**Figure Captions**

Figure 1 SEM images of Ti (a, c) and Nano-Ti (b, d). Low magnification images (a, b) with scale bars of 10 μm, and high magnification images (c, d) with scale bars of 1 μm. The arrow in the image (d) indicates the orientation of the incident beam polarization.

Figure 2 AFM images of Ti (a) and Nano-Ti (b) with scanning area of 2.5 μm x 2.5 μm.

Figure 3 (Color) Fluorescence micrographs of cells cultured on Ti (a-d) and Nano-Ti (e-h) for 1 h. Actin filaments (a, e), vinculin (b, f), nuclei (c, g), and merged images (d, h) are shown with scale bars of 50 μm.

Figure 4 (Color) Fluorescence micrographs of cells cultured on Ti (a-d) and Nano-Ti (e-h) for 6 h. Actin filaments (a, e), vinculin (b, f), nuclei (c, g), and merged images (d, h) are shown with scale bars of 50 μm.

Figure 5 (Color) Fluorescence micrographs of cells cultured on Ti (a-d) and Nano-Ti (e-h) for 24 h. Actin filaments (a, e), vinculin (b, f), nuclei (c, g), and merged images (d, h) are shown with scale bars of 50 μm.

Figure 6 Low magnification SEM images of cells cultured on Ti (a, b, c) and Nano-Ti (d, e, f) for 1 h (a, d), 6 h (b, e), and 24 h (c, f) with scale bars of 5 μm (a, b, d, e) and 10 μm (c, f).

Figure 7 High magnification SEM images of cells cultured on Ti (a, b, c) and Nano-Ti (d, e, f) for 1 h (a, d), 6 h (b, e), and 24 h (c, f) with scale bars of 1 μm.

Figure 8 Cell adhesion areas on Ti and Nano-Ti cultured for 1 h, 6 h, and 24 h. Averaged data from 10 samples ($n$=10) are shown with standard deviation, with statistically significant differences set at 0.05.

Figure 9 Aspect ratios of adhered cells on Ti and Nano-Ti cultured for 1 h, 6 h, and 24 h. Averaged data from 10 samples ($n$=10) are shown with standard deviation, with statistically significant differences set at 0.05. The dotted line is just "1" indicating a shape of true circle.

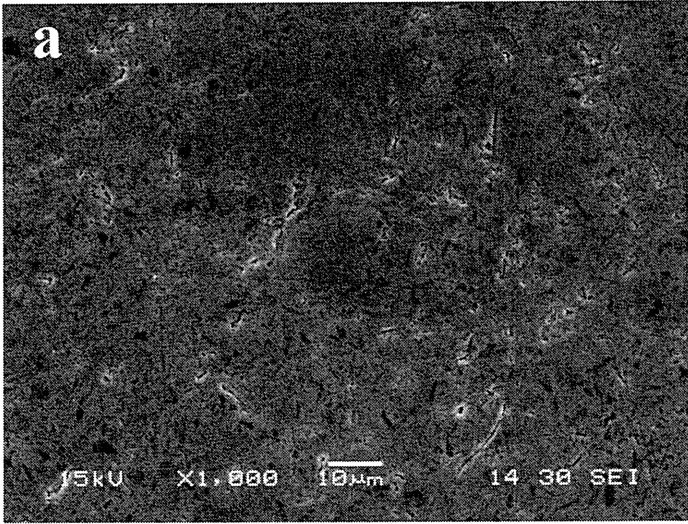
Figure 10 Distribution of cells in the range of each orientation angle cultured for 24 h on Ti (a) and Nano-Ti (b) with sample number of 50 ($n$=50).

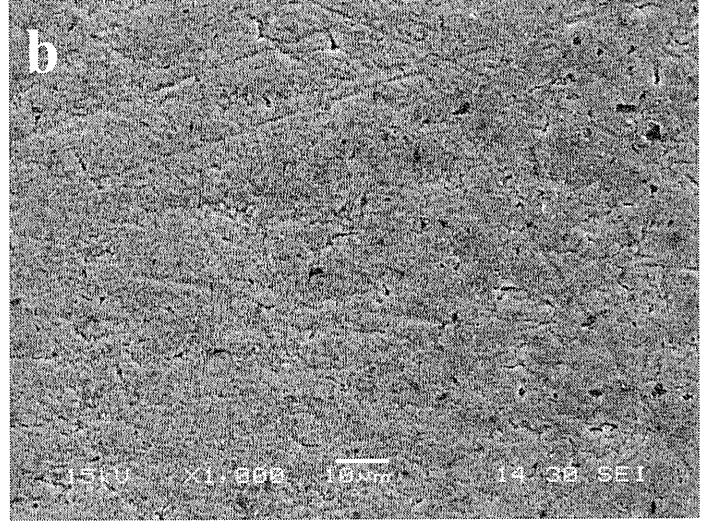
Table I Roughness parameters on Ti and Nano-Ti.

