



Original article

Roles of hypoxia inducible factor-1 α in the temporomandibular joint

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ABSTRACT

Objective: Temporomandibular joint osteoarthritis (TMJ-OA) is a degenerative disease characterized by permanent cartilage loss. Articular cartilage is maintained in a low-oxygen environment. The chondrocyte response to hypoxic conditions involves expression of hypoxia inducible factor 1 α (HIF-1 α), which induces chondrocytes to increase expression of vascular endothelial growth factor (VEGF). Here, we investigated the role of HIF-1 α in mechanical load effects on condylar cartilage and subchondral bone in heterozygous HIF-1 α -deficient mice (HIF-1 α ^{+/-}).

Design: Mechanical stress was applied to the TMJ of C57BL/6Ncr wild-type (WT) and HIF-1 α ^{+/-} mice with a sliding plate for 10 days. Histological analysis was performed by HE staining, Safranin-O/Fast green staining, and immunostaining specific for articular cartilage homeostasis.

Results: HIF-1 α ^{+/-} mice had thinner cartilage and smaller areas of proteoglycan than WT controls, without and with mechanical stress. Mechanical stress resulted in prominent degenerative changes with increased expression of HIF-1 α , VEGF, and the apoptosis factor cleaved Caspase-3 in condylar cartilage. **Conclusion:** Our results indicate that HIF-1 α may be important for articular cartilage homeostasis and protective against articular cartilage degradation in the TMJ under mechanical stress condition, therefore HIF-1 α could be an important new therapeutic target in TMJ-OA.

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1. Introduction

Articular cartilage is an avascular tissue found in synovial joints that produces angiogenic inhibitors (Horner, Bord, Kelsall, Coleman, & Compston, 2001). The nutrition and oxygen for articular cartilage are supplied by diffusion from the synovial fluid and the blood vessels in subchondral bone (Zhang, Luo, & Lei, 2015). Replacement of cartilage by bone in skeletal growth and regeneration requires angiogenesis (Gerber et al., 1999). Chondrocytes in the hypertrophic cartilage layer produce vascular endothelial growth factor (VEGF), a potent angiogenic peptide with specific mitogenic and chemotactic actions, especially during growth periods (Garcia-Ramirez, Toran, Andaluz, Carrascosa, & Audi, 2000; Aoyama et al., 2004). VEGF is also expressed in the articular cartilage of osteoarthritic joints, suggesting that VEGF may be involved in the formation of new vessels in cartilage and

the onset of osteoarthritis (OA) (Pufe et al., 2004; Tanaka et al., 2005).

VEGF expression in OA cartilage is upregulated in response to strong mechanical loading (Freemont et al., 1997; Shirakura et al., 2010; Tanaka et al., 2005). It has been reported that the induction of VEGF in hypertrophic cartilage in response to mechanical overload is involved in upstream activation of hypoxia-induced transcription factor-1 α (HIF-1 α) (Forsythe et al., 1996). Moreover, the induction of VEGF leads to increased levels of matrix metalloproteinases (MMPs), which are important mediators of extracellular protein degradation (Pufe et al., 2004). These prior studies indicate that VEGF induced by mechanical overload might facilitate hypoxia-associated tissue destruction in OA in an autocrine manner.

Differing etiologically from rheumatoid arthritis, temporomandibular joint OA (TMJ-OA) is primarily of non-inflammatory origin (Zarb & Carlsson, 1999). The pathological process is characterized by deterioration and abrasion of articular cartilage and local thickening and remodeling of the subchondral bone (Kuroda et al., 2009). Frequently, these changes are accompanied by the superimposition of secondary inflammatory changes.

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Chondrocytes appear to have evolved mechanoresponsive mechanisms (Ohashi, Robling, Burr, & Turner, 2002; Wong, Siegrist, & Goodwin, 2003). Mechanical stimulation of chondrocytes may lead to increases in their metabolic activity and activation of pathological, irreversible cartilage degradation processes (Abramson, Attur, & Yazici, 2006).

The development of strategic treatment options for TMJ-OA will require a better understanding of the onset and developmental mechanisms of TMJ-OA. However, little information is available regarding the supposed HIF-1 α induction and activation in the mandibular condylar cartilage cells of osteoarthritic TMJ. The aim of this study was to investigate the role of HIF-1 α induction in OA cartilage of the TMJ in heterozygous HIF-1 α -deficient mice.

2. Materials and methods

2.1. Animals and body weight measurement

Fifteen wild-type C57BL/6NCr (WT) mice purchased from SLC Japan (Tokyo, Japan) and 15 heterozygous HIF-1 α -deficient (HIF-1 α ^{+/-}) mice provided by Dr. Shuhei Tomita (formerly Tottori University) were used in this study. After weaning at 3 weeks of age, the mice were fed a conventional diet with water *ad libitum*. Body weight for each mouse was recorded weekly, until 13 weeks of age, to monitor its growth and health. This study was approved by the Animal Care and Use Committee of Tokushima University (No. 12131).

