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The Role of Osteopontin in Tendon Tissue Remodeling after Denervation-Induced Mechanical Stress Deprivation

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Abstract

It has been shown that musculoskeletal tissues undergo dynamic tissue remodeling by a process that is quite sensitive to the mechanical environment. However, the detailed molecular mechanism underlying this process remains unclear. We demonstrate here that after denervation-induced mechanical stress deprivation, tendons undergo dynamic tissue remodeling as evidenced by a significant reduction of the collagen fibril diameter. Importantly, the transient up-regulation of osteopontin (OPN) expression was characteristic during the early phase of tendon tissue remodeling. Following this dynamic change of OPN expression, matrix metalloproteinase (MMP)-13 expression was induced, which presumably accounts for the morphological changes of tendon by degrading tendon collagen fibrils. The modulation of MMP-13 expression by OPN was specific, since the expression of MMP-2, which is also known to be involved in tissue remodeling, did not alter in the tendons under the absence nor presence of OPN. We also demonstrate that the modulation of MMP-13 expression by OPN is due to the signaling through cell surface receptors for OPN. Thus, we conclude that OPN plays a crucial role in conveying the effect of denervation-induced mechanical stress deprivation to the tendon fibroblasts to degrade the extracellular matrices by regulating MMP-13 expression in tendon fibroblasts.

1. Introduction

Bone and muscle provide mechanical support for movement under gravitational and mechanical stress. The structure, organization, and remodeling of bone and muscle are sensitive to the mechanical environment as evidenced by the bone loss and muscle atrophy in bedridden patients as well as the increase in bone and muscle mass in athletes participating in high impact sports. Tendon tissues connect muscle to bone and allow transmission of forces generated by muscle to bone and provide motion and mechanical support to the joints. As in bone and muscle, mechanical loading improves the tensile strength, stiffness, weight and cross-section area of tendons, and these effects can be explained by an increase in collagen and extracellular matrix synthesis by tendon cells (Kannus et al., 1992, 1997; Majima et al., 1996, 2003). However, the molecular mechanism coupling mechanical stress to tendon tissue remodeling remains unclear.

Osteopontin (OPN), also called early T lymphocyte activation-1 (Eta-1) or secreted phosphoprotein 1 (Spp1), was originally identified as a noncollagenous matrix protein in bone (Franzen and Heinegard, 1985; Singh et al., 1990). This cytokine and mineral matrix protein plays an important role in a number of physiological and pathological events,

including tissue repair, regulation of bone metabolism, and inflammation and immunity (Denhardt et al., 2001b; Diao et al., 2004; Liaw et al., 1995; O'Regan and Berman, 2000; Sodek et al., 2000; Uede et al., 1997). Recent studies have shown that OPN is a crucial factor triggering bone remodeling (Asou et al., 2001; Denhardt et al., 2001a; Ishijima et al., 2001; Meazzini et al., 1998; Rittling et al., 1998; Terai et al., 1999). In addition, disorganization of collagen and decreases in collagen type I content were observed in mice lacking OPN in the model of skin incision/wound healing (Liaw et al., 1998). Furthermore, OPN was highly up-regulated during the muscle regeneration process induced by injection of the snake venom, cardiotoxin (Hirata et al., 2003).

Musculoskeletal tissue cells, including osteoblasts, chondrocytes, myoblasts and skin and tendon fibroblasts, arise from differentiated mesenchymal stem cells (Salingcarnboriboon et al., 2003; Sharma and Maffulli, 2005). Thus, the musculoskeletal tissues are categorized as the same functional unit developed from the mesenchymal stem cells. This theoretical background and the data mentioned above led us to hypothesize that OPN could regulate tendon tissue remodeling in response to mechanical stress. To test this hypothesis, a denervation-induced mechanical stress deprivation model of the patellar tendon was used to evaluate the role of OPN during tendon tissue remodeling. The specific objectives of this

study were 1) to detect OPN protein expression in normal tendon, 2) to examine the kinetics of OPN mRNA expression in the patellar tendon after mechanical stress deprivation, 3) to analyze the fine structure of patellar tendon after mechanical stress deprivation either in the presence or absence of OPN, and 4) to clarify a mechanism underlying OPN-induced tendon remodeling. The results shown in this paper provide the essential information on the role of OPN in musculoskeletal soft tissue remodeling responding to mechanical stress. Furthermore, this knowledge can be applied to the development of novel tactics for the treatment of various musculoskeletal soft tissue diseases involving abnormal tissue remodeling.

2. Results

2.1. Detection of OPN Expression in Tendon Fibroblasts

In reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, OPN mRNA expression was clearly detected in normal patellar tendon of wild-type (WT) mice, which had been kept under a physiological gravitational environment (Fig. 1A).

Immunohistochemical analysis demonstrated that OPN protein expression was detected in tendon fibroblasts, which existed in the interstitial space of the tendons of WT mice (Fig. 1B-D). OPN protein expression was not detected in the patellar tendon of osteopontin-deficient (OPN^{-/-}) mice (Fig. 1E). We further examined whether cultured tendon fibroblasts could secrete OPN *in vitro*. The cultured tendon fibroblasts from the normal patellar tendon secreted significant amounts of OPN protein (608.7 ± 86.5 ng/ml) into the culture medium. As expected, no OPN protein was found in culture supernatants of the tendon fibroblasts from mice lacking OPN.

2.2. Denervation-Induced Stress Deprivation of Patellar Tendon

Physical tension of tendon is controlled by contraction of quadriceps femoris muscle, which is innervated by femoral nerve. Thus, it is expected that the contraction of quadriceps femoris muscle can be abolished by transection of the femoral nerve, which leads to mechanical stress deprivation of patellar tendon. Surgery was performed under general anesthesia with pentobarbital sodium via intraperitoneal injection at 25 mg/kg body weight. A longitudinal skin incision was made over the proximal part of right medial

thigh. To eliminate the effect of femoral nerve regeneration and inflammation on patellar tendon, a 1.0-cm segment of right femoral nerve was microscopically excised at the inguinal region (Fig. 2A,B). Before the excision of femoral nerve, electromyography showed that the stimuli of right femoral nerve elicited electric activity in right quadriceps femoris muscle. We confirmed that the stimuli of femoral nerve did not elicit the electric activity in quadriceps femoris muscle after denervation. We also examined whether the transection of femoral nerve induces inflammatory reactions in tendon. Histologically, there was no inflammatory cell infiltration in tendon at day 7 (Fig. 2D). This was confirmed by absence of F4/80-positive macrophages in tendon tissues at days 1, 3, 7 and 14 after mechanical stress deprivation (Fig. 2D,E). At day 42 after stress deprivation, we showed that atrophy of right quadriceps femoris muscle was induced (Fig. 2F) as described previously (Zhang et al., 2006). Thus, patellar tendon load is selectively deprived by denervation-induced muscle atrophy. Using this model, we evaluated the role of OPN during the course of tendon tissue remodeling after stress deprivation.

2.3 Lack of OPN Prevents Stress Deprivation-Induced Decrease in Collagen Fibril Diameter of Patellar Tendon

The morphological analysis of tendons by transmission electron microscopy (TEM) demonstrated that the diameter of the collagen fibrils significantly decreased in WT mice at 42 days after the denervation-induced stress deprivation of the tendon, as compared to those of the control contralateral tendon of the same mouse (Fig. 3A). In contrast, in mice lacking OPN, the stress deprivation-induced decrease in diameter of tendon collagen fibril was not detected. At day 42, the diameter of the tendon collagen fibrils was very similar between the stress deprived right tendon and the left tendon that was still responsive to mechanical stress (Fig. 3A).

To quantify the morphological change of tendon collagen fibrils after stress deprivation, a detailed analysis was performed. Although the distribution of collagen fibril diameters at 42 days after stress deprivation was shifted to smaller diameters in WT mice, no such alteration was found in mice lacking OPN (Fig. 3B). The apparent left shift in the collagen fibril size suggests the loss of large fibers and a gain of small fibers with stress deprivation in WT mice.