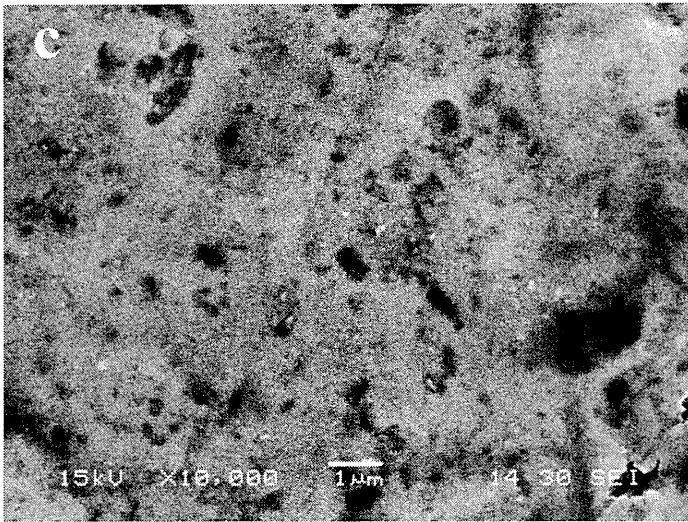
| | Ra (nm) | Rq (nm) | Rz (nm) | Rv (nm) |
|---|---|---|---|---|
| Ti | 20 ± 7 | 54 ± 21 | 155 ± 64 | 100 ± 48 |
| Nano-Ti | 57 ± 18 | 190 ± 54 | 390 ± 72 | 201 ± 34 |

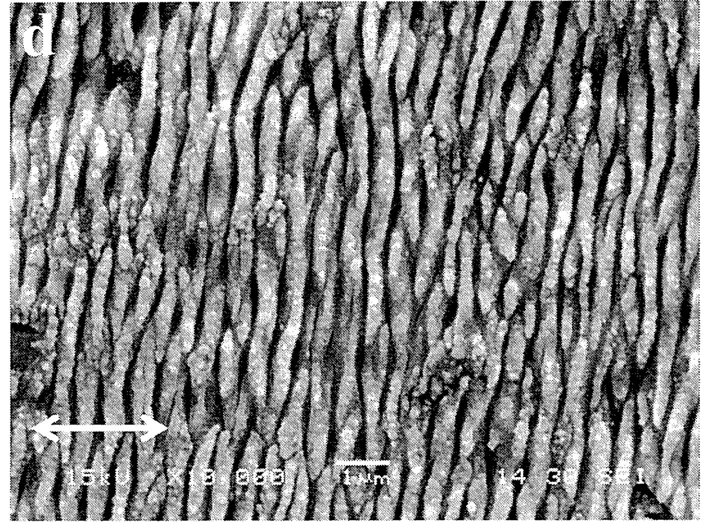
The data are mean ± SD; *n*=3. Five points per specimen were measured.

Figure1



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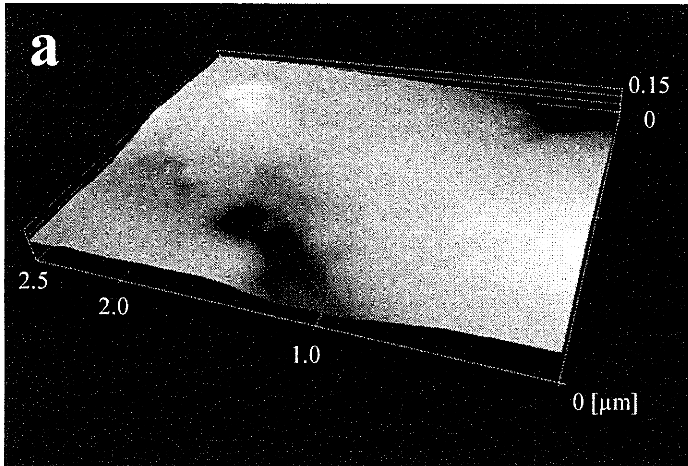