2.2. Real-time polymerase chain reaction (PCR) analysis

Mandibular condylar cartilage specimens were resected from three of WT and HIF-1 α ^{+/-} mice each (13 weeks old) and minced. Total RNA was extracted from the cartilage samples with a Pure Link RNA Mini Kit (Ambion; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentrations were estimated with NanoDropND-2000 (Nano Drop Technologies, Wilmington, DE, USA). Total RNA was converted to cDNA with a high-capacity RNA to c-DNA Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. HIF-1 α , VEGF, and MMP9 mRNA levels were examined by a real-time PCR with StepOnePlus and TaqMan Fast Advanced Master Mix (both from Applied Biosystems). The following TaqMan probe mixtures were used: HIF-1 α , Mm00468869_m1; VEGF, Mm01281449_m1; MMP9, Mm00442991_m1 (Applied Biosystems). The cycling conditions included: 20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C. Detection of β -actin was used as an internal control. Expression of HIF-1 α , VEGF, and MMP9 were calculated by the Ct method.

2.3. Mechanical stress application

At 13 weeks of age, the remaining 12 WT and 12 HIF-1 α ^{+/-} mice were divided randomly into control and experimental mechanical stress groups (N=6 per genotype-treatment group). Temporomandibular joints of the experimental groups were repetitively loaded during a period of 10 days by application of sliding plates on upper incisor to keep the mandible position posterior by biting (Shirakura et al., 2010). The control groups were subjected to a sham experiment without application of mechanical stress.

2.4. Tissue preparation and histology

At the conclusion of the 10-day mechanical stress procedures, the animals were killed with an overdose of sodium pentobarbital (Nembutal; Dinabott, Osaka, Japan). The right TMJs were removed carefully, fixed in 4% paraformaldehyde, decalcified in EDTA

solutions for 4 weeks, dehydrated in ethanol, and embedded in paraffin. Serial sagittal sections (7 μ m) were cut from paraffin-embedded TMJ tissue blocks with a microtome (Carl Zeiss HM360, Jena, Germany). Serial sections of each condyle were stained with hematoxylin and eosin (HE) for histology, with 1% Safranin-O (red in color) to delineate cartilage tissue borders and 0.02% Fast Green to detect proteins. Tartrate-resistant acid phosphatase (TRAP) staining was also used in accordance with the manufacturer's instructions to identify active bone-absorbing osteoclasts.

2.5. Histomorphometric analysis

The modified Mankin scoring system (Xu et al., 2003) was used to assess the osteoarthritic state of articular cartilage. Scoring was based on pericellular and background Safranin-O staining, chondrocyte arrangement, and cartilage structural condition. A score of 0 signifies normal cartilage, with higher scores indicating greater degeneration up to a maximum score of 10. The scoring was conducted by three independent experts who were blinded to the group identities of the samples analyzed.

2.6. Immunohistochemistry

After deparaffinization and blocking in 5% skim milk in phosphate buffered saline (PBS), sections were incubated with primary rabbit polyclonal antibodies against HIF-1 α , VEGF, aggrecan, MMP9, and cleaved Caspase-3 diluted in PBS containing 0.1% bovine serum albumin overnight at 4 °C. The sections were then washed in PBS Tween, incubated with anti-rabbit horse-radish-peroxidase-conjugated secondary antibodies at room temperature for 1 h, and washed in PBS Tween again. Antibody binding was visualized by reaction with 3,3-diaminobenzidine (2.5 mg/mL), followed by counterstaining with 0.1% methyl green. Negative control sections were incubated with nonimmune IgG antibodies. The stained sections were mounted and analyzed under a BioRevo BZ-9000 microscope (KEYENCE, Osaka Japan).

2.7. TUNEL staining

The distribution of apoptotic chondrocyte cells was assessed with the TdT-mediated dUTP-digoxigenin nick-end labeling (TUNEL) method. TUNEL assay was performed with Apoptosis In Situ Detection Kit (Wako Pure Chemical, Osaka, Japan), according to the manufacturer's directions. TUNEL positive cells were observed by microscopy (KEYENCE).

2.8. Statistical analysis

All experiments were performed at least in triplicate for each set of conditions, and each experiment was independently repeated at least two or three times. The results were presented as the means \pm SD. These data were statistically analyzed with one-way analysis of variance with post hoc Tukey honest significant difference test, as appropriate for each case. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Body weight

As shown in Fig. 1A, the body weight of the HIF-1 α ^{+/-} mice increased steadily week over week in parallel with those of WT mice until 11 weeks of age. At the 12-week-old and 13-week-old time points, the HIF-1 α ^{+/-} mice were significantly lighter than WT mice ($p < 0.05$).

