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Deficiency of Macrophage Migration Inhibitory Factor Gene Delays Healing of the Medial Collateral Ligament: A Biomechanical and Biological Study

Soichiro Kitayama[1], Shin Onodera[2*], Eiji Kondo[1], Takumi Kobayashi[1], Shin Miyatake[1], Nobuto Kitamura[1], Harukazu Tohyama[1], Kazunori Yasuda[1]

[1]Department of Sports Medicine and Joint Reconstruction Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan

[2]Research Center for Cooperative Projects, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Running Title: MIF deficiency impairs MCL healing

*Corresponding author: Shin Onodera

Research Center for Cooperative Projects, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo, 060-8638, Japan.

Tel: +81-11-706-7211 Fax: +81-11-706-7822 E-mail:onodera@med.hokudai.ac.jp

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ABSTRACT

The role played by macrophage migration inhibitory factor (MIF) in the process of wound healing is controversial. Besides, there have been no reports that investigated the expression or the role of MIF in the repair process after ligament injury. In this study, we hypothesized that the deficiency in MIF gene might delay ligament healing in mice. The aim of this study was to clarify this hypothesis using MIF gene-deficient mice (MIFKO) and murine model of injury to the medial collateral ligament (MCL). Biomechanical testing showed that the levels of mechanical properties were significantly lower in MIFKO than in wild-type mice (WT) on day 28 after injury. Levels of matrix metalloproteinase (MMP)-2 and -13 mRNA in the healing tissue were significantly lower in MIFKO than in WT on day 28 and on day 7, respectively. Histologically, healing tissues in MIFKO exhibited prolonged hypertrophy, poor vascularity, and prolonged increase in cell number compared with those in WT. Taken together, it was suggested that MIFKO exhibited delayed healing of the MCL, which might be caused by lower mRNA expression of MMP-2 and -13.

Keywords: Medial Collateral Ligament; Macrophage Migration Inhibitory Factor; Healing; Mouse; Matrix Metalloproteinase

1. Introduction

Ligament healing has been one of the greatest foci in the field of biomechanics (Woo et al., 2008), and medial collateral ligament (MCL) injury of the knee has been commonly used as a model to study ligament healing (Frank et al., 1999). Commonly, ligament healing occurs very slowly, and it takes a few years or more. Therefore, a number of investigators have studied to develop methods to enhance ligament healing, such as growth factor application, cell therapy, gene therapy, and so on. For example, MCL healing can be enhanced *in vivo* by local administration of platelet-derived growth factor (PDGF)-BB, growth/differentiation factor (GDF)-5, and nerve growth factor (NGF) (Hildebrand et al., 1998, Tashiro et al., 2006, Mammoto et al., 2008). In addition, Cerovecki et al has reported that oral administration of pentadecapeptide BPC157, an anti-ulcer peptide, could enhance MCL healing in a rat model (Cerovecki et al., 2010).

Macrophage migration inhibitory factor (MIF) was initially identified as a soluble factor in culture medium of activated T cells (Bloom, Bennet, 1966; David, 1966). Following the cloning of MIF complementary deoxyribonucleic acid (cDNA) (Weiser al., 1989), previously unrecognized biological functions of MIF have been revealed. MIF is released as a proinflammatory cytokine and a glucocorticoid-induced immunomodulator in response to a variety of inflammatory stimuli (Calandra et al.,

1994). On the other hand, the fact that MIF is strongly expressed in highly-proliferative tissues including basal layer of the skin, the eye lens, and the developing brain (Shimizu et al., 1996; Wistow et al., 1993; Suzuki et al., 1999), and that it plays an important role in cellular proliferation (Nishihira, 2002) have suggested a role of MIF in tissue repair after injury. Concerning this, it still remains controversial whether MIF is promotional or suppressive for wound repair. Zhao et al. reported that mice deficient in MIF gene showed delayed skin wound healing, while Emmerson et al. recently reported the opposite results (Zhao et al., 2005; Emmerson et al., 2009).

Ligament healing undergoes a similar form of wound healing process. Therefore, there is a strong possibility that MIF plays a significant role in ligament healing process. However, no studies have been conducted to clarify the role of MIF in this process. We have hypothesized that deficiency of MIF gene might delay healing of the MCL after injury. The purpose of this study was to test this hypothesis by biomechanically evaluating the difference of ligament healing process between Wild-type mice (WT) and MIF gene-deficient mice (MIFKO) at the early phase of healing. Moreover, biological evaluation was performed to support the biomechanical results.

2. Materials and Methods

2.1. *Animals*

In the present study, we used MIFKO from a mouse strain (bred onto a Balb/c background) deficient in the MIF gene, which was established by Honma et al. (Honma et al., 2000) WT Balb/c mice were purchased from Sankyo Lab (Tokyo, Japan). WT and MIFKO, aged 10 weeks, were used in this study. All experiments were performed according to the protocol approved by the Animal Care and Use Committee of Hokkaido University Graduate School of Medicine.

2.2. Murine model of MCL injury

Thirty-six female MIFKO mice and 36 female WT mice, both at 10 weeks of age, were used. Under general anesthesia with pentobarbiturate (0.5mg/10g body weight, Sigma), the right hind limb was shaved and disinfected. A 10-mm incision was made longitudinally over the medial aspect of the right knee, and a blunt dissection of the subcutaneous tissue was made to expose the MCL. Under microscopic observation using a magnifier (2.5x, Keeler Co., PA, USA), the MCL was carefully dissected from the capsule, and was sharply transected in full-thickness at the level of menisco-tibial joint space with a microsurgical scalpel (AESCULAP, Tuttlingen, Germany). After irrigation with saline, the skin was sutured with 5-0 nylon. No external fixation was

used, and the animals were allowed unrestricted activity as well as diet and water *ad libitum*.