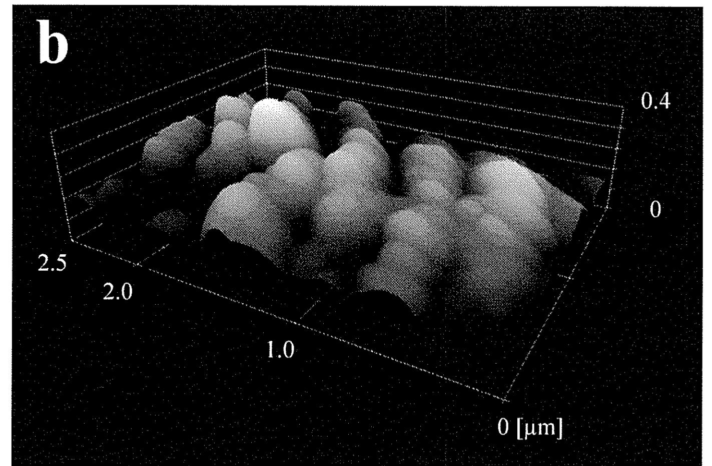

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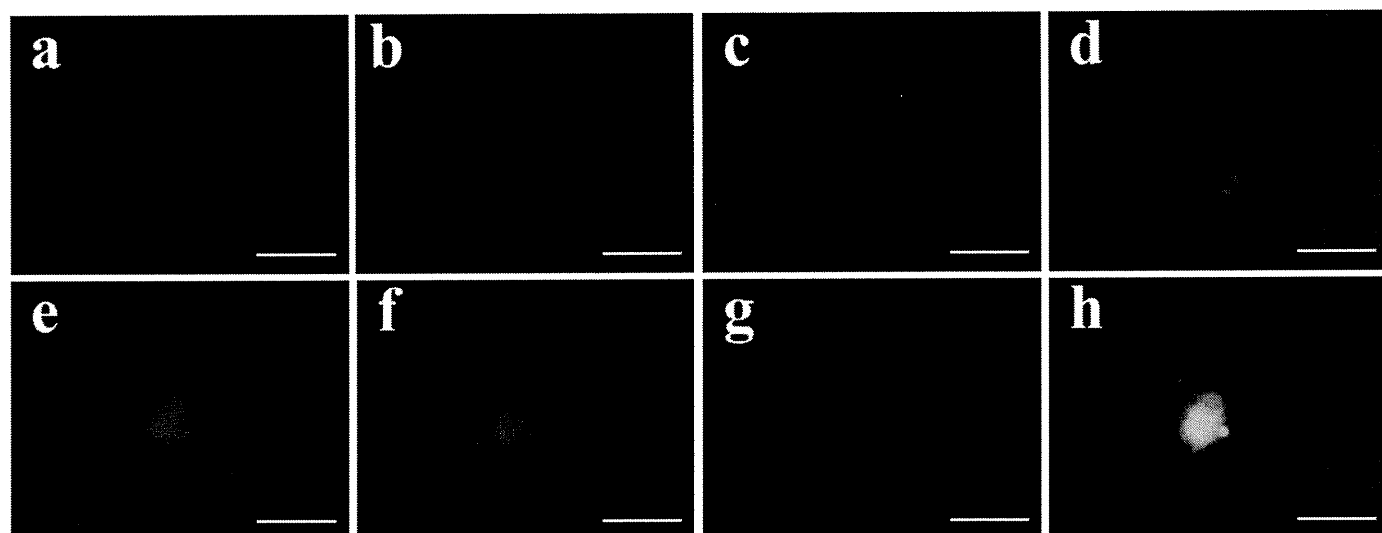