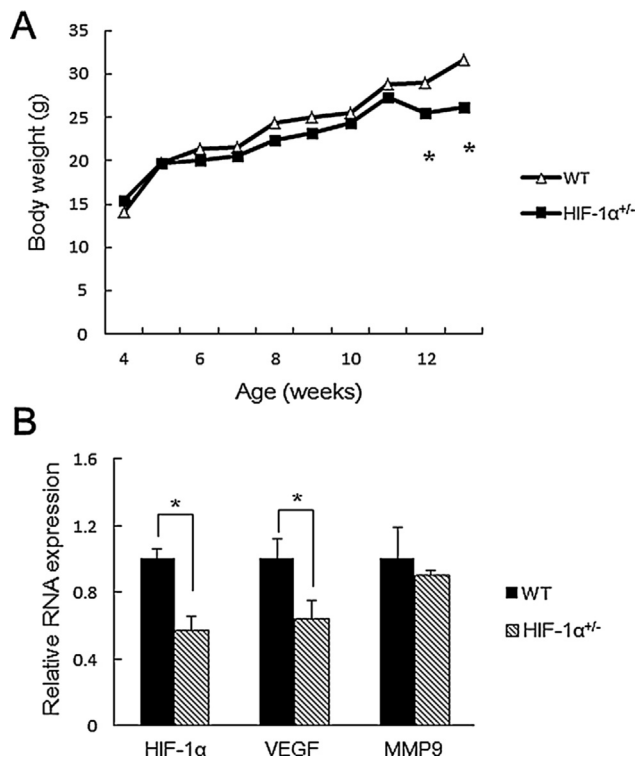


Fig. 1. (A) Body weight curves of WT (+/+, N=13) and heterozygous HIF-1 α deficient mice (+/–, N=10). (B) Real-time PCR analysis of HIF-1 α , VEGF and MMP9 mRNAs in WT and HIF-1 $\alpha^{+/-}$ condylar cartilage. * $p < 0.05$.

3.2. Gene expression of HIF-1 α , VEGF and MMP9 in HIF-1 $\alpha^{+/-}$ condylar cartilage

Real-time PCR analysis showed lower mRNA levels (by ~40%) of HIF-1 α and VEGF in mandibular condylar cartilage from HIF-1 $\alpha^{+/-}$ mice than in those from WT mice ($p < 0.05$), but no group difference in MMP9 mRNA levels (Fig. 1B).

3.3. Histology

Four distinct layers of articular cartilage with regularly arranged cells were clearly observed in tissue specimens from WT mice (Fig. 2A). The articular cartilage especially hypertrophic layer of HIF-1 $\alpha^{+/-}$ mice appeared markedly decreased in thickness relative to the WT cartilage (Fig. 2B).

HIF-1 $\alpha^{+/-}$ cartilage subjected to 10 days of mechanical stress showed evidence of destruction with vertical clefts scattered on the surface of the tissue (Fig. 2A, arrowheads) and a roughening of the tissue surface characteristic of TMJ-OA. Other OA-like lesions were also observed in the condylar cartilage of the HIF-1 $\alpha^{+/-}$ mice, including reduced cartilage thickness, increases in local cell-free area, irregular arrangement of chondrocytes, deterioration of the smooth articular cartilage surface, and fibrillation of the superficial layer. The areas of Safranin-O staining in HIF-1 $\alpha^{+/-}$ cartilage were smaller ($p < 0.05$) than those in WT cartilage, with or without mechanical stress. And the proteoglycan staining areas were reduced in response to TMJ mechanical stress in both groups ($p < 0.05$; Fig. 2C). Modified Mankin morphological scores were significantly higher ($p < 0.01$) for mandibular condylar cartilage samples from the experimental groups than for those from the respective no-stress control groups for both WT and HIF-1 $\alpha^{+/-}$ mice (Fig. 2D).

Opposing effects of HIF-1 α deficiency and mechanical stress on osteoclastogenesis in subchondral bone

TRAP-positive osteoclasts were observed in the mineralized subchondral bone layers of condyles in control and HIF-1 $\alpha^{+/-}$ mice (Fig. 3A). Higher numbers of TRAP-positive, and thus presumably bone-absorbing, cells ($p < 0.01$) were observed in the subchondral bone of WT experimental mechanical stress group than in that of either WT control group or HIF-1 $\alpha^{+/-}$ mechanical stress group (Fig. 3B). The pattern of TRAP-positive osteoclast counts across the four groups in subchondral bone was similar to the pattern of HIF-1 α -positive chondrocyte counts observed in the cartilage specimens (Figs. 3 B and 4B).