2.3. Biomechanical testing

For biomechanical testing, the mice were sacrificed on day 28 postoperatively (n=6 for MIFKO and WT, respectively). This period was chosen because, in our preliminary study, all the specimens harvested on days 7 and 14 were ruptured during preconditioning in both WT and MIFKO so that we could not perform biomechanical testing. The harvested specimens were wrapped in saline-soaked gauze, and placed in a -80℃ freezer. Following thawing, they were subjected to biomechanical testing as described below. After removal of remaining soft tissues from the samples except for the MCL, the resected ends of both the femur and the tibia were embedded in polymethylmethacrylate in an aluminum pot. Two parallel line markers were drawn on the most proximal and distal parts of the MCL. After we applied a 0.015 N load to the femur-MCL-tibia complex, we measured the width and thickness of the MCL at a midpoint between the two line markers using a digital caliper (IP67 ABS Coolant Proof Caliper, Mitsutoyo Co, Kanagawa, Japan), the accuracy of which device was 0.02mm. The measurements were repeated three times, and the mean value was used as a

representative value. The cross-sectional area was calculated by multiplying the measured width and thickness together, assuming that the cross-section was rectangular.

Biomechanical testing was performed using a specially designed micro-tensile tester (Fig. 1) (Yamamoto et al., 1999). The femur-MCL-tibia complex specimen was fixed in specially-designed clamps. One of the clamps was attached to a load cell (LVS-1KA, Kyowa, Tokyo, Japan) having a capacity of 9.8 N and a resolution of 0.001 N, and the other one was attached to the crosshead of the linear stage (LU09200AKL1-P5Z0, Nihonseiko, Tokyo, Japan). The elongation of the MCL midsubstance between the two line markers was measured using a video dimension analyzer (C3160, Hamamatsu Photonics, Shizuoka, Japan) and a CCD camera (WV-BD400, Panasonic, Osaka, Japan). Prior to tensile testing, each specimen was preconditioned with a static preload of 0.1 N, followed by 10 cycles of cyclic loading (The maximal load was 0.2 N and the minimal load was 0 N) with a crosshead speed of 10 mm/min. Thus, preconditioning was performed under load control. We could convert these load values to the strain and the crosshead speed to the strain rate, after we determined the initial length of the MCL between the two markers in the next phase of this testing, For example, when the maximal load of 0.2 N was applied, the strain of the ligament averaged 1.7 %. The crosshead speed of 10 mm/min was equivalent to the

strain rate of approximately 1 %/sec. After preconditioning, the initial length between the two markers was determined under a 0 N load. Then, the femur-MCL-tibia complex specimen was stretched to failure at a crosshead speed of 10 mm/min, monitoring the length changes between the two markers. The strain was calculated with the above-described initial length. Outcomes measured included maximal load, stiffness, tensile strength, and tangent modulus. Data acquired from injured MCL were normalized by dividing injured side outcome by uninjured side outcome, which was reported as percentage.

2.4. RNA isolation and real time RT-PCR

For ribonucleic acid (RNA) extraction, the mice were sacrificed on days 3, 7, 14, and 28 postoperatively. The injured MCLs were harvested (n=3 at each time point for MIFKO and WT, respectively). The uninjured MCLs were also harvested to obtain normal control data (n=3 for MIFKO and WT, respectively). The harvested MCL were immediately soaked in RNAiso (TakaraBio, Shiga, Japan) and subsequently crushed using a microsmasher (Tomy medico, Tokyo, Japan) set at 4000rpm for 1 min using zirconia beads. Total RNA extraction was carried out using the RNeasy mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA qualities

from each sample were assured by the A260/280 absorbance ratio. One microgram of total RNA was reverse transcribed into single strand cDNA using PrimeScript® RT regent Kit (TakaraBio, Shiga, Japan). The reverse transcription (RT) reaction was carried out for 15 min at 37℃ and then for 5 sec at 85℃. All oligonucleotide primer sets were designed based upon the published messenger RNA (mRNA) sequence. The expected amplicon lengths ranged from 85 to 180 base pair (bp). Oligonucleotide primers used in this study are listed in Table 1. The real time polymerase chain reaction (PCR) was performed in Thermal Cycler Dice® TP800 (TakaraBio, Shiga, Japan) by using SYBR SYBR® Premix Ex Taq^{TM} II (TakaraBio, Shiga, Japan). One microliter of cDNA template was used for real time PCR in a final volume of 25μl. The cDNA template was amplified according to the following condition: 95℃ for 5 s and 60℃ for 30 s at 40 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A dissociation curve analysis was performed (0.5℃/s increase from 60 to 95℃ with continuous fluorescence readings) at the end of cycles to ensure that single PCR products were obtained. The amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. The results were evaluated using the Thermal Cycler Dice® Real Time System software program. Glyceroaldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to

normalize samples.