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Figure4

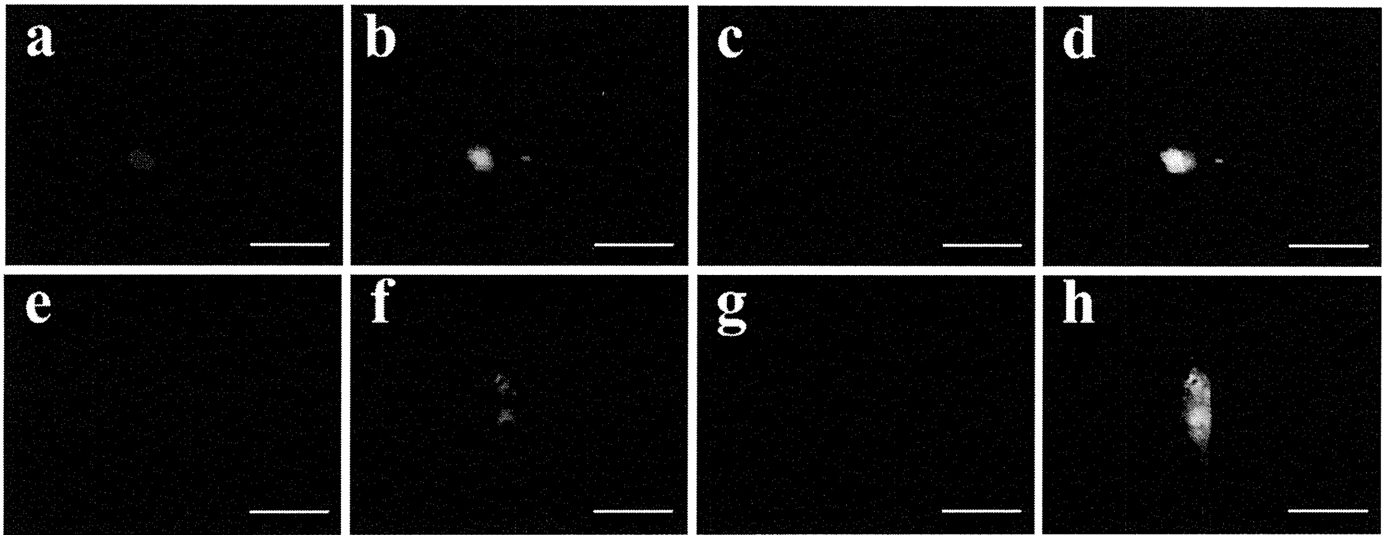


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Figure5