In this regard, it should be noted that number of fibrils was not significantly different between stress deprived and control loaded tendons in WT mice (Fig. 4A), indicating that

appearance of small diameter collagen fibrils newly synthesized may not account for the apparent left shift in the collagen fiber size. In addition, mean collagen fibril diameter and fibril occupation ratio per given area were significantly decreased in stress deprived tendons at day 42, as compared to those of control loaded tendons (111.6 ± 5.2 nm versus 74.4 ± 4.6 nm (Fig. 4B) and 61.2 ± 2.3 % versus 42.6 ± 2.7 % (Fig. 4C), respectively). These results indicated that pre-existed collagen fibers were forced to reduce diameter of collagen fibrils in WT mice. It is also possible that control contralateral tendon is subjected to mechanical overloading, which leads to increase of collagen fibril diameter. This overloading may explain the difference in collagen fibril diameter between stress deprived and control loaded tendons. Therefore, we examined whether diameter of collagen fibrils in control tendons differ between before and after stress deprivation. We found that collagen fibril diameter did not differ significantly between day 0 and day 42 tendons (Fig. 4D). Nevertheless, in mice lacking OPN, there were no significant differences in collagen fibril diameter and fibril occupation ratio between stress deprived tendon and control tendon at 42 days (Fig. 4B,C). In order to further clarify the time point when fibril diameter change occurs, we additionally obtained tendons of WT mice at 10 and 20 days post-operation. We found that there was significant differences in collagen

diameter at 20 days, but not 10 days after operation (Fig. 4B). These data demonstrate that OPN is involved in the process of tendon remodeling after stress deprivation. In order to clarify the mechanism how OPN regulates the process of tendon remodeling, we examined the kinetics of OPN mRNA expression in tendon after unloading.

2.4. The Kinetics of OPN Gene Expression in Tendon after Denervation-Induced Stress Deprivation

At days 0, 1, 3, 5, 7, 14, and 42 after stress deprivation, the expression of OPN mRNA in patellar tendon was examined. By quantitative real-time PCR analysis, we found that normal tendon expressed OPN mRNA. Importantly, OPN expression was up-regulated at day 1 and day 3 ($n = 3, P < 0.001$) after stress deprivation and then declined significantly at day 5 ($n = 3, P < 0.001$). The level of OPN expression at day 5 was not up-regulated, but rather lower than that of normal tendon ($n = 3, P < 0.001$), and it remained suppressed even up to day 42 after stress deprivation. In control contralateral tendon, such dynamic changes in OPN expression were not detected (Fig. 5A). Consistent with these data, we demonstrated that OPN protein expression was detected in normal tendon and tendon

tissues at day 1 and 3 after stress deprivation. However, at day 5 OPN protein expression was not detected (Fig. 5B).

2.5. Lack of Significant Contribution of Collagen Synthesis and Apoptosis for the Decrease of Collagen Fibril Diameter of Tendon after Mechanical Stress Deprivation

OPN might modulate the process of tendon remodeling in various ways. It has been shown that OPN regulates collagen synthesis and accumulation during myocardial remodeling (Trueblood et al., 2001). We therefore tested whether a decrease in collagen synthesis could explain the decrease in collagen fibril diameter in tendon of WT mice after stress deprivation. The real-time PCR analysis demonstrated that collagen mRNA synthesis decreased at days 3 and 14 in both stress deprived tendon and control contralateral loaded tendons, as compared to that in tendon at day 0 (without surgery). However, importantly, the values do not differ significantly between stress deprived tendon and contralateral loaded tendon (Fig. 6A), indicating that the decrease of collagen mRNA synthesis may not account for the decrease in collagen fibril diameter of tendon after stress

deprivation.

OPN is also involved in the regulation of cell apoptosis(Khan et al., 2002; Weintraub et al., 2000; Zohar et al., 2004). As shown in Fig. 5A, a dynamic change of OPN expression was observed during the early phase of tissue remodeling in stress deprived tendon. Therefore, we examined for the presence of TUNEL-positive cells at days 0, 3, and 7 after stress deprivation. As shown in Fig. 6B, apoptotic cells were not detected in WT mice. Results suggested that an increase in apoptosis of tendon fibroblasts does not significantly contribute to the decrease in collagen fibril diameter in stress deprived tendon.

2.6. The Dynamic Regulation of MMP-13 Expression during the Course of Tendon Remodeling after Stress Deprivation

MMPs play a critical role during the course of tissue remodeling. Among the major factors in the remodeling process, collagenases are considered to predominantly degrade the native interstitial collagens in tendon tissue (Chung et al., 2004; Cunningham et al., 1999; Jain et al., 2002). In addition, it has been shown that OPN negatively and positively regulates MMPs expression (D'Alonzo et al., 2002; Philip et al., 2001; Rangaswami et al.,

2004). Therefore, we analyzed the expression level of the genes encoding collagenase-3 (MMP-13) and as a control, MMP-2 in WT mice. MMP-13 mRNA expression was very low up to day 3 after stress deprivation, and then dramatically increased up to day 14 (20 fold increase at day 14). At day 21, the degree of MMP-13 expression returned to the basal level (Fig. 7A). It should be noted that augmentation of MMP-13 expression was not seen in control contralateral loaded tendons. This dynamic change in gene expression was not found for MMP-2 (Fig. 7A). It should be noted that prior to the augmentation of MMP-13 expression, OPN expression was transiently up-regulated at day 3 and sharply declined thereafter (Fig. 5A). Therefore, we investigated whether OPN was involved in MMP-13 gene expression during tendon remodeling after stress deprivation, by comparing the expression level of MMP-13 in tendons of WT and OPN^{-/-} mice. First, we performed RT-PCR analysis to detect MMP-2 and MMP-13 expression in stress deprived tendons of WT mice and OPN^{-/-} mice. MMP-2 expression at stress deprivation day 14 was not significantly augmented in both WT and OPN^{-/-} mice, compared to those at control day 0. In contrast, MMP-13 expression was augmented at day 14 as expected in WT mice, while this augmentation was not detected in OPN^{-/-} mice (Fig. 7B). To quantitate the level of MMP-13 expression, we carried out real-time PCR analysis. We found that MMP-13 gene

expression was increased 20.7-fold at day 14 after stress deprivation in WT mice but only 4.1-fold in OPN^{-/-} mice (Fig. 7C).

The interpretation of data shown in Figs. 5A and 7A is ambivalent. Naturally, the high concentration of OPN may up-regulate MMP-13 expression. However, one can argue that the sharp decline of OPN concentration is required for the up-regulation of MMP-13 expression. In this scenario, exogenous OPN may down-regulate MMP-13 expression. In order to analyze how OPN regulate MMP expression, we set up an *in vitro* system using cultured tendon fibroblasts. Cultured tendon fibroblasts secreted OPN as described above and expressed a low level of MMP-13 (Fig. 8A). We assumed that secreted OPN bound to its cell surface receptor on fibroblasts. Importantly, MMP-13 expression was significantly augmented by the addition of synthetic peptides, GRGDS, which interfere with the binding of OPN to its receptor, but not GRGES (Fig. 8A). Furthermore, an anti- α_v integrin antibody that interferes with the binding of OPN to its receptor $\alpha_v\beta_3$ could also augment MMP-13 expression (Fig. 8A). We also inhibit the interaction of OPN and its integrin receptor by anti-OPN antibody (M5). M5, but not control M3 antibody augmented MMP-13 expression (Fig. 8A). To maximize the OPN-mediated signaling, we added exogenous OPN to the culture and found that the MMP-13 expression by tendon fibroblasts

was down-regulated by exogenous OPN (Fig. 8A). Cultured tendon fibroblasts of OPN-deficient mice also expressed very low level of MMP-13 (Fig. 8B). Since endogenous OPN-mediated signaling is missing in those cells, we added exogenous OPN to the culture and found that MMP-13 expression was further down-regulated (Fig. 8B). Importantly, the interference of interaction with exogenous OPN and its receptor on fibroblasts of OPN-deficient mice by M5 antibody resulted in significant up-regulation of MMP-13 expression, but not by control M3 antibody (Fig. 8B). Taken together, OPN negatively regulated MMP-13 expression through its integrin receptor.