3.4. Expression of HIF-1 α , VEGF, aggrecan, and MMP9 in articular cartilage

Immunohistochemical cell count analyses showed that both HIF-1 α and VEGF protein expression levels were lower in HIF-1 $\alpha^{+/-}$ than in WT articular cartilage without mechanical stress ($p < 0.05$). Mechanical stress resulted in significant increases in the numbers of HIF-1 α -positive cells ($p < 0.01$) and VEGF-positive cells ($p < 0.05$) in both WT and HIF-1 $\alpha^{+/-}$ mice, relative to their respective no stress controls (Fig. 4A–C). High magnification HIF-1 α immunohistochemical staining of WT mice with or without mechanical stress revealed that mechanical stress might up-regulate expression of HIF-1 α not only in chondrocytes but in osteoclasts. Aggrecan expression was decreased in HIF-1 $\alpha^{+/-}$, relative to WT, cartilage in both the control and mechanical stress conditions, and was also decreased by mechanical stress in cartilage in both WT and HIF-1 $\alpha^{+/-}$ mice, relative to the respective control condition groups (Fig. 4A, D). MMP9 was expressed more strongly after mechanical stress application, relative to non-stress controls, in both WT and HIF-1 $\alpha^{+/-}$ cartilage surface and subchondral bone.

3.5. Chondrocyte apoptosis

As shown in Fig. 5A and B, the numbers of cells immunopositive for cleaved Caspase-3, an apoptosis marker, were greater in HIF-1 $\alpha^{+/-}$ than in WT cartilage, both with and without mechanical stress ($p < 0.01$). Additionally, mechanical stress produced increases in cleaved Caspase-3 immunopositive cell counts in both WT and HIF-1 $\alpha^{+/-}$ groups ($p < 0.01$). Moreover, in Fig. 5A and C, TUNEL staining was performed to detect cell death in degraded cartilage. In HIF-1 $\alpha^{+/-}$ mice, obviously increase in the number of TUNEL positive cells were observed compared to WT mice. Mechanical stress made increase in TUNEL positive cells as well as cleaved Caspase-3 in both WT and HIF-1 $\alpha^{+/-}$ groups ($p < 0.05$).

4. Discussion

To our knowledge, this study is the first to examine the effect of HIF-1 α deficiency on the development of TMJ-OA in heterozygous HIF-1 α deficient mice. We obtained histological, mRNA expression, and protein expression findings suggesting that HIF-1 α signaling may be involved in mandibular condylar chondrocyte apoptosis via activation of Caspase signaling cascades.

The breakdown of cartilage in OA is associated with loss of extracellular matrix (ECM) and chondrocyte apoptosis (Kühn, D’Lima, Hashimoto, & Lotz, 2004). It has been suggested that VEGF and HIF-1 α contribute to apoptosis of hypertrophic growth plate chondrocytes by inducing the liberation of pro-apoptotic factors from blood vessels. Consistent with this hypothesis, blood vessel invasion from subchondral bone into articular cartilage has been documented in advanced OA (Pfander et al., 2001).