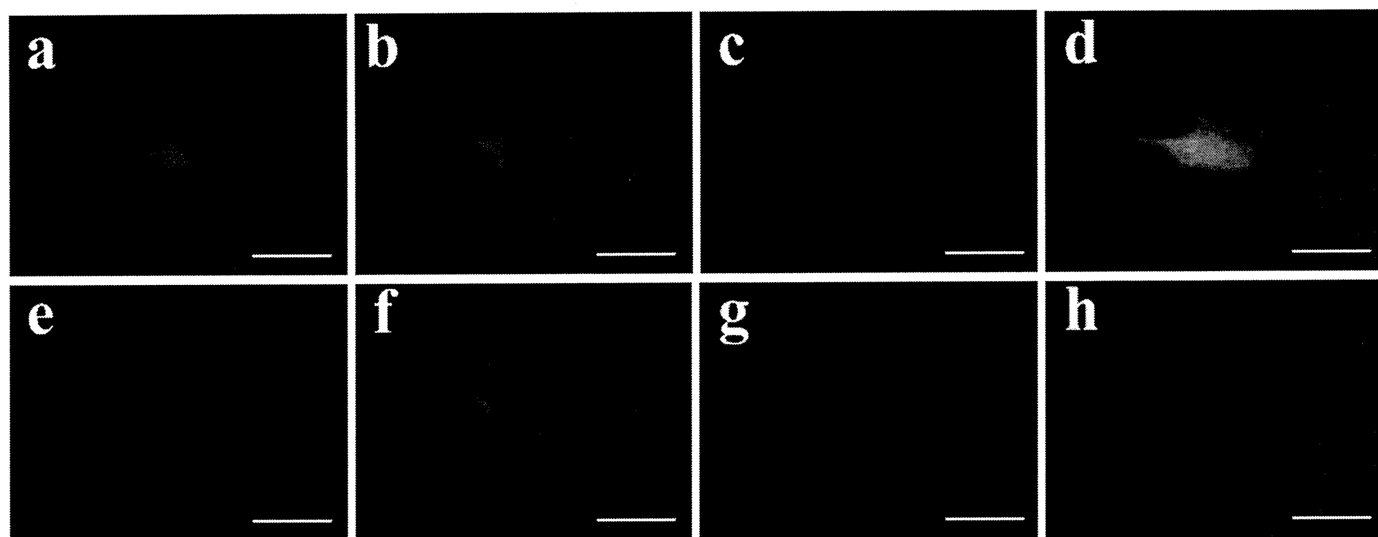


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Figure6

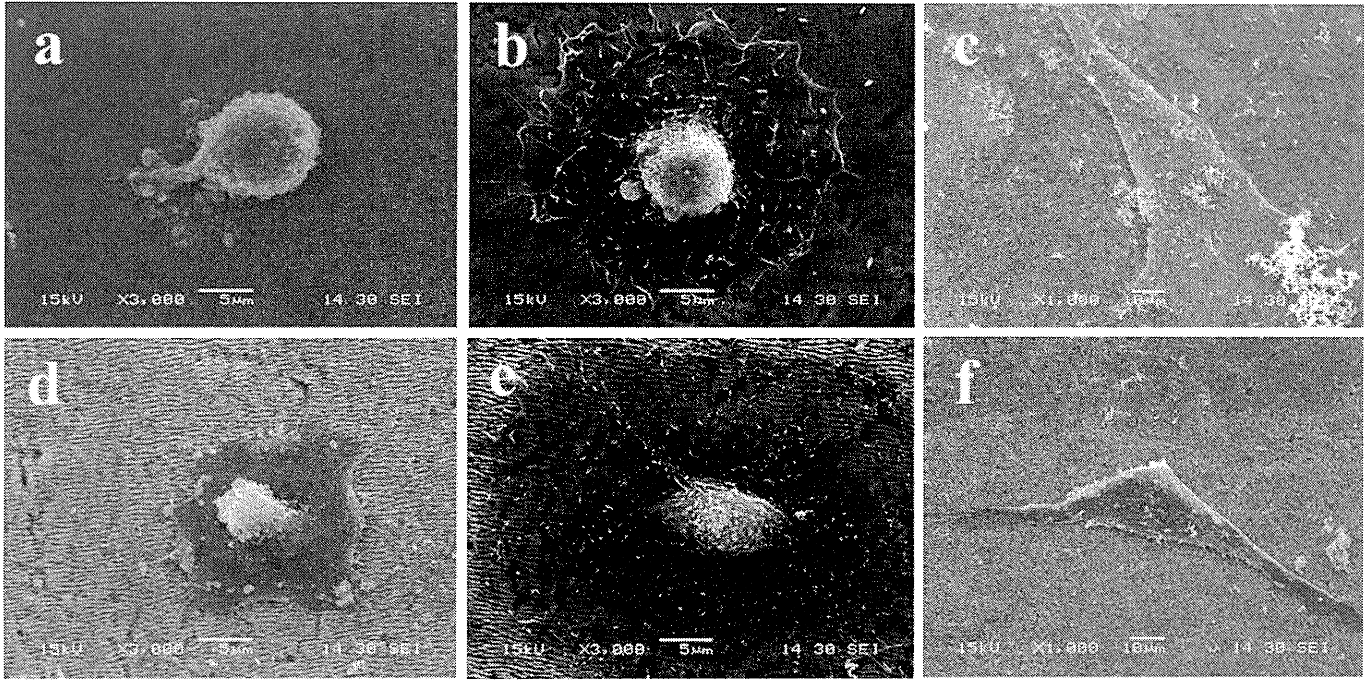
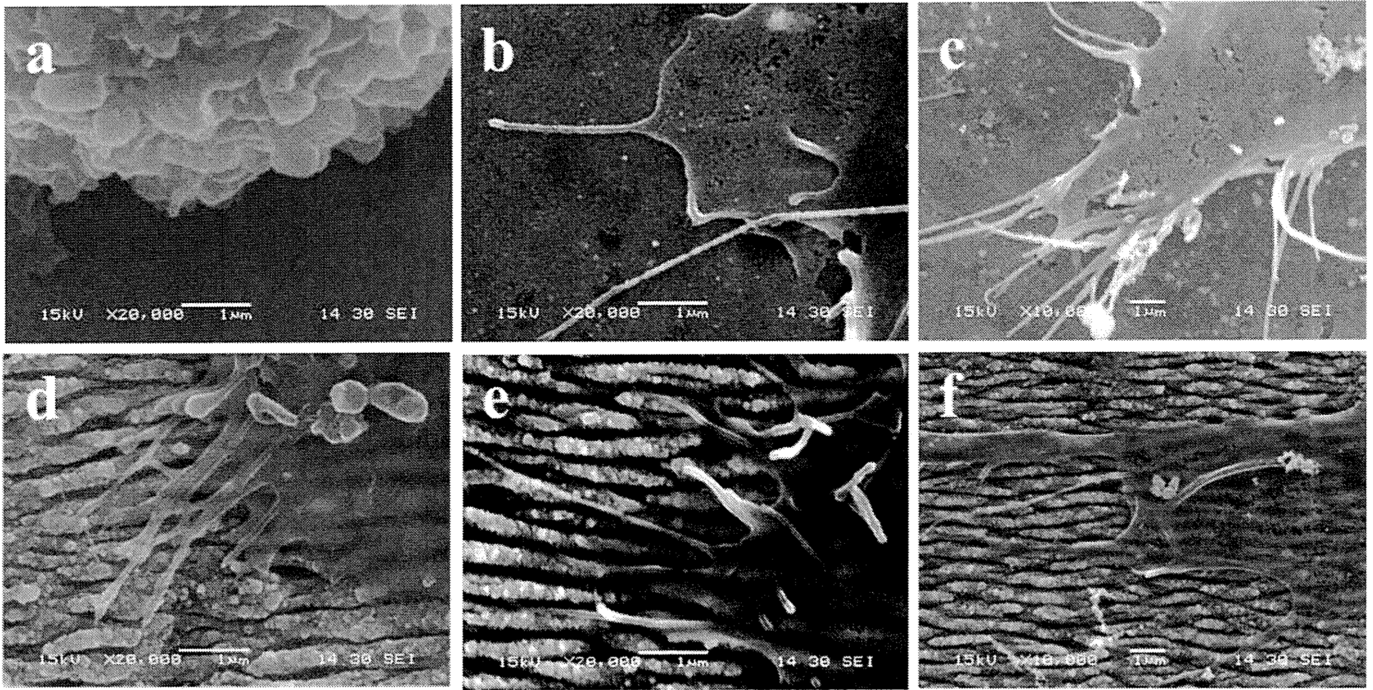