3. Discussion

This study demonstrated that, after denervation-induced stress deprivation, tendons underwent dynamic tissue remodeling as evidenced by the significant reduction of the constituent collagen fibril diameter. However, in the absence of OPN, such morphological alterations were not evident. Our results indicate that OPN plays a crucial role in regulating tendon remodeling induced by stress deprivation. It should be noted that augmentation of OPN mRNA expression lasted only for 3 days, indicating that OPN is

involved in the initial early molecular events during the course of tendon remodeling. Since tendon is categorized as the same functional compartment, including bones and muscles, and shares the common stem cells with these tissues, our findings are consistent with the previous reports, where OPN gene expression was detected in early phases of bone remodeling and muscle regeneration (Denhardt et al., 2001a; Hirata et al., 2003; Nomura and Takano-Yamamoto, 2000; Terai et al., 1999). The tissue remodeling involves both degradation of extracellular matrix proteins and synthesis of new matrix components. In addition, denervation-induced stress deprivation results in the reduction of mechanical strength of stress deprived patellar tendon and the reduction of elastic modulus is associated with the up-regulation of interstitial collagenases (MMP-1, -8 and -13) in tendon (Arnoczky et al., 2004; Lavagnino et al., 2005; Majima et al., 2000). Importantly, these collagenases (MMP-1, -8 and -13) are capable of unwinding and degrading type I collagen, the major component (> 90%) of tendon tissue (Chung et al., 2004; Cunningham et al., 1999; Jain et al., 2002). MMP-8 is mainly produced by neutrophils under inflammatory condition (Balbin et al., 1998; Hasty et al., 1990). We showed that our model did not involve neutrophil infiltration into tendons. It has been shown that rodents lack MMP-1 (collagenase-1) gene (Balbin et al., 2001; Henriot et al., 1992; Vincenti et al., 1998).

Recently OPN has been linked to activation of MMP-2, MMP-3 and MMP-9 (Agnihotri et al., 2001; Fisher et al., 2004; Ogbureke and Fisher, 2004; Philip et al., 2001; Rangaswami et al., 2004), of which only MMP-2 and MMP-9 degrade type IV collagen, but not type I collagen. Therefore, we focused on MMP-13 and tested whether OPN could regulate MMP-13 (collagenase-3) in stress deprived tendon. We found that the mice lacking OPN did not augment MMP-13 expression during the course of tendon remodeling. However, importantly, we demonstrated that stress deprivation induced a transient, but significant up-regulation of OPN expression that preceded the subsequent up-regulation of MMP-13 gene expression in tendon of WT mice. It should be pointed out that collagen diameter thinning in tendon did not occur prior to the elevation of MMP-13 expression. Thus, it is reasonable to speculate that high OPN expression stimulates or is required for the subsequent up-regulation of MMP-13 expression and tendon remodeling. It is important to note that decreased collagen fibril diameter could be at least partially explained by altered collagen assembly in the presence of higher OPN levels in areas of new matrix synthesis in the tendons as reported previously (Liaw et al., 1998). The defected collagen assembly may lead to secretion of excess non-fibrillar (soluble) collagen into the matrix, which then interact with cell surface receptors and cause the up-regulation of MMP-13 as

it has been shown in chondrocytes (Ronziere et al., 2005).

However, our results argued against above hypothesis. We demonstrated that cultured tendon fibroblasts of WT mice secreted OPN and expressed low levels of MMP-13. It is possible that secreted OPN binds to its receptor expressed by fibroblasts, thereby negatively regulating the expression of MMP gene in autocrine and paracrine fashion. In this regard, it should be pointed out that addition of exogenous OPN down-regulated the MMP-13 expression in cultured tendon fibroblasts of WT mice. Similarly, the addition of OPN to cultured tendon fibroblast of OPN-deficient mice resulted in the down-regulation of MMP-13 expression. Thus, we favored the scenario in which sharp reduction of OPN expression at day 5 after stress deprivation played a critical role in subsequent induction of MMP-13 expression. We reasoned that the reduction of OPN expression in tendon tissue of WT mice made it difficult to maintain the interaction between tendon fibroblasts and OPN in situ, thus inevitably resulting in the abrogation of interaction between OPN and its receptor on fibroblasts. To mimic this environment, we added synthetic peptide GRGDS, which interferes with the interaction of OPN with its receptor, to the cultured fibroblasts of WT mice and found that MMP-13 expression was significantly up-regulated, however, control peptide GRGES was without effect. In accordance with our results, it was

previously reported that IL-1 stimulated MMP-2 expression in cultured cardiac fibroblasts and this stimulation was significantly reduced by OPN. Importantly, the OPN effect was abrogated by an integrin antagonist, indicating that OPN's interaction with its receptor inhibited IL-1-stimulated MMP expression (Xie et al., 2003). We further demonstrated that the anti- α v integrin antibody, which also interferes with the binding of OPN to its α v β 3 integrin receptor, also significantly augmented MMP-13 expression when added to the cultured tendon fibroblasts of WT mice. One may argue that anti-integrin antibodies and antagonistic peptides may activate integrins (Carron et al., 2000; D'Alonzo et al., 2002; Humphries et al., 2005). Therefore, we attempted to inhibit the interaction of OPN and its receptor by using anti-OPN antibodies. We found that anti-OPN antibody (M5), which interferes with the binding to integrin, but not the control antibody (M3), up-regulate MMP-13 expression. It should be reminded that OPN can be specifically recognized by numbers of cell surface receptors including CD44, RGD-recognizing integrins such as α 5 β 1 and α v β 3 and non-RGD recognizing integrins such as α 4 β 1 and α 9 β 1 (Weber et al., 1996; Uede et al., 1997; Katagiri et al., 1999; Yamamoto et al., 2003; Diao et al., 2004). Other MMPs might be involved in this remodeling process after stress deprivation. The lack of OPN interaction with its receptor, due to the decreased OPN protein expression by

anti-sense OPN, led to the increased MMP-2 expression in mammary epithelial cells (Ronziere et al., 2005). However, we found that MMP-2 expression did not differ in the absence or presence of OPN during the course of tendon tissue remodeling.

One critical issue that should be discussed here is the rationale why level of MMP-13 expression in cultured fibroblasts of OPN-deficient mice was not significantly higher as compared to those of wild-type fibroblasts if OPN negatively regulated MMP-13 expression. It is possible that cultured fibroblasts of OPN-deficient mice was stimulated by OPN in calf serum in culture, although we changed culture condition to serum-starved condition prior to MMP-13 assay. In accordance with this hypothesis, MMP-13 expression in cultured fibroblasts of OPN-deficient mice was further down-regulated by addition of exogenous OPN. Nevertheless, the abrogation of this interaction by M5 antibody, significantly up-regulated MMP-13 expression in cultured fibroblasts of OPN-deficient mice. More importantly, *in vivo* MMP-13 expression was up-regulated in tendon of OPN-deficient mice at day 14 after denervation-induced stress deprivation, although this up-regulation was considerably low as compared to those in WT mice. Taken together, these data suggest that the reduction of OPN expression, thus abrogation of pre-existing interaction between OPN and tendon fibroblasts may play a key role during

tendon remodeling by regulating MMP-13 expression.

Osteopontin could also suppress collagen synthesis or induce fibroblast apoptosis, thereby modulating remodeling process. We demonstrated that collagen synthesis as defined by type I collagen gene expression was significantly reduced in tendon after stress deprivation. However, importantly, the collagen synthesis did not differ significantly between stress deprived tendon and contralateral loaded tendon, indicating that suppression of collagen synthesis may not account for the decrease in collagen fibril diameter after stress deprivation. We were also unable to detect apoptotic cells during the course of tendon remodeling.

Based on these results, we postulate following scenario for tendon remodeling caused by denervation-induced mechanical stress deprivation. Stress deprivation of the tendon initiates dynamic change of OPN expression in fibroblasts, which induces MMP-13 gene expression, resulting in the degradation of the tendon ECM. Thus in tendon remodeling, OPN may act as a transducer of the mechanical stress to the tendon fibroblasts (Denhardt et al., 2001a). It is therefore possible to control the remodeling process of musculoskeletal soft tissues by manipulating OPN local expression or interfering in the interaction of OPN with its receptor.

4. Experimental procedures

4.1. Animals

Osteopontin-deficient ($OPN^{-/-}$) mice were generated according to the previous study (Rittling et al., 1998). Six-week-old male $OPN^{-/-}$ mice backcrossed 9 times to C57BL/6 mice and age-matched B6 male WT mice were used in the experiments. The animal experiments were carried out in the Institute of Animal Experimentation, Hokkaido University School of Medicine, under the Rules and Regulations of the Animal Care and Use Committee, Hokkaido University School of Medicine.