2.5. Histological analyses

For histological analyses, we sacrificed the mice on days 7, 14, 28, and 56, postoperatively (n=3 at each time point for MIFKO and WT, respectively), and then, harvested the injured and uninjured MCLs from the bilateral knees. The samples were fixed in formalin, decalcified in 10% ethylenediaminetetraacetic acid (EDTA), embedded in paraffin, and were sectioned in a mid-frontal direction. For histological analysis, the sections were stained with hematoxylin/eosin (HE). We evaluated the changes in the thickness of the MCL over time under microscopic observation (AX-80, Olympus, Tokyo). Namely, the most thickened part close to the transected portion of the ligament was identified under x200 magnification, and the thickness was measured using the scaling function of DPC controller (Olympus, Tokyo, Japan). In addition, we evaluated the number of cells and vessels observed in the MCL at each period. Namely, we counted the number of cells within an area of 300×300 μm^2, which was randomly set at the center of the transected portion of the MCL, using image J software (National Institutes of Health, Bethesda, Maryland, USA). We defined the cell number within a unit area of 100x100 μm^2 as the cell density. The number of vessels regenerated in the

whole MCL was counted under x200 magnification, defining a tubular structure with contained blood cells as a vessel.

2.6. Statistical analysis

The data were evaluated for statistical significance by analysis of variance (ANOVA) using Fisher's test as a post hoc test, or by Student's t-test. A p-value of < 0.05 was considered to be statistically significant.

3. Results

3.1. The levels of mechanical properties of the ligament healing tissues in WT and MIFKO

Concerning the thickness measured by digital caliper, MIFKO was significantly greater than WT (WT; 140±43.59 μm, MIFKO; 240±36.06 μm, p=0.038). The cross sectional area was significantly larger in MIFKO than in WT (WT; 0.15±0.05mm^2, MIFKO; 0.26±0.07mm^2, p=0.0077). The mode of failure was mid-substance tear in all cases. Load-deformation curve and stress-strain curve in both groups are shown in Fig. 2A. To assess the difference in mechanical properties of the MCL in both groups, we first compared the material and structural properties of the contralateral control MCL,

which showed no significant differences between WT and MIFKO (Fig. 2B). This endorsed the appropriateness of comparing the mechanical properties of healing MCL of MIFKO with those of WT. Next, we evaluated the differences in mechanical properties of the ligament healing tissues between WT and MIFKO on day 28 postoperatively (Fig. 2C). Concerning structural properties, maximal load was significantly lower in MIFKO than in WT (WT; 4.71±0.94N, MIFKO; 2.81±0.99N, p=0.0065). Stiffness was also significantly lower in MIFKO than in WT (WT; 4.44±0.26N/mm, MIFKO; 2.83±0.74N/mm, p=0.0005). Concerning material properties, tensile strength was significantly lower in MIFKO than in WT (WT; 33.15±11.34MPa, MIFKO; 11.06±4.47MPa, p=0.013). Tangent modulus was also significantly lower in MIFKO than in WT (WT; 339.90±77.19MPa, MIFKO; 116.38±15.92MPa, p<0.0001). Finally, we calculated injured/uninjured ratio of each parameter in WT and MIFKO. Maximal load, stiffness, tensile strength, and tangent modulus were all significantly lower in MIFKO than in WT (Fig. 2D, p=0.0079, p=0.0105, p=0.0027, and p=0.0003, respectively）.

3.2. Levels of mRNA expression for TNF-α, VEGF, MMP-2, MMP-9, and MMP-13 in the ligament healing tissue of WT and MIFKO

We examined mRNA expression of MIF and factors that might participate in the healing process and could be influenced by endogenous MIF, namely, tumor-necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2, MMP-9, and MMP-13 (Fig. 3). In WT after surgery, MIF mRNA significantly increased on day 3 after transection compared with normal ($p=0.0101$). There were no statistical differences in mRNA levels for TNF-α and MMP-9 between WT and MIFKO. VEGF mRNA levels, which decreased once on day 14 and increased again on day 28 in WT, were significantly lower in MIFKO than in WT on day 28 ($p<0.0001$). MMP-2 mRNA levels, which gradually increased until day 14 in both WT and MIFKO, were significantly lower in MIFKO than in WT on day 28 ($p=0.014$). MMP-13 mRNA levels, which peaked on day 7 and then gradually decreased in WT, were significantly lower in MIFKO than in WT on day 7 ($p<0.0001$).

3.3. Histological observation of the healing of the MCL in WT and MIFKO.

In both WT and MIFKO, the uninjured ligament consisted of closely packed collagen bundles oriented longitudinally and fibroblasts with a rod-shaped nucleus were sparsely scattered between the collagen fibers (Fig. 4A a, b). On day 7, the injured portion in the MCL was filled with loosely packed, irregularly oriented fibrous tissues

rich in cells with a small round or spindle-shaped nucleus in both WT and MIFKO (Fig. 4A-c, d). In WT, the thickness and the cell density of the injured portion further increased on day 14 (Fig. 4A-e), while they obviously decreased on days 28 and 56 (Fig. 4A-g, i). Collagen bundles appeared to be closely packed with nearly longitudinal orientation on day 56. In MIFKO, the thickness and the cell density of the injured portion further increased on day 14 (Fig. 4A-f), and they remained increased on days 28 and 56 (Fig. 4A- h, j).