Figure7



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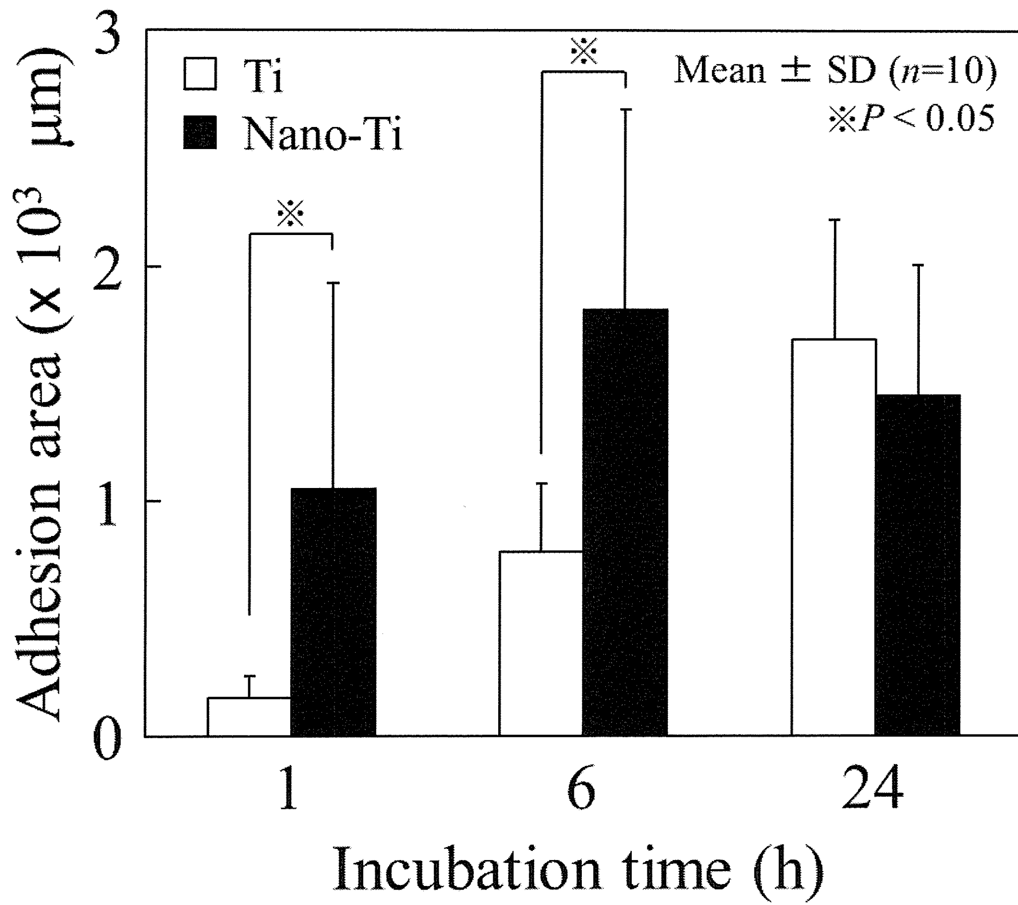