4.2. Real-time Quantitative RT-PCR Analysis of *OPN*, *MMP*, and *Type I Collagen Transcript Expression*

To examine the change of *OPN* mRNA expression during the course of tissue remodeling, the patellar tendons from the stress deprived right knee in WT mice were removed at day 0, 1, 3, 5, 7, 14, and 42 after surgery. Similarly, the patellar tendons from the control left

knee in WT mice were removed at day 0, 3, 7, 14, and 21. To clarify the alteration of MMP-2, -13, and type I collagen mRNA, the patellar tendons from bilateral knees in WT mice were removed at day 0, 3, 7, 14, and 21 after surgery. Total RNAs were prepared from patellar tendons, and first-strand cDNA was generated with a First-strand cDNA synthesis kit (Amersham Biosciences, Uppsala, Sweden). Real-time quantitative RT-PCR was performed on LightCycler-FastStart DNA Master SYBR Green 1 Systems (Roche Diagnostics, Basel, Switzerland). The specific primers used in this study were as follows; the sense primer for OPN was 5'-ACGACCATGAGATTGGCAGTG-3' and the anti-sense primer was 5'-TTAGTTGACCTCAGAAGATGA-3'. The sense primer for MMP-2 was 5'-AGATCTTCTTCTTCAAGGACCGGT-3' and the anti-sense primer was 5'-GGCTGGTCAGTGGCTTGGGGTA-3'. The sense primer for MMP-13 was 5'-CATCCATCCCGTGACCTTAT-3' and the anti-sense primer was 5'-GCATGACTCTCACAATGCGA-3'. The sense primer for type I collagen was 5'-TTTGTGGACCTCCGGCTC-3' and the anti-sense primer was 5'-AAGCAGAGCACTCGCCCT-3'. The sense primer for G3PDH was 5'-ACCACAGTCCATGCCATCAC-3' and the anti-sense primer was 5'-TCCACCACCCTGTTGCTGTA-3'.

Values in gene expression level of OPN, MMPs, and type I collagen were normalized to those of G3PDH in each tendon sample. For kinetics of gene expression values at day 0 (before surgery) are expressed as 1. Preliminary study indicated that G3PDH gene expression levels were not altered after denervation.

4.3. Immunohistochemistry

Four-micrometer-sections of formalin-fixed and paraffin-embedded mouse tendon tissues were made and deparaffined in xylene, treated with 0.3% hydrogen peroxidase to block endogenous peroxidase activity. For OPN staining, these sections were then stained with anti-mouse OPN Rabbit IgG (O-17; IBL, Gunma, Japan), followed by a DAKO EnVision+ system (DAKO, Carpinteria, CA) as specified by the manufacturers. For macrophage staining, the sections were stained with anti-macrophage rat monoclonal, F4/80 antibody (Serotec Ltd, Oxford, UK) and then biotinylated anti-rat IgG (H+L) (Vector Laboratories, Inc., Burlingame, CA). After washing, the sections were treated with ABC complex (Vector Laboratories, Inc.). Positive staining was visualized by the peroxidase-diaminobenzidine (DAKO, Carpinteria, CA) reaction and the sections were

counterstained with Meyer`s hematoxylin.

4.4. Terminal Deoxynucleotidyltransferase-Mediated dUTP End Labeling (TUNEL)

TUNEL assay was conducted by using an *In situ* Apoptosis Detection Kit according to the manufacturer`s instruction (Takara Bio Inc., Otsu, Japan). Briefly, the sections were incubated with 15 µg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H₂O₂ for 5 min at room temperature and then washed with PBS. Multiple fragmented 3`-OH ends in the sections were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyltransferase (TdT) in a humid atmosphere at 37°C for 90 min, and then washed with PBS. Peroxidase-conjugated anti-digoxigenin antibody was then reacted with the sections at room temperature for 30 min. Apoptotic nuclei were visualized using the peroxidase-DAB reaction. The sections were then counterstained with methyl green. TUNEL-positive cells in the sections were counted under a light microscope (× 200).

4.5. OPN Protein Secretion

To determine the secretion of OPN protein from tendon fibroblasts, OPN protein levels in culture supernatant were measured with an OPN ELISA kit (Kon et al., 2000) according to the manufacturer's instructions (IBL, Gumma, Japan). Briefly, patellar tendons of WT mice and OPN^{-/-} mice were plated in the medium and cultured for 20 days. Culture supernatants of 2nd passage fibroblasts (1×10^4 cells/ml) were collected and subjected to an ELISA analysis. Absorbance at 450 nm was measured with a microplate reader (Bio-Rad, Richmond, CA).

4.6. Quantitative Analyses by Transmission Electron Microscopy (TEM)

WT mice and OPN^{-/-} mice were used for this analysis. At 10, 20 and 42 days after femoral nerve transection, the patellar tendons from both knees in each mouse were removed. A 1 mm-thick specimen sliced perpendicular to longitudinal axis of the patellar tendon axis was cut from each block, and fixed in glutaraldehyde and osmium tetroxide. The specimen was dehydrated and embedded in epoxy resin. Ultrathin sections,

approximately 80 nm, were cut perpendicular to the longitudinal axis of each specimen. The sections were stained with uranyl acetate and lead citrate for transmission electron microscopy (JEM-100CX, Nihon Denshi, Tokyo, Japan). For quantitative analyses, a randomly selected electron micrograph was taken at a final magnification of $\times 30,000$. The diameters of all collagen fibrils in a $4 \mu\text{m}^2$ area chosen from each micrograph were measured using an image analysis software (Win Roof; Mitani Corporation, Tokyo, Japan). A histogram of the diameters of the collagen fibrils for each patellar tendon was obtained from summation of the results of the analysis. For each group, an average histogram was made by calculating the histogram data obtained. The ratio of the total cross sectional area of collagen fibrils to the whole visualized area was defined as the fibril occupation ratio, and this value was calculated for each patellar tendon. The fibril occupation ratio, and the number of collagen fibrils in a $1 \mu\text{m}^2$ were also measured using image analysis software.

4.7. Purification of OPN protein

Chinese hamster ovary (CHO) cells stably transfected with murine OPN cDNA were

established. Culture supernatant of transfectants was applied to a formyl-cellulofine column (Seikagaku Kogyo, Tokyo, Japan) coupled with purified IgG from rabbit immunized with synthetic peptide, KRSFQVSDEQYPDATDE (referred to as M3 antibody). Eluted fraction with 0.2 M glycine-HCl, pH 2.5 was immediately neutralized and dialyzed against PBS and was referred to as mOPN/CHO. OPN concentration was quantified with OPN ELISA kit (IBL, Fujisaki, Japan).

4.8. Cell Culture of Fibroblasts

Fibroblasts were isolated from the patellar tendons of B6 male WT mice and OPN^{-/-} mice at various time points after surgery as described previously (Naginei et al., 1992). The culture medium used in the present study was made from TIL medium (IBL, Gunma, Japan) supplemented with 10% fetal calf serum. The cells (2×10^4 cells/ml) were serum-starved and GRGDS peptide (100 µg/ml, ASAHI TECHNO GLASS Co., Tokyo, Japan), GRGES peptide (100 µg/ml, ASAHI TECHNO GLASS Co., Tokyo, Japan), anti- α integrin antibody (Ab) (RMV7) (50 µg/ml, PharMingen, San Diego, CA), control anti-Rat IgG (50 µg/ml, West Grove, PA), M5 Ab (30 µg/ml), M3 Ab (30 µg/ml), or mOPN/CHO

protein (10 µg/ml) was added and incubated for 48h. Total RNA from cells was extracted and MMP-13 mRNA expression was measured by real-time PCR.

4.9. Anti-OPN Antibodies

The M5 Ab was generated by immunizing rabbit with a synthetic peptide, VDVPNGRGRGDSLAYGLRS, corresponding to the internal sequence of mouse OPN and could inhibit the interaction of OPN with its receptors as described previously (Yamamoto et al., 2003). The M3 Ab does not interfere with the binding of OPN to its receptors.

4.10. Statistical Analysis

All data were represented as mean ± SE. Significant differences between two groups were determined with using an unpaired Student's *t*-test. The significance level was set at 0.05. **P* < 0.05 versus control; ***P* < 0.001 versus control; NS, not significantly different.

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Legends for Figures

Fig. 1. Expression of OPN in normal patellar tendon in wild-type (WT) mice. (A) Total RNA was isolated from the homogenized samples of 21 patellar tendons from WT mice. RT-PCR shows the expression of OPN mRNA in normal loaded patellar tendon. The RT-PCR for G3PDH was used as a control. The number indicates the relative expression level of OPN mRNA against G3PDH. (B) Immunohistochemical staining of the sections obtained from the patellar tendon tissues of WT mice. (C) Magnified view of OPN-positive fibroblasts in the boxed region in B. (D) Hematoxylin-eosin (HE) staining of respective sections in B. (E) Immunohistochemical staining of the sections obtained from patellar tendon tissues of OPN^{-/-} mice. Scale bars indicate 50 μ m.