Quantitative evaluation of the thickness and the cell density of the injured portion showed that an apparent peak was on day 7 in WT, while the peak was on day 14 in MIFKO (Fig. 4B and C). In MIFKO, these parameters remained significantly increased even on day 56, while they decreased to the level close to the normal control in WT on days 28 and 56. Concerning the histological thickness, MIFKO was significantly greater than WT on day 28 (WT: 94.95±21.28 μm, MIFKO; 233±4.95 μm, $p<0.0001$). Regarding the cell density, MIFKO was significantly greater than WT on day 14 ($p=0.0014$) and day 28 ($p=0.0084$). The regenerated vessels were scattered around the marginal area of the injured portion of the ligament, which were significantly more in WT than in MIFKO on day 14 (Fig. 4D, $p=0.0073$).

4. Discussion

In this study, we revealed for the first time that MIFKO mice exhibited delayed ligament healing. Biomechanical testing showed that the mechanical properties of the healing tissue were significantly lower in MIFKO on day 28 after surgery, which suggested the important role played by MIF in improving mechanical properties of healing tissues in the repair process after MCL injury. We believed that these biomechanical differences with statistical significance were enough to show the differences in ligament healing process between WT and MIFKO, although we could not perform biomechanical testing on days 7 and 14 because the harvested specimens were too weak to undergo the tensile test, as described in the Method section.

MIF mRNA level was significantly up-regulated on day 3 post-injury, which suggests that MIF plays an important role in the early phase of the healing process after MCL injury. It is already reported that MIF up-regulates MMP-1, -3, -9, and -13 mRNA in several lineage of cells including fibroblasts (Onodera et al., 2000, 2002). The healing of injured MCL was reported to be delayed in MMP-12 gene-deficient mice (Wright et al., 2006). Our results showed significantly suppressed mRNA expression of MMP-13 on day 7, and that of MMP-2 on day 28 in MIFKO compared with WT. The temporal changes in mRNA expression of MMP-2 and -13 in WT after MCL injury are

similar to those after flexor tendon injury in rat (Oshiro et al., 2003), which might support the validity of our results.

MMPs have been known to play important roles in the repair process after tissue damage. Firstly, MMPs are absolutely necessary for angiogenesis. While degrading ECM components to open up an avenue for migrating pericytes and endothelial cells is an essential requirement for MMPs in angiogenesis, MMPs contribute in many other ways to pro-angiogenic process (Rundhaug et al., 2005). In our study, stronger mRNA expression of MMP-13 could be observed in WT than in MIFKO on day 7. Histological evaluation on day 14 showed significantly less vessels within the healing tissues of MIFKO compared with those in WT. These suggested that in WT, enhanced expression of MMP-13 played essential roles in the angiogenesis of injured MCL, which was less obvious in MIFKO due at least in part to the suppressed expression of MMP-13 on day 7. In relation to this, it is noteworthy that there was no difference in the number of vessels on day 28, the only time point when the expression of VEGF mRNA showed statistical difference between WT and MIFKO. Although the underlying mechanism remains unknown how the mRNA expression of VEGF in MIFKO was suppressed on day 28, i.e., a relatively late phase of ligament healing, the suppressed expression did not cause difference in angiogenesis.

Secondly, the role of MMPs in the remodeling of healing tissues of MCL should be considered. Ligament scar maturation requires a large amount of tissue remodeling, also involving a balance of matrix removal and matrix molecule secretion. At the molecular level, specific proteinases, particularly MMPs, and their inhibitors regulate this process (Wright et al., 2006). Our results of histological examination clearly demonstrated that on day 28, healing tissues in WT exhibited almost normal thickness, whereas those in MIFKO still exhibited hypertrophy. This suggested that the deficiency in MIF gene caused the delay in remodeling of healing tissues after MCL injury. Hellio Le Graverand et al. (Hellio Le GraverandP et al., 2000) reported that in the healing process after MCL injury in rabbits, MMP-13 expression peaked prior to the remodeling phase, which might relate to the rates of collagen turnover and remodeling. Also in our results, MMP-13 mRNA expression in WT peaked on day 7. It is possible that the suppressed MMP-13 mRNA expression in MIFKO on day 7 might have caused the delay in the remodeling of healing tissues. Besides, MMP-2 also has been reported to play an important role in the remodeling of healing tissues at the remodeling phase after various tissue damage including laceration wound of flexor tendon (Oshiro et al., 2003) or scar after burn wound of skin (Yang et al., 2005). Our results showed significantly suppressed expression of MMP-2 mRNA in MIFKO than in WT on day 28, which

might also have caused the delay in remodeling of healing tissues in MIFKO.

Histological findings indicated poor vascularity, prolonged swelling, and prolonged increase of the cell density within the healing tissue of MIFKO, specifically on day 28. These findings suggested that the remodeling of the healing tissue was significantly delayed in MIFKO in comparison with WT, leading to the significant deterioration of structural and material properties of the healing tissue harvested on day 28 in MIFKO compared with WT. It has been suggested that in murine model of MCL injury, the ablation of MIF gene caused impairment of angiogenesis that was required for tissue proliferation and maturation, due at least in part to the suppressed expression of MMP-13 on day 7. In addition, suppressed mRNA expression of MMP-13 and MMP-2 might have caused the impairment of tissue remodeling, leading to the deterioration of structural and material properties of the healing tissues on day 28 after MCL injury in MIFKO compared with WT. This study presented for the first time that MIF was one of important molecules that profoundly contributed to the healing of MCL, which was worthy of note when considering the therapeutic approaches to control and regulate the healing process of injured ligament.