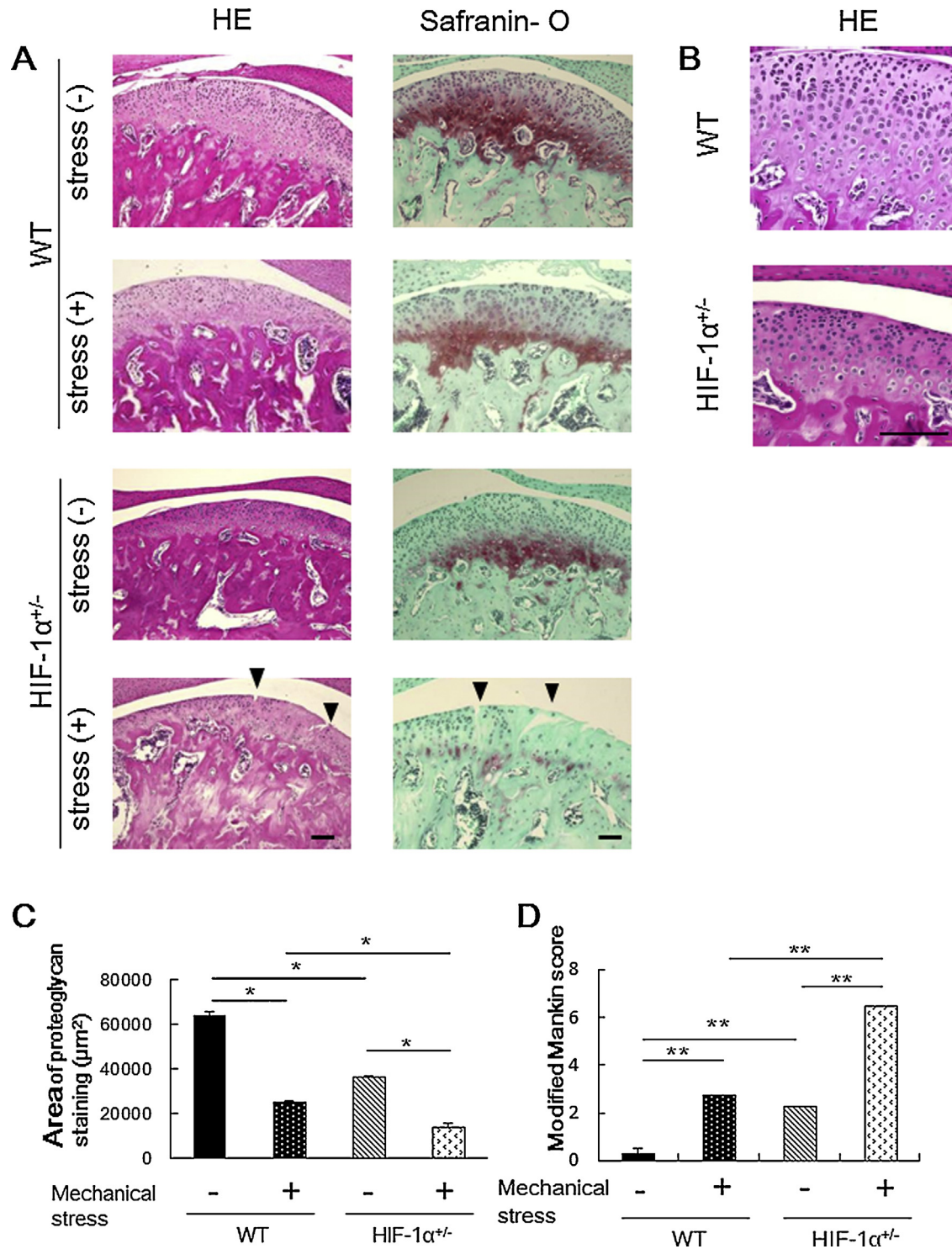


Fig. 2. Effects of HIF-1α and repeated mechanical stress on articular cartilage and subchondral bone. (A) Representative HE-stained (right) and Safranin-O/Fast Green-stained (left) sections of condylar cartilage. Arrows demonstrate the thickness of each articular cartilage. Arrowheads indicate the locations of vertical clefts characteristic of TMJ-OA. (B) HE staining of articular cartilage in WT and HIF-1α^{+/-} mice without mechanical stress with high magnification. (C) Significant decreases in Safranin-O proteoglycan staining areas were observed after repeated mechanical stress in WT and HIF-1α^{+/-} mice. (D) Analysis of Modified Mankin scores confirmed that mechanical stress and HIF-1α deficiency each disrupt the morphology of mandibular condylar cartilage. Bars = 100 μm, **p* < 0.05, ***p* < 0.01.

Conditional knockout of VEGF in chondrocytes causes cell death in the center of mutant growth plates, indicating that VEGF is critical for chondrocyte survival during endochondral bone development (Zelzer et al., 2004). This cell death phenotype is similar to that observed in HIF-1α deficient chondrocytes,

suggesting that HIF-1α and VEGF may form a common pathway that supports chondrocyte survival in endochondral bone development (Araldi & Schipani, 2010). In our study, the mandibular condylar cartilage of HIF-1α^{+/-} mice showed thinner condylar cartilage layer, lower expression of HIF-1α, VEGF, and aggrecan