Fig. 2. Denervation-induced stress deprivation model of the patellar tendon. (A)

Macroscopic anatomy of the proximal part of mouse lower extremity. (B) Right panel, an enlarged view of the region in the circled portion in left panel, shows femoral nerve exposed by small incision over the proximal part of the right medial thigh. The femoral nerve was microscopically transected at the inguinal region. (C) Electromyographic investigation to confirm the patellar tendon of denervation-induced stress deprivation. When femoral nerve is stimulated before denervation (upper panel), electrical activity is recorded in the quadriceps femoris muscle, while that is not detected after denervation (lower panel). FN, femoral nerve; Quad, quadriceps femoris muscle. (D) No inflammatory cell infiltration in stress deprived tendon after denervation. HE staining (left) and F4/80 staining (right) of tendon sections at day 7 after stress deprivation. (E) F4/80 staining of tendon sections at day 1, 3, 14 after stress deprivation. Inflammatory synovial tissue of mice was used as a positive control and arrowheads indicate F4/80-positive cells. Scale bars indicate 50 μm . Data are representative of several independent experiments. (F) Light micrographs of the transverse sectioned quadriceps femoris muscle obtained from WT (left panels) and $\text{OPN}^{-/-}$ mice (right panels) at day 42 after stress deprivation. Sections were stained with hematoxylin-eosin (HE). Scale bars indicate 50 μm . Macroscopic views of right and control left quadriceps muscles in both

WT and OPN^{-/-} mice were also shown.

Fig. 3. Transmission electron micrographs (TEM) of collagen fibril morphology in normal loaded and stress deprived patellar tendon. (A) Upper panel shows the micrographs of stress deprived patellar tendon (left) and contralateral normal patellar tendon (right) in WT mice at day 42 after stress deprivation. Lower panel shows micrographs of stress deprived patellar tendon (left) and contralateral normal patellar tendon (right) in OPN^{-/-} mice at day 42. Scale bars indicate 1 μm . (B) Histogram profiles of the collagen fibril diameter in WT mice ($n = 8$) and OPN^{-/-} mice ($n = 10$) at day 42.

Fig. 4. Quantitative analyses from ultrastructural observations of patellar tendons in WT mice ($n=5, 5,$ and 8 at day 10, 20 and 42, respectively) and OPN^{-/-} mice ($n = 10$) at day 10, 20, or 42 after stress deprivation. (A) Number of fibrils/ μm^2 at day 42. (B) Mean collagen fibril diameter (nm) at day 10, 20, or 42. (C) Fibril occupation ratio (%) at day 42. (D) Left panel is preoperative normal loaded patellar tendon and right panel is control patellar tendon at day 42 after surgery in WT mice. Scale bars indicate 1 μm . * $P < 0.05$ versus day 0; ** $P < 0.001$ versus day 0; NS, not significantly different.

Fig. 5. Changes in the expression level of OPN in tendon of WT mice after stress deprivation. (A) Real-time PCR was conducted at indicated times after stress deprivation of the tendon. Expression of OPN mRNA was normalized by G3PDH. Total RNA was isolated from the homogenized samples of ten stress deprived patellar tendons at day 0, 1, 3, 5, 7, and 42 after surgery and ten control patellar tendons at day 0, 3, 7, and 14 after surgery. $n = 3$, $**P < 0.001$ versus day 0. (B) Immunohistology of OPN expression by patellar tendon fibroblasts at days 0, 1, 3, and 5 after stress deprivation. Arrowheads indicate positive staining. Scale bars indicate 50 μm .

Fig. 6. Lack of significant contribution of collagen mRNA synthesis and apoptosis for the decrease of collagen fibril diameter after stress deprivation. (A) Real-time PCR analysis of type I collagen mRNA expression in WT mice was conducted at day 0, 3, and 14 after mechanical stress deprivation ($n = 4$, NS, not significantly different). Expression of OPN mRNA was normalized by G3PDH. (B) Terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL) assay was conducted at day 0, 3, 7 after stress deprivation. Mammary ground tissue in mice was used as a positive control. Arrowheads indicate TUNEL-positive cells. Scale bars indicate 50 μm .

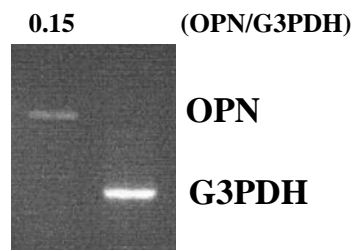
Fig. 7. Change of the expression level of MMP-13 and -2 genes in stress deprived tendons of WT mice and OPN^{-/-} mice. (A) Real-time PCR analyses of MMP-13 (left panel) and -2 (right panel) mRNA expression in stress deprived and control loaded tendons of WT mice at indicated time points ($n = 4$, $*P < 0.05$ versus day 0. NS, not significantly different). (B) RT-PCR analysis of MMP-13 (left panel) and -2 (right panel) expression at day 0 and day 14 after stress deprivation in both WT and OPN^{-/-} mice. The numbers indicate the relative expression level of OPN mRNA against G3PDH. (C) Quantitative real-time PCR analysis of MMP-13 mRNA expression in tendon at day 0 day 14 after stress deprivation ($n = 4$, $*P < 0.05$) in both WT and OPN^{-/-} mice.

Fig. 8. Up-regulation of MMP-13 expression by abrogation of interaction between OPN and its receptors. (A) The tendon fibroblasts of WT mice (2×10^4 cells/ml) were cultured under serum-starved conditions with 100 $\mu\text{g/ml}$ GRGDS, 100 $\mu\text{g/ml}$ GRGES peptide, 50 $\mu\text{g/ml}$ anti- αv integrin antibody, 50 $\mu\text{g/ml}$ control Rat IgG, 30 $\mu\text{g/ml}$ M5 antibody, 30 $\mu\text{g/ml}$ M3 antibody, or 10 $\mu\text{g/ml}$ purified mouse OPN (mOPN/CHO) for 48h. Total RNA was extracted and MMP-13 mRNA expression was measured by real-time PCR ($n = 3$, $*P < 0.05$ versus control; $**P < 0.001$ versus control; NS, not significantly different). (B) The

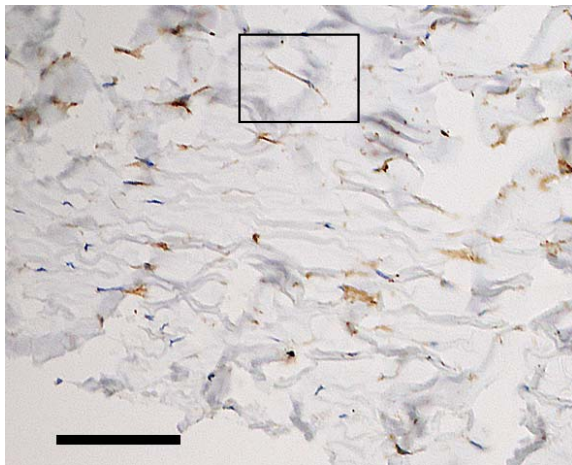
tendon fibroblasts of OPN^{-/-} mice (2×10^4 cells/ml) were cultured under serum-starved conditions in the absence or presence of 10 µg/ml purified mouse OPN (mOPN/CHO) for 48 h. In some experiments, 30 µg/ml M5 or 30 µg/ml M3 antibody was included in the culture. Total RNA was extracted and MMP-13 mRNA expression was measured by real-time PCR ($n = 3$, $**P < 0.001$ versus control).