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Figure Legends

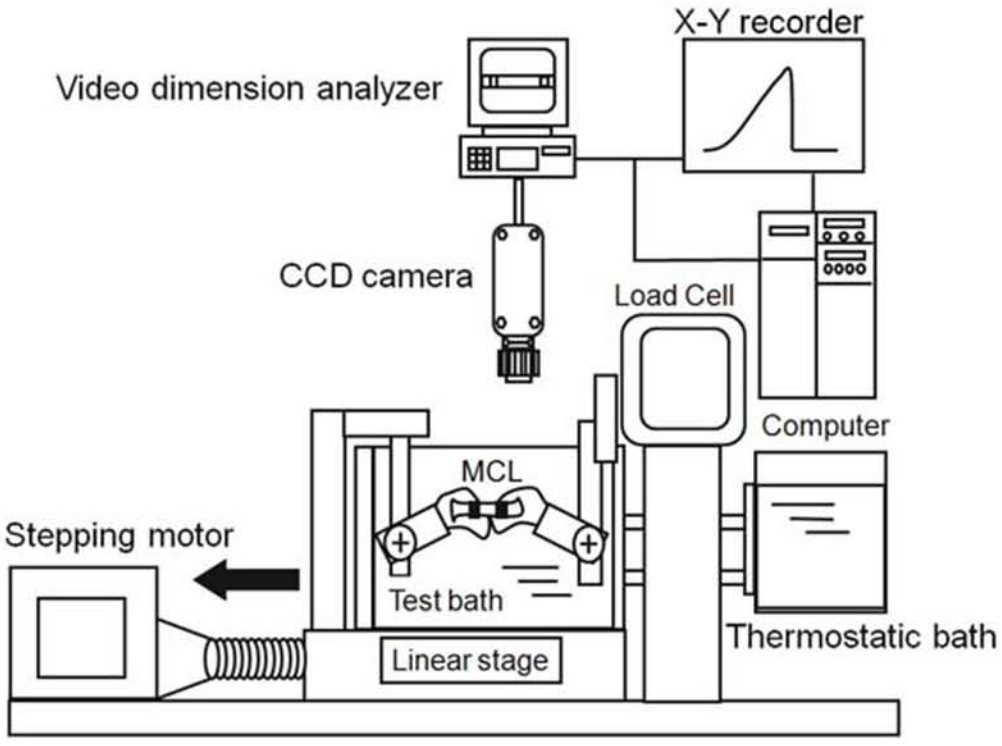
Fig. 1. A micro-tensile tester used to determine the mechanical properties of the MCL.

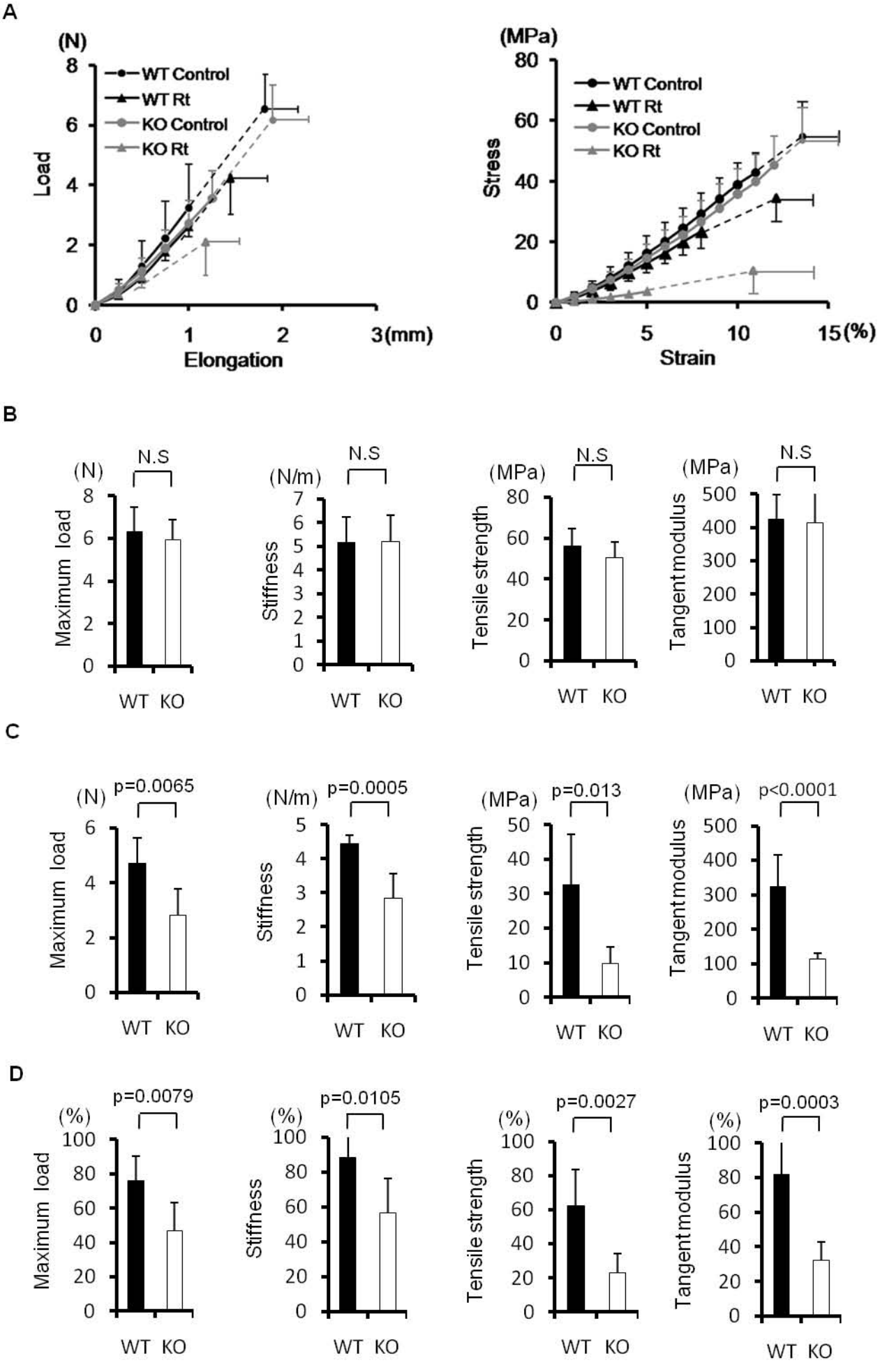
Fig. 2. Biomechanical properties of the injured MCL of WT and MIFKO collected on day 28 after surgery. (A) Load-elongation curve (left) and stress-strain curve (right) of injured right MCL and uninjured left MCL of both WT and MIFKO are shown. Rt: Right. (B) The actual values of maximal load, stiffness, tensile strength, and tangent modulus of uninjured left MCL are shown. (C) The actual values of maximal load, stiffness, tensile strength, and tangent modulus of injured right MCL are shown. (D) The relative ratios of maximal load, stiffness, tensile strength, and tangent modulus of the injured right MCL against those of uninjured left MCL are shown. Data are expressed as means±SD. n=6 per each bar.