Figure9

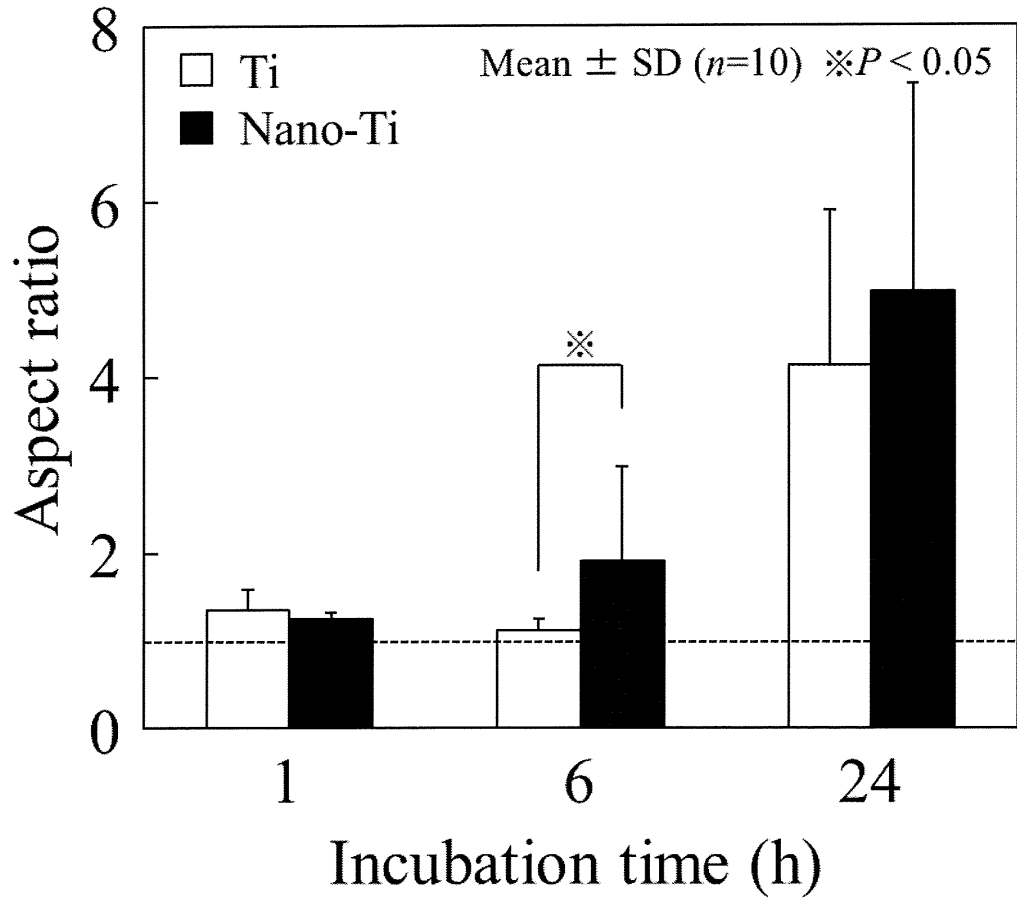
