or four of *Mkx* null or wild-type mice.

Western Blot Analysis and Collagen Measurements. Total soluble protein was obtained from Achilles tendon and tail tendon by homogenization in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% DOC, 0.1% SDS, 1% Nonidet P-40; pH 8.0) and used for Western blot analysis and collagen measurements. For Western blotting, total soluble protein was separated by SDS/PAGE followed by a semidry transfer to PVDF. Membranes were blocked for 30 min with Blocking One (Nacalai Tesque), incubated with anti-collagen I antibody (ab292; Abcam) or anti- β -actin antibody (A5316; SIGMA) at 4 °C overnight, rinsed, and then incubated for 1 h with HRP-conjugated anti-rabbit IgG (IgG) antibody (A6154; SIGMA) or HRP-conjugated anti-mouse IgG antibody (A2304; SIGMA). The blot was then developed with Chemi-Lumi One (Nacalai Tesque). Total soluble collagen measurements of homogenate in Achilles and tail tendons were performed using the Sircol Soluble Collagen Assay (Biocolor) according to the manufacturer's instructions. Total soluble protein was measured using the DC Protein Assay (Bio-Rad) and used the data for normalization. These experiments have been repeated at least three times to confirm reproducibility.

Electron Microscopy. Small pieces (approximately 1 mm³) were excised from Achilles tendons of wild-type and *Mkx* null mice, and fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight. The samples were then washed in 0.1 M cacodylate buffer and rinsed with physiological saline. They were dehydrated through an ethanol series, embedded in epoxy resin, and cut into ultrathin (approximately 100 nm) sections. Sections were mounted on copper grids, contrasted with aqueous uranyl acetate and lead citrate and examined by transmission electron microscope (H-7100; Hitachi).

RNA Isolation, RT-PCR, and Quantitative Real-Time PCR. Total RNA was isolated from Achilles tendons, tail tendons and femoral muscles using ISOGEN (Nippongene), and reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). RT-PCR was performed with Go-Taq polymerase (Promega). Quantitative real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems). The expression of *Gapdh* was used as a control for mRNA expression. Gene expression changes were quantified using the delta-delta C_T method. This experiment was performed with three independent samples and confirmed reproducibility. Primer sequences for RT-PCR and real-time PCR are described in Table S2.

Statistical Analysis. The two-tailed independent Student's *t*-test was used to calculate all *P* values. Asterisks in figures indicate differences with statistical significance as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

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1. Birk DE, Trelstad RL (1986) Extracellular compartments in tendon morphogenesis: Collagen fibril, bundle, and macroaggregate formation. *J Cell Biol* 103:231–240.
2. Sharma P, Maffulli N (2006) Biology of tendon injury: Healing, modeling and remodeling. *J Musculoskelet Neuronal Interact* 6:181–190.
3. Bi Y, et al. (2007) Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 13:1219–1227.
4. Kannus P (2000) Structure of the tendon connective tissue. *Scand J Med Sci Sports* 10: 312–320.
5. Yoon JH, Halper J (2005) Tendon proteoglycans: Biochemistry and function. *J Musculoskelet Neuronal Interact* 5:22–34.
6. Williams IF, McCullagh KG, Silver IA (1984) The distribution of types I and III collagen and fibronectin in the healing equine tendon. *Connect Tissue Res* 12:211–227.
7. Docheva D, Hunziker EB, Fässler R, Brandau O (2005) Tenomodulin is necessary for tenocyte proliferation and tendon maturation. *Mol Cell Biol* 25:699–705.
8. Benjamin M, Ralphs JR (2000) The cell and developmental biology of tendons and ligaments. *Int Rev Cytol* 196:85–130.
9. Kjaer M (2004) Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev* 84:649–698.
10. Danielson KG, et al. (1997) Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol* 136:729–743.
11. Jepsen KJ, et al. (2002) A syndrome of joint laxity and impaired tendon integrity in lumican- and fibromodulin-deficient mice. *J Biol Chem* 277:35532–35540.
12. Ameye L, et al. (2002) Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. *FASEB J* 16:673–680.
13. Chakravarti S, et al. (1998) Lumican regulates collagen fibril assembly: Skin fragility and corneal opacity in the absence of lumican. *J Cell Biol* 141:1277–1286.
14. Mao JR, Bristow J (2001) The Ehlers-Danlos syndrome: On beyond collagens. *J Clin Invest* 107:1063–1069.
15. Schweitzer R, et al. (2001) Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 128:3855–3866.
16. Cserjesi P, et al. (1995) Scleraxis: A basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. *Development* 121:1099–1110.
17. Murchison ND, et al. (2007) Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* 134:2697–2708.
18. Léjard V, et al. (2007) Scleraxis and NFATc regulate the expression of the pro- $\alpha 1(I)$ collagen gene in tendon fibroblasts. *J Biol Chem* 282:17665–17675.
19. Anderson DM, et al. (2006) Mohawk is a novel homeobox gene expressed in the developing mouse embryo. *Dev Dyn* 235:792–801.
20. Selleri L, et al. (2001) Requirement for Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation. *Development* 128:3543–3557.
21. Brendolan A, et al. (2005) A Pbx1-dependent genetic and transcriptional network regulates spleen ontogeny. *Development* 132:3113–3126.
22. Moens CB, Selleri L (2006) Hox cofactors in vertebrate development. *Dev Biol* 291: 193–206.
23. van Tuyl M, et al. (2006) Iroquois genes influence proximo-distal morphogenesis during rat lung development. *Am J Physiol Lung Cell Mol Physiol* 290:L777–L789.
24. dilorio P, Alexa K, Choe SK, Etheridge L, Sagerström CG (2007) TALE-family homeodomain proteins regulate endodermal sonic hedgehog expression and pattern the anterior endoderm. *Dev Biol* 304:221–231.
25. Liu H, Liu W, Maltby KM, Lan Y, Jiang R (2006) Identification and developmental expression analysis of a novel homeobox gene closely linked to the mouse Twirler mutation. *Gene Expr Patterns* 6:632–636.
26. Takeuchi JK, Bruneau BG (2007) *Irx1*, a divergent Iroquois homeobox family transcription factor gene. *Gene Expr Patterns* 7:51–56.
27. Yokoyama S, et al. (2009) A systems approach reveals that the myogenesis genome network is regulated by the transcriptional repressor RP58. *Dev Cell* 17:836–848.
28. Nagai T, et al. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol* 20:87–90.
29. Brent AE, Schweitzer R, Tabin CJ (2003) A somitic compartment of tendon progenitors. *Cell* 113:235–248.
30. Anderson DM, Beres BJ, Wilson-Rawls J, Rawls A (2009) The homeobox gene Mohawk represses transcription by recruiting the sin3A/HDAC co-repressor complex. *Dev Dyn* 238:572–580.
31. Pryce BA, et al. (2009) Recruitment and maintenance of tendon progenitors by TGF β signaling are essential for tendon formation. *Development* 136:1351–1361.
32. Tallquist MD, Soriano P (2000) Epiblast-restricted Cre expression in MORE mice: A tool to distinguish embryonic vs. extra-embryonic gene function. *Genesis* 26:113–115.
33. Yokoyama S, et al. (2008) Dynamic gene expression of Lin-28 during embryonic development in mouse and chicken. *Gene Expr Patterns* 8:155–160.
34. Gentleman E, et al. (2003) Mechanical characterization of collagen fibers and scaffolds for tissue engineering. *Biomaterials* 24:3805–3813.