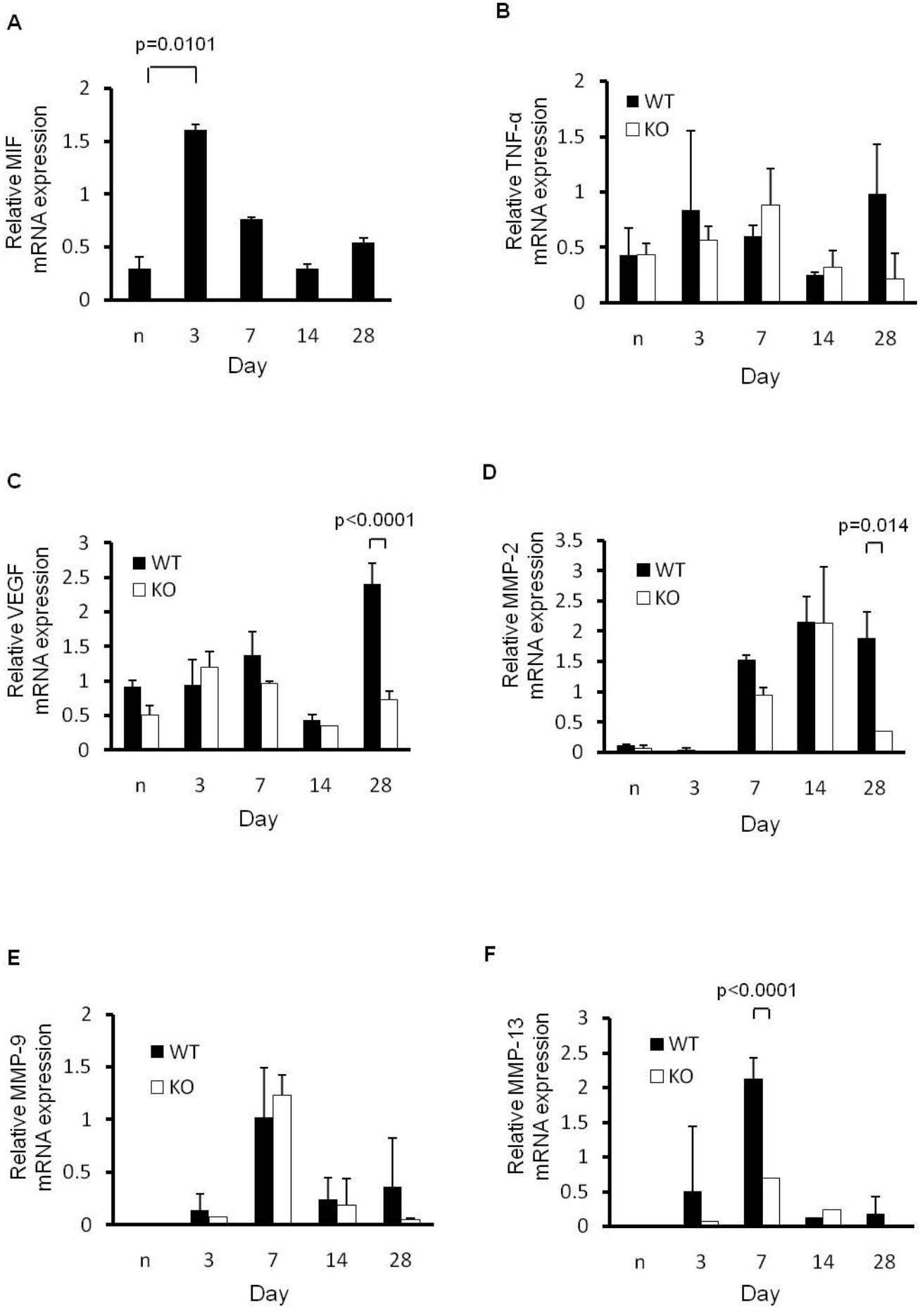
Fig. 3. Real-time PCR analysis of mRNA for (A) MIF, (B) TNF-α, (C) VEGF, (D) MMP-2, (E) MMP-9, and (F) MMP-13 in the normal (n) and healing tissues of MCL in WT and MIFKO retrieved on days 3, 7, 14, and 28 postoperatively. Data are normalized by respective GAPDH mRNA levels, and are expressed as means±SD. n=3 per each bar.