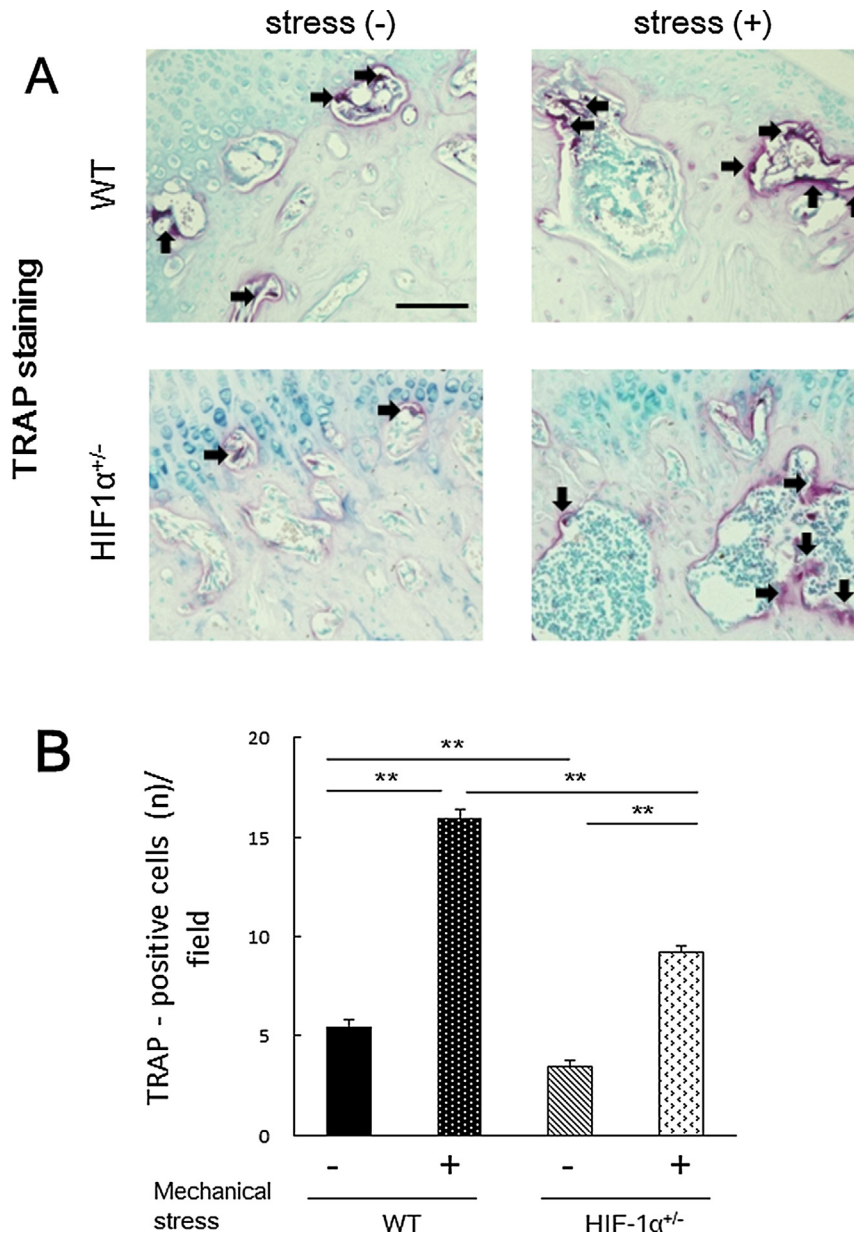


Fig. 3. (A) TRAP staining in mouse TMJ condyle subchondral bone. Arrows indicate TRAP-positive osteoclasts. (B) Quantitative analysis revealed a reducing effect of HIF-1 α deficiency and an increasing effect of mechanical stress on TRAP-immunopositivity in subchondral bone. Bars = 100 μ m, ** p < 0.01.

than WT cartilage, suggesting that HIF-1 α may be essential for articular cartilage homeostasis and beneficial in protection of articular cartilage.

Meanwhile, HIF-1 α ^{+/-} subchondral bone had fewer TRAP-positive (bone-absorbing) cells than WT subchondral bone. Mechanical stress led to increase of TRAP-positive cell number in both WT and HIF-1 α ^{+/-} subchondral bone. These results suggest that HIF-1 α may positively control osteoclast-mediated bone resorption, potentially protecting condylar subchondral bone from degenerative changes. The present findings are consistent with the data of Knowles et al. (2010) showing that HIF-1 α can increase osteoclastogenesis *in vivo* as well as the data of Shirakura et al. (2010) data showing TRAP-positive osteoclast counts in subchondral bone correlate with HIF-1 α positivity in rat mandibular condylar cartilage. Moreover, the findings of Gelse et al. (2008) suggest that the physiological function of HIF-1 α may depend

upon its activity being well-balanced, with surplus levels not necessarily providing further protection of bone tissue. Thus, HIF-1 α may exert paradoxical roles in bone (pro-absorption) versus cartilage (anti-apoptotic, discussed below).

Our mechanical stress manipulation resulted in decreased expression of aggrecan in articular cartilage, with the effect adding to already sub-WT levels in HIF-1 α ^{+/-} cartilage, in which it was associated with the development of surface discontinuities in the form of vertical fissures. Aggrecan determines water content in cartilage ECM, and changes in aggrecan levels can result in reduced tissue resiliency and hydration (Madej et al., 2016), such that the tissue becomes more rigid. Through regulation of matrix protein expression, articular cartilage is able to perform a critical biomechanical function while maintaining mechanosensitivity. On the other hand, increased expression of MMP9 was observed in HIF-1 α cartilage under mechanical stress condition. Taken