Figure 1

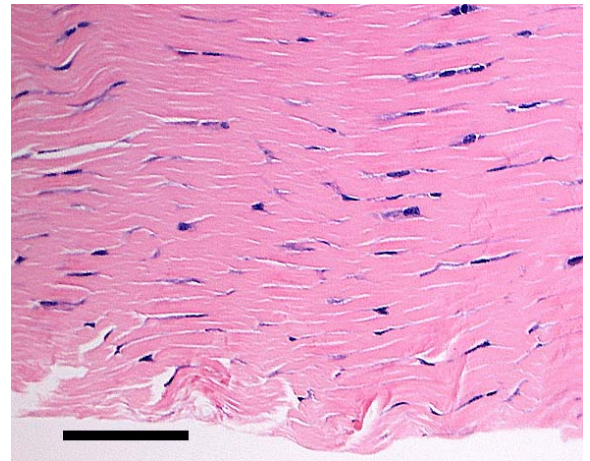
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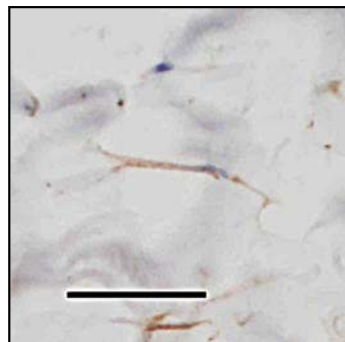
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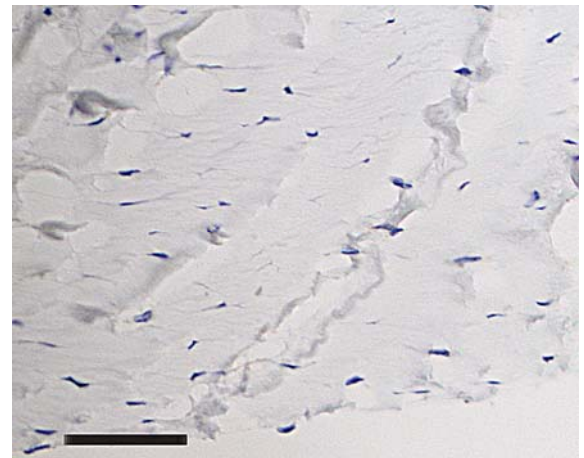


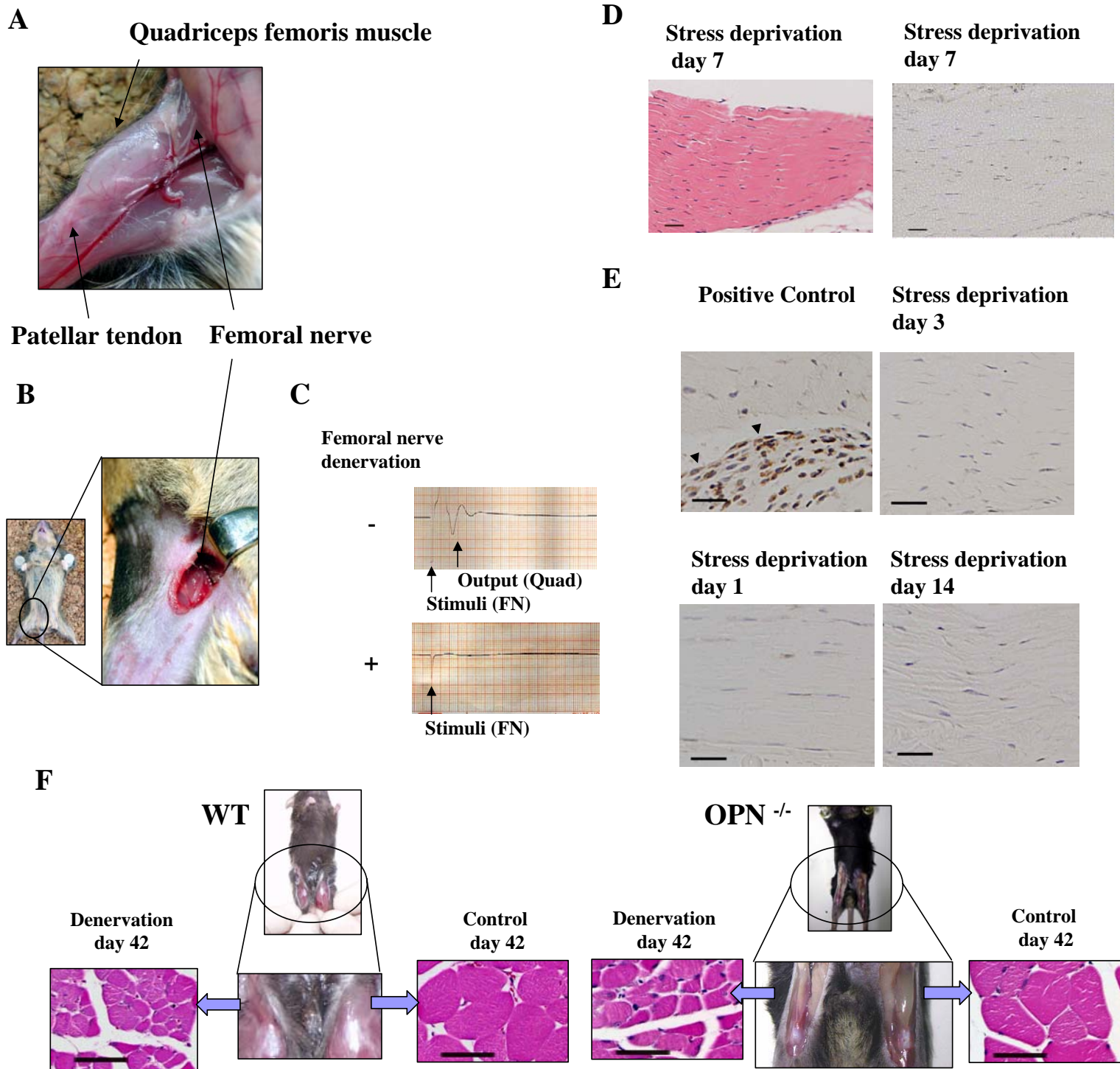
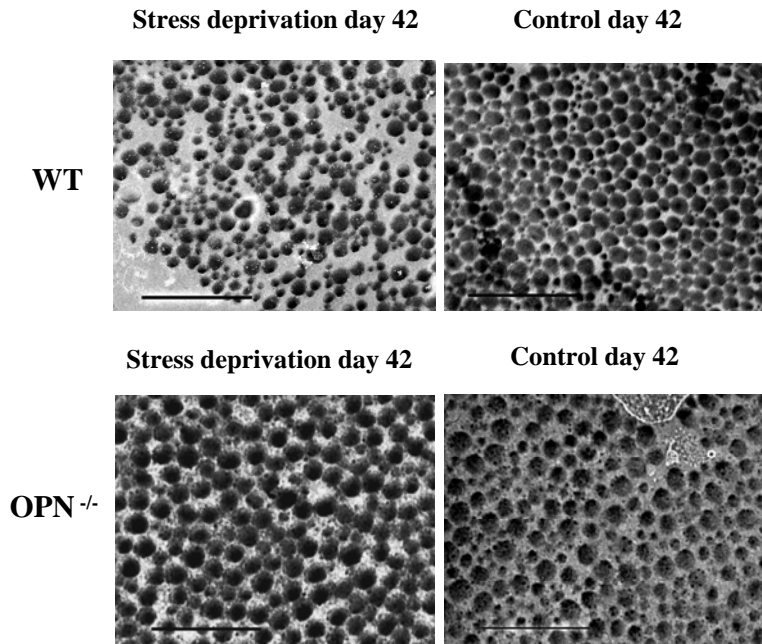
Figure 2