Fig. 4. (A) Histological analysis of the injured MCL of WT (left) and MIFKO (right). The normal and injured MCL of WT (a, c, e, g, i) and MIFKO (b, d, f, h, j). The normal MCL (a, b) and injured MCL harvested on days 7 (c, d), 14 (e, f), 28 (g, h), and 56 (i, j) postoperatively, were stained with hematoxylin and eosin. The observation under x40 magnification is shown left (scale bar : 500μm), while that under x200 magnification is shown right (scale bar : 100μm). (B) The thickness of normal and transected MCL on days 7, 14, and 28, and 56, postoperatively. (C) The cell density of normal and transected MCL on days 7, 14, and 28, and 56, postoperatively. (D) The number of vessels within the normal and transected MCL on days 7, 14, and 28, and 56, postoperatively. Data are expressed as means±SD.

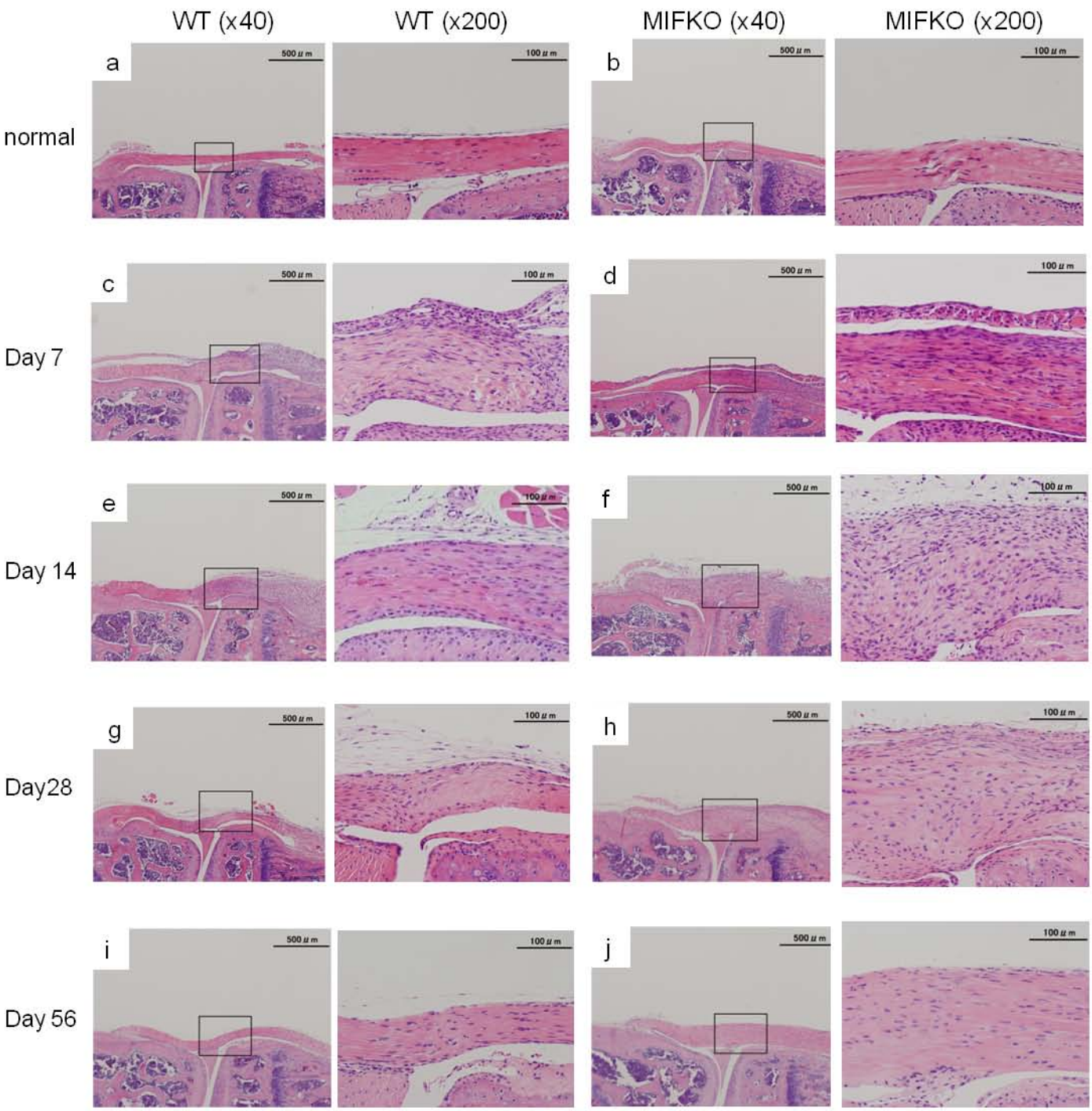
Fig. 1 Kitayama et al.