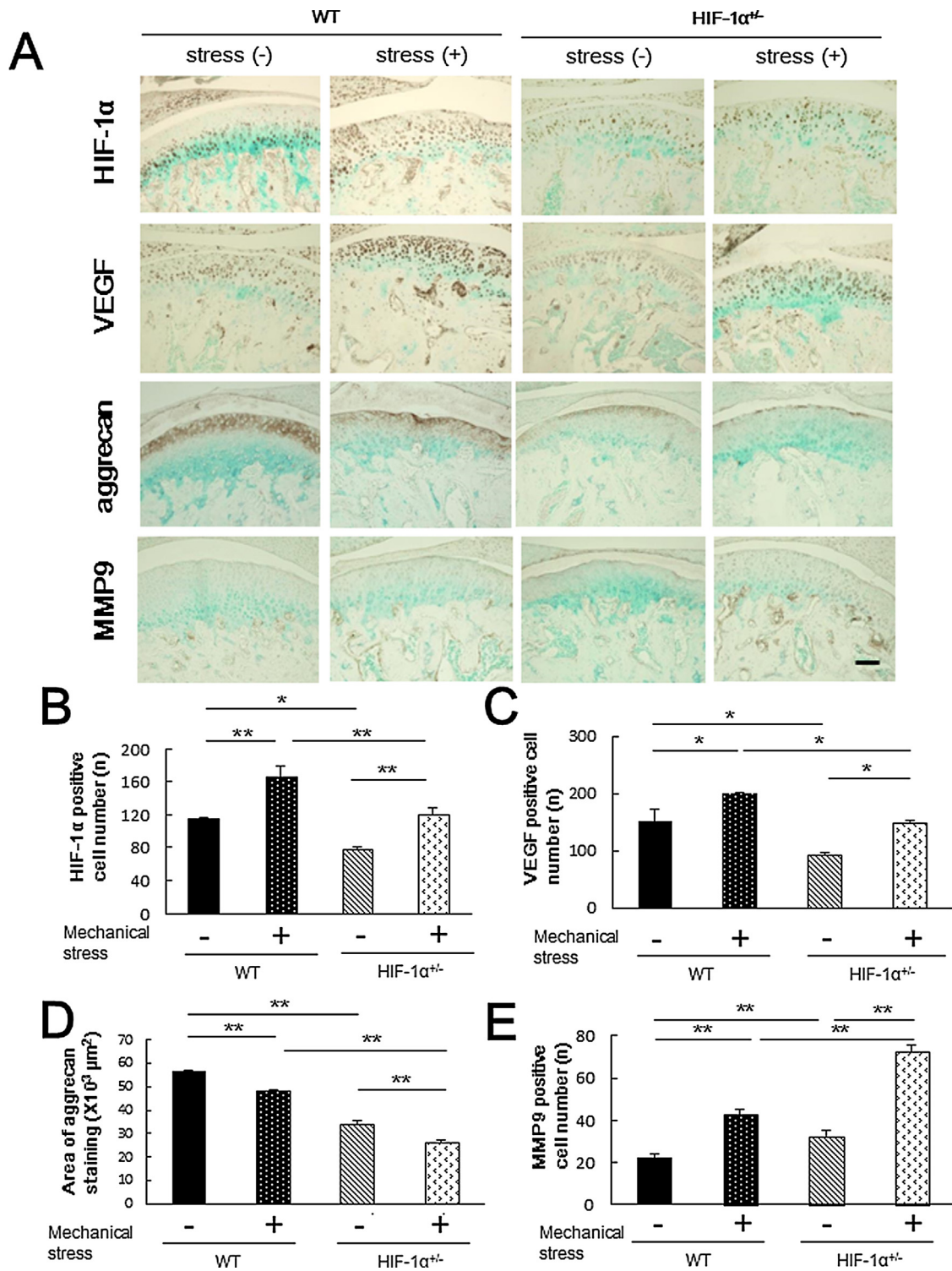


Fig. 4. (A) Immunohistochemical labeling of HIF-1α, VEGF, aggrecan, and MMP9 in mandibular articular cartilage samples obtained from WT and HIF-1α^{+/-} mice before and after extensive mechanical stress application. (B, C) Significant increases in HIF-1α and VEGF-positive cells were observed after repeated mechanical stress. (D) A significant decrease in aggrecan labeling area was observed after repeated mechanical stress in both WT and HIF-1α^{+/-} mice. (E) A significant increase in MMP9-positive cells were observed after repeated mechanical stress especially in HIF-1α^{+/-} mice. Bars = 100 μm, *p < 0.05, **p < 0.01.

together, our findings suggest that, in HIF-1α-deficient cartilage, mechanical stress impairs the tissue's ability to regulate ECM components and growth factors, resulting in fragility. Further investigation should be conducted to clarify the mechanism by which HIF-1α-deficiency influences tissue mechanosensitivity in articular cartilage.

Our cleaved Caspase-3 immunohistochemistry and TUNEL staining results indicate that mechanical stress to articular cartilage induces chondrocyte apoptosis and the property of apoptosis increment is more markedly in HIF-1α^{+/-} condylar cartilage than in WT. An increase in the rate of apoptosis in articular cartilage could play an important role in the OA disease