Figure 3

A



B

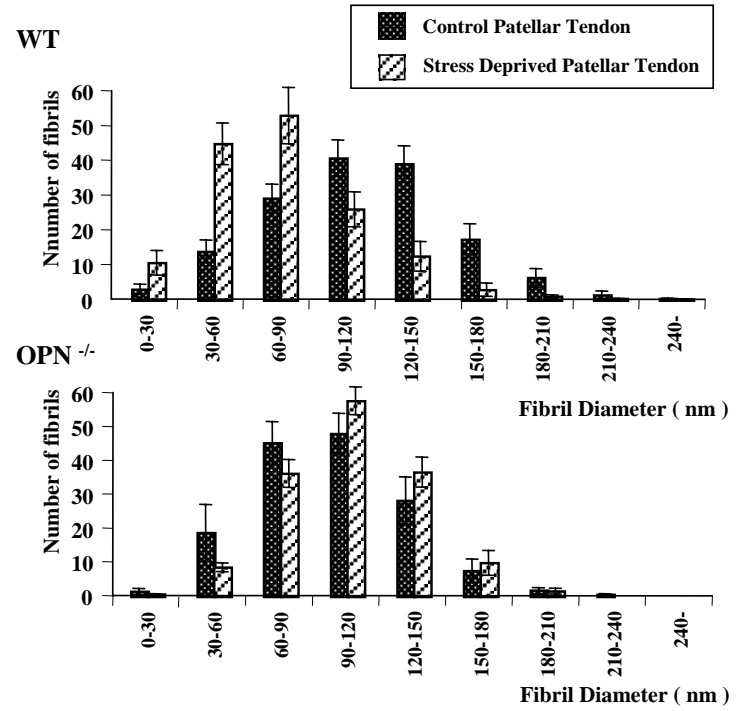


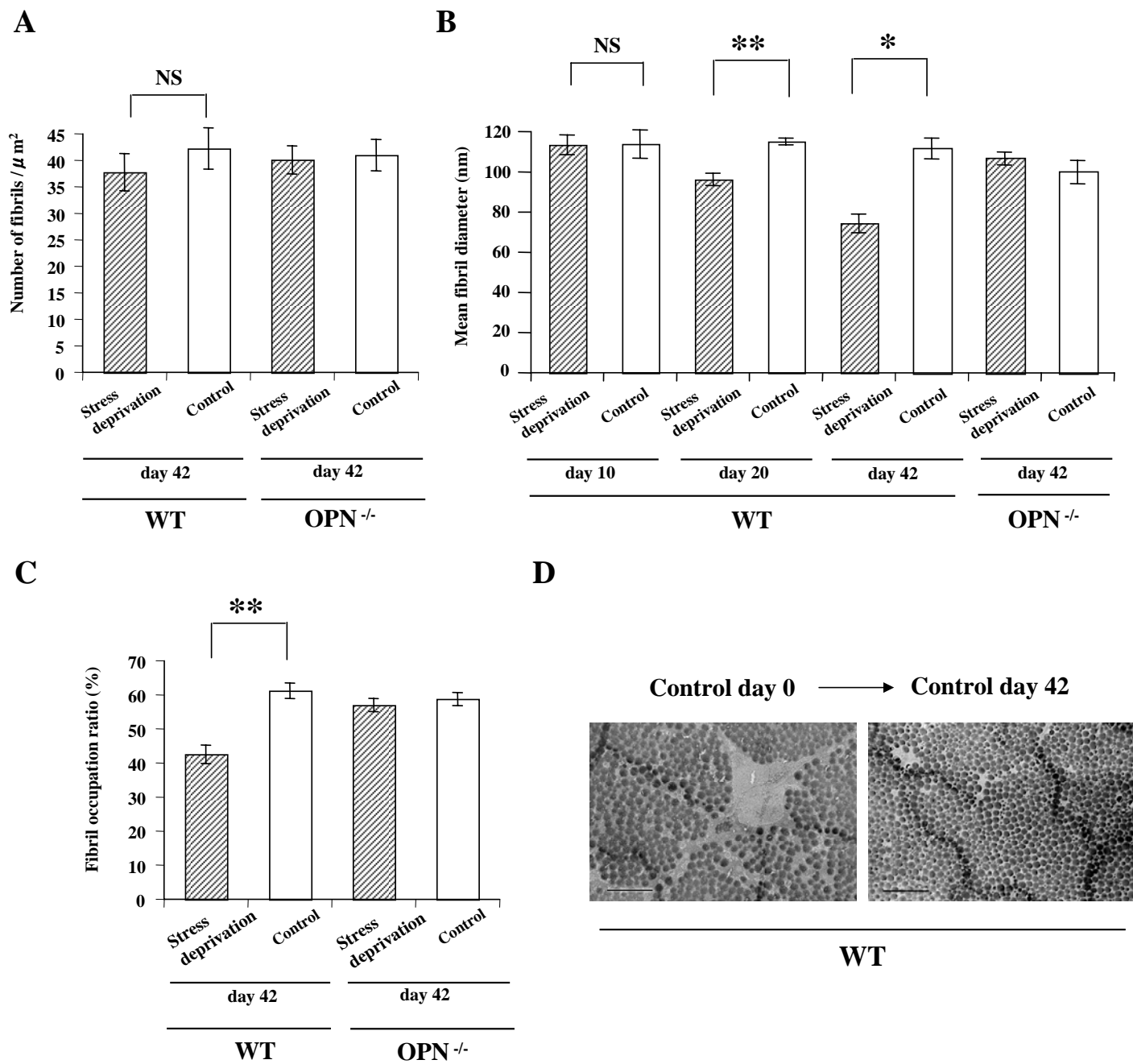
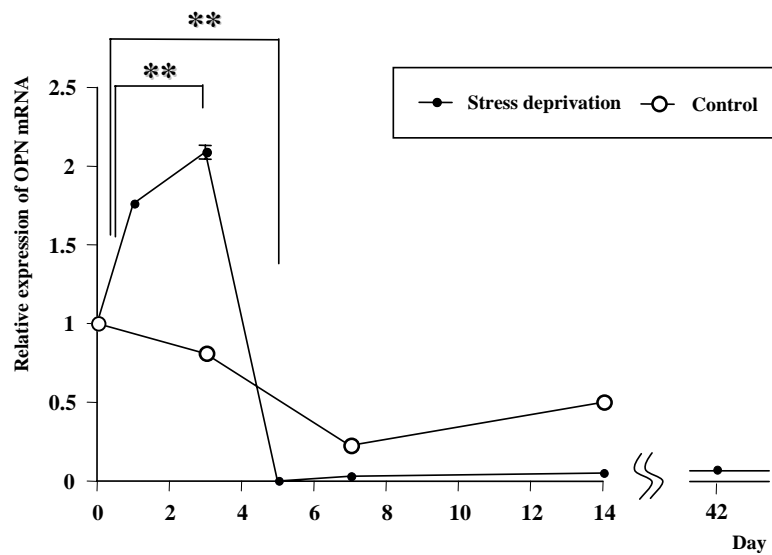
Figure 4