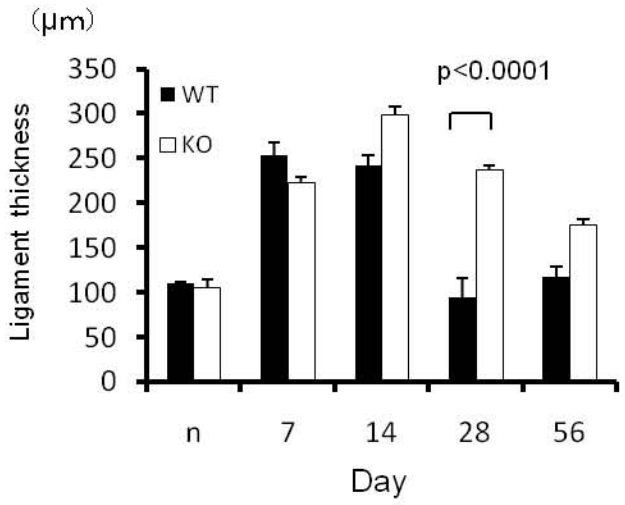

A
(N)
WT Control
WT Rt
KO Control
KO Rt
Load
Elongation
3(mm)
(MPa)
Stress
Strain
15(%)
B
N.S
Maximum load
Stiffness
Tensile strength
Tangent modulus
WT KO
C
p=0.0065
p=0.0005
p=0.013
p<0.0001
D
p=0.0079
p=0.0105
p=0.0027
p=0.0003
(%)


A
p=0.0101
Relative MIF mRNA expression
2
1.5
1
0.5
0
n
3
7
14
28
Day
B
WT
KO
Relative TNF-α mRNA expression
2
1.5
1
0.5
0
n
3
7
14
28
Day
C
p<0.0001
WT
KO
Relative VEGF mRNA expression
3
2.5
2
1.5
1
0.5
0
n
3
7
14
28
Day
D
p=0.014
WT
KO
Relative MMP-2 mRNA expression
3.5
3
2.5
2
1.5
1
0.5
0
n
3
7
14
28
Day
E
WT
KO
Relative MMP-9 mRNA expression
2
1.5
1
0.5
0
n
3
7
14
28
Day
F
p<0.0001
WT
KO
Relative MMP-13 mRNA expression
3
2.5
2
1.5
1
0.5
0
n
3
7
14
28
Day

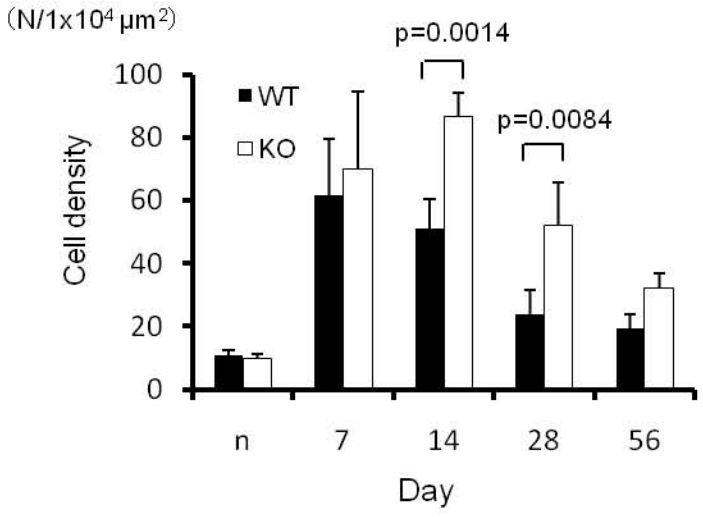
A


WT (x40)
WT (x200)
MIFKO (x40)
MIFKO (x200)
normal
Day 7
Day 14
Day28
Day 56
a
b
c
d
e
f
g
h
i
j
500 μm
100 μm

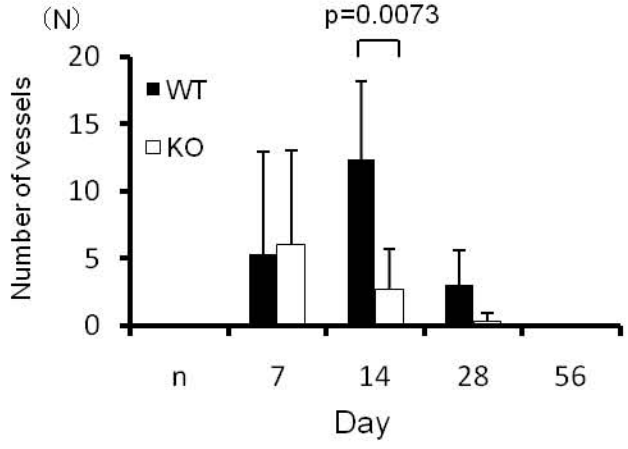
B


(μm)
Ligament thickness
350
300
250
200
150
100
50
0
WT
KO
p<0.0001
n
7
14
28
56
Day

C


(N/1x10⁴ μm²)
Cell density
100
80
60
40
20
0
WT
KO
p=0.0014
p=0.0084
n
7
14
28
56
Day

D


(N)
Number of vessels
20
15
10
5
0
WT
KO
p=0.0073
n
7
14
28
56
Day