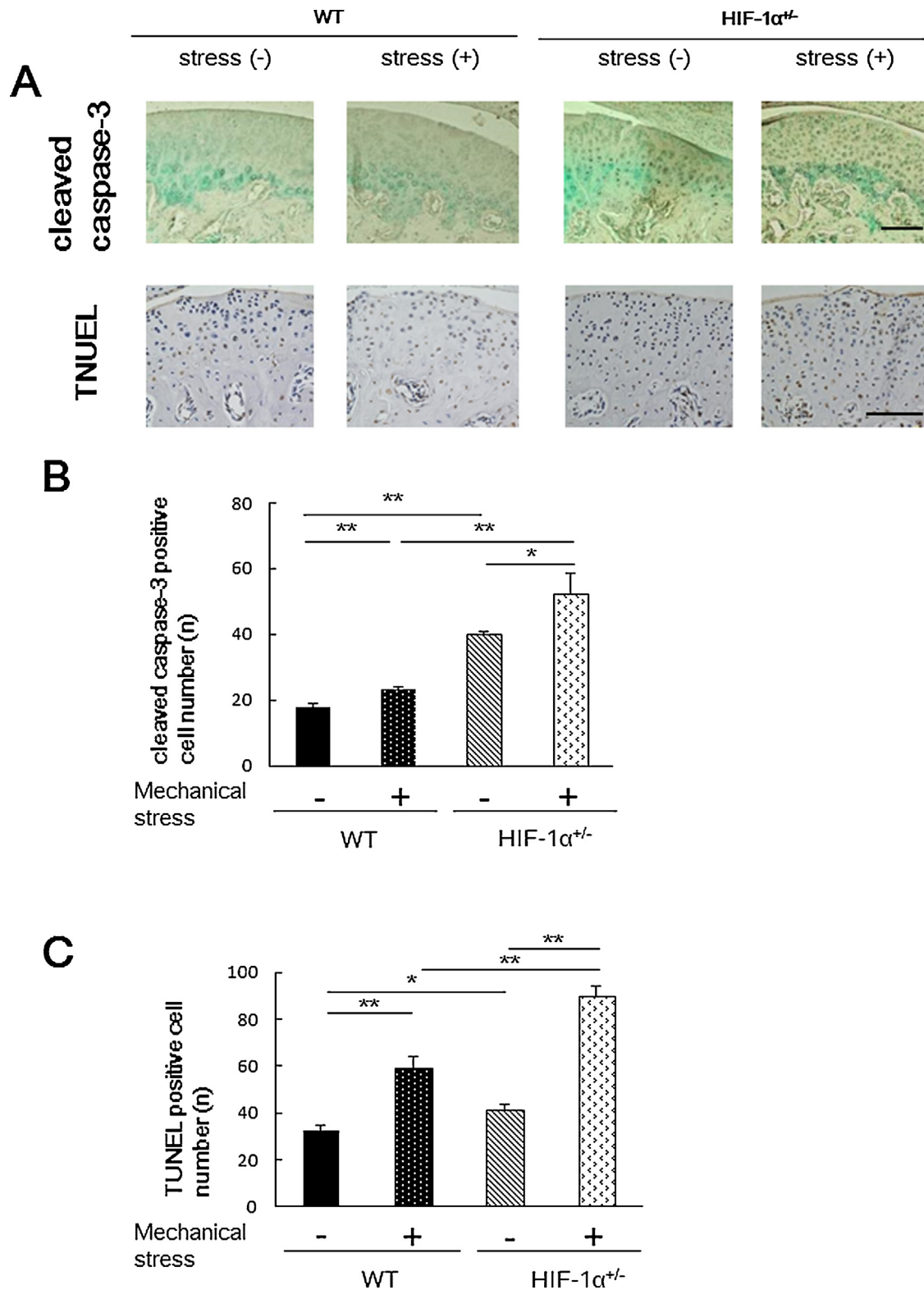


Fig. 5. (A) Serial sections of condylar cartilage from WT and HIF-1 α ^{+/-} mice immunolabeled with cleaved Caspase-3, and TUNEL staining of WT and HIF-1 α ^{+/-} mice with or without mechanical stress. (B,C) Condylar cartilage in HIF-1 α ^{+/-} mice had significantly more cleaved Caspase-3-positive and TUNEL positive cells than that in WT mice. Bar = 100 μ m, * P < 0.05, ** p < 0.01.

process (Sharif, Whitehouse, Sharman, Perry, & Adams, 2004). Notably, Yudoh et al. (2005) showed that IL-1 β -induced apoptosis was also increased in HIF-1 α deficient chondrocytes *in vivo* under hypoxic conditions. Cartilage breakdown in OA is related not only

to ECM loss, but also chondrocyte death (Kühn et al., 2004). Therefore, HIF-1 α may be involved in Caspase signaling pathway and essential for condylar cartilage homeostasis via regulation of chondrocyte apoptosis.

5. Conclusions

To our knowledge, this is the first study to investigate the effects of HIF-1 α deficiency on the development of TMJ-OA in heterogeneous HIF-1 α deficient mice. The presently observed increases in HIF-1 α and VEGF expression and in the numbers of cleaved Caspase-3-immunopositive chondrocytes in mechanically loaded TMJs suggest that HIF-1 α may be essential for condylar cartilage homeostasis and protective against TMJ-OA owing to its regulatory influence on chondrocyte apoptosis in the TMJ. Further studies need to elucidate the molecular mechanisms of HIF-1 α in cartilage. Then HIF-1 α might be an important target for TMJ-OA treatment.

Conflict of interest

None.

Ethical approval

This work was approved by the Ethical Committee of Tokushima University.

Acknowledgments

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