Figure 5

A



B

Normal

Stress deprivation day 1

Stress deprivation day 3

Stress deprivation day 5

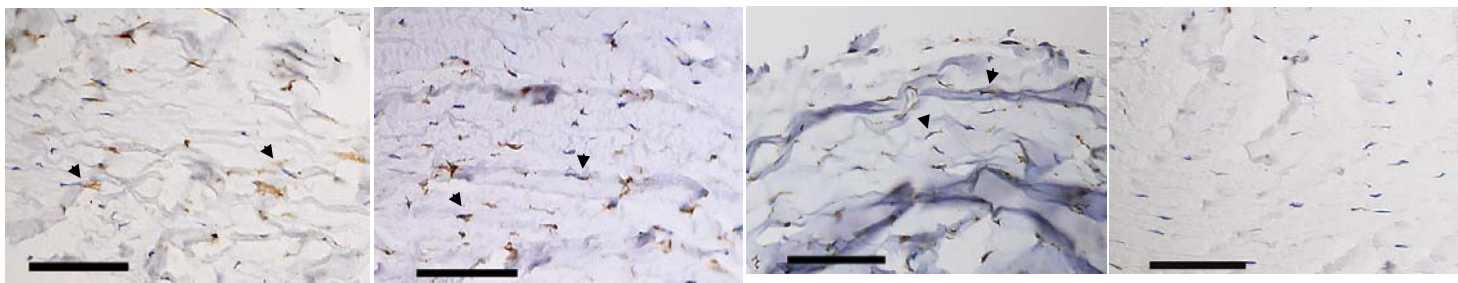


Figure 6

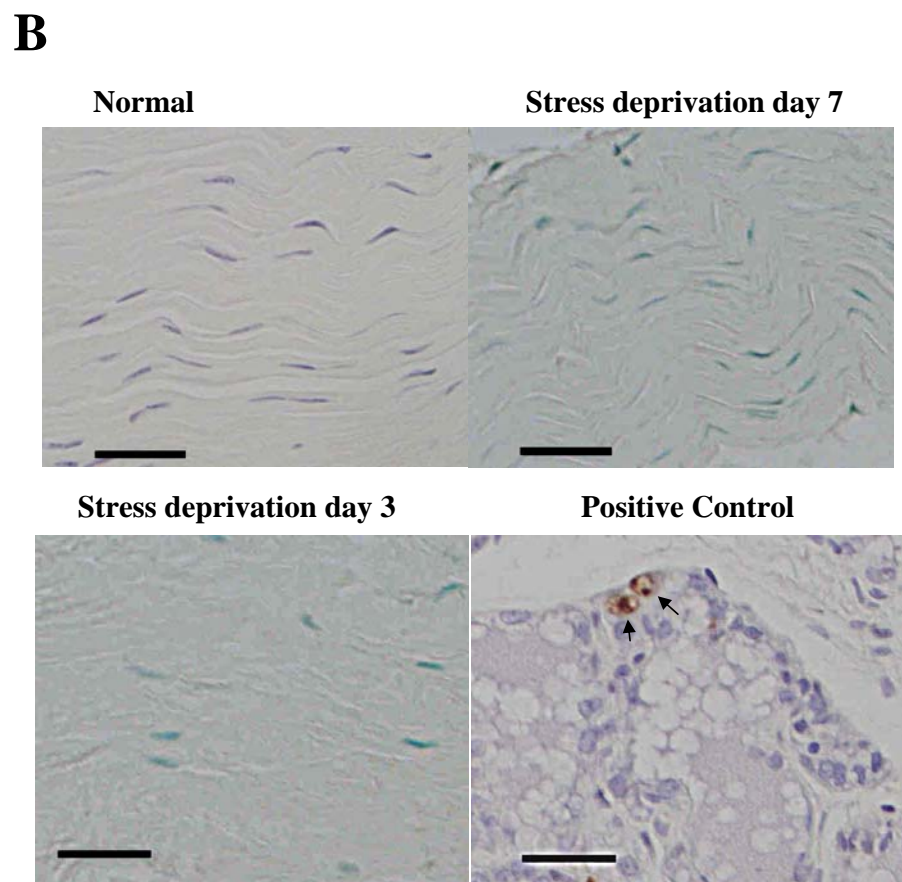
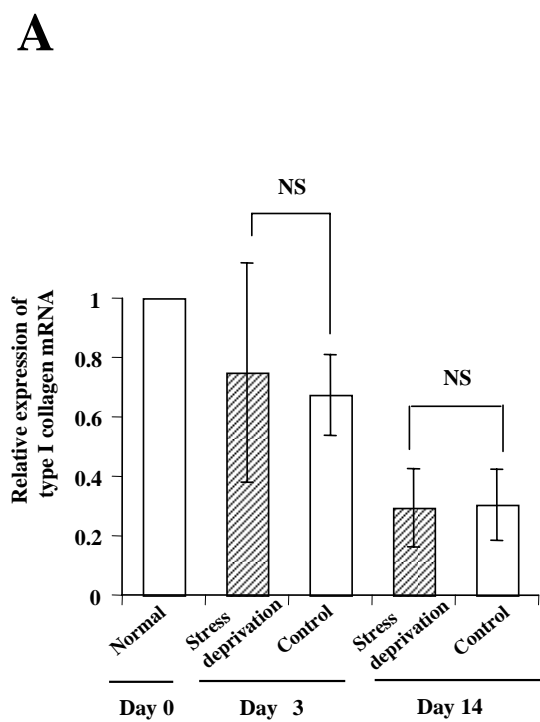


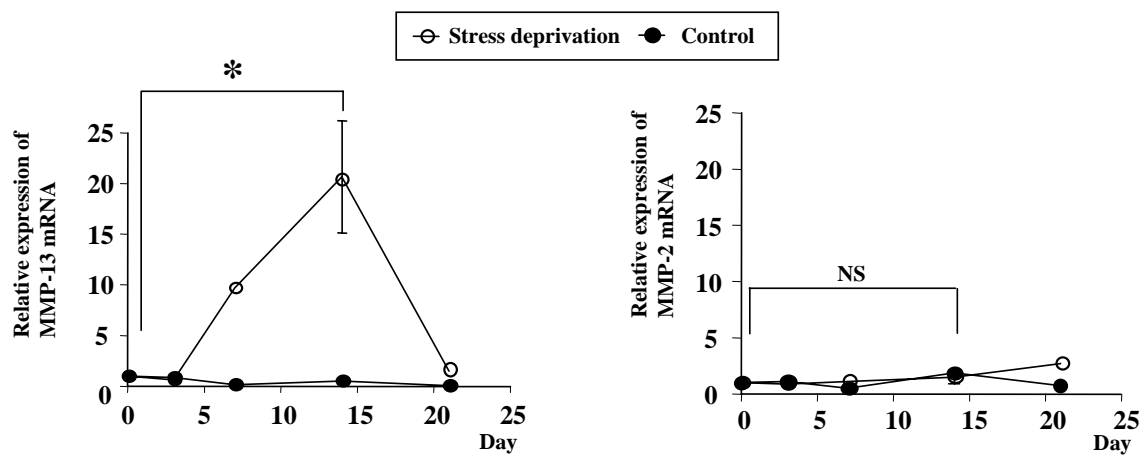
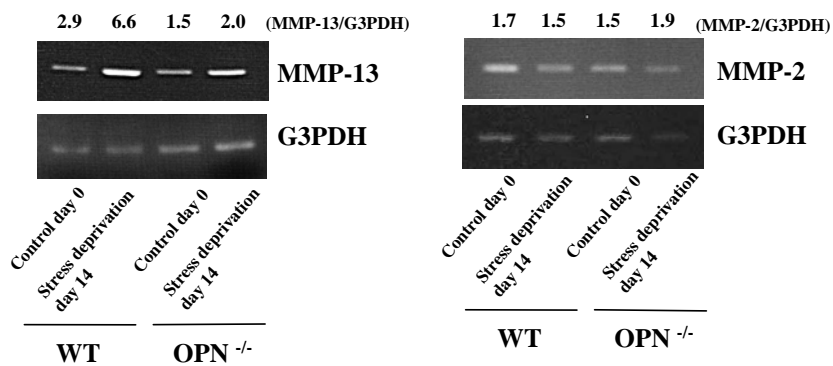
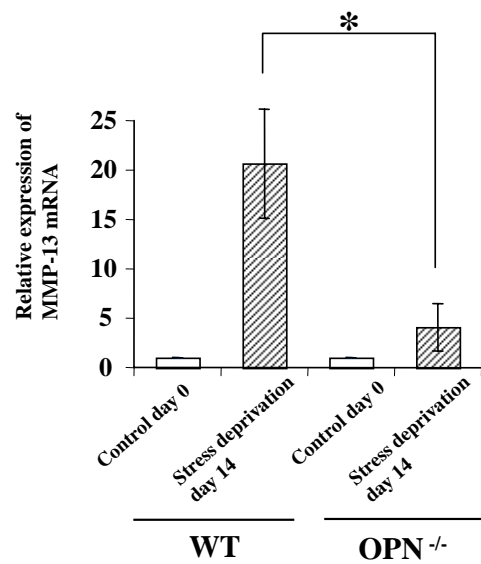
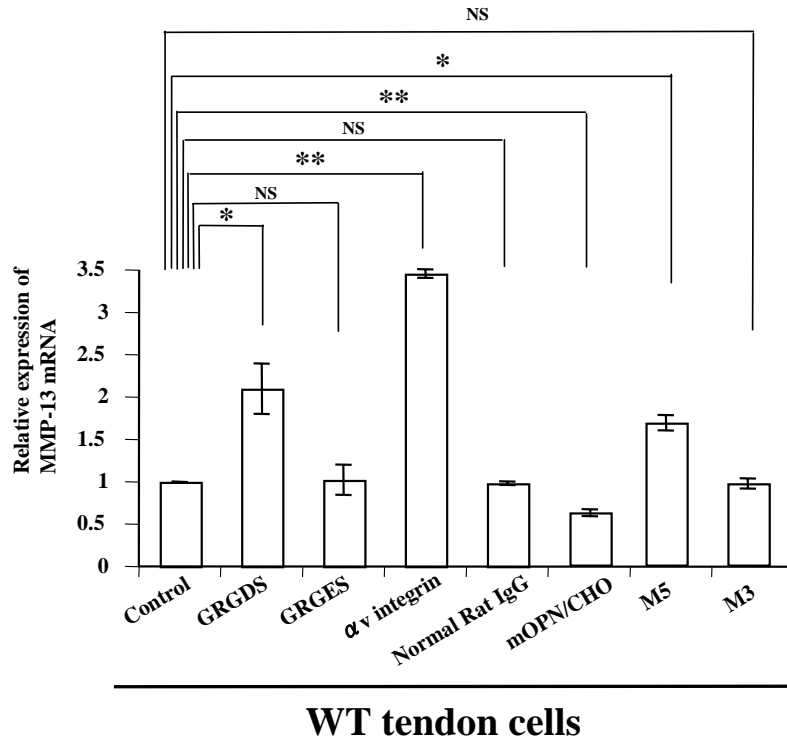
Figure 7**A****B****C**

Figure 8

A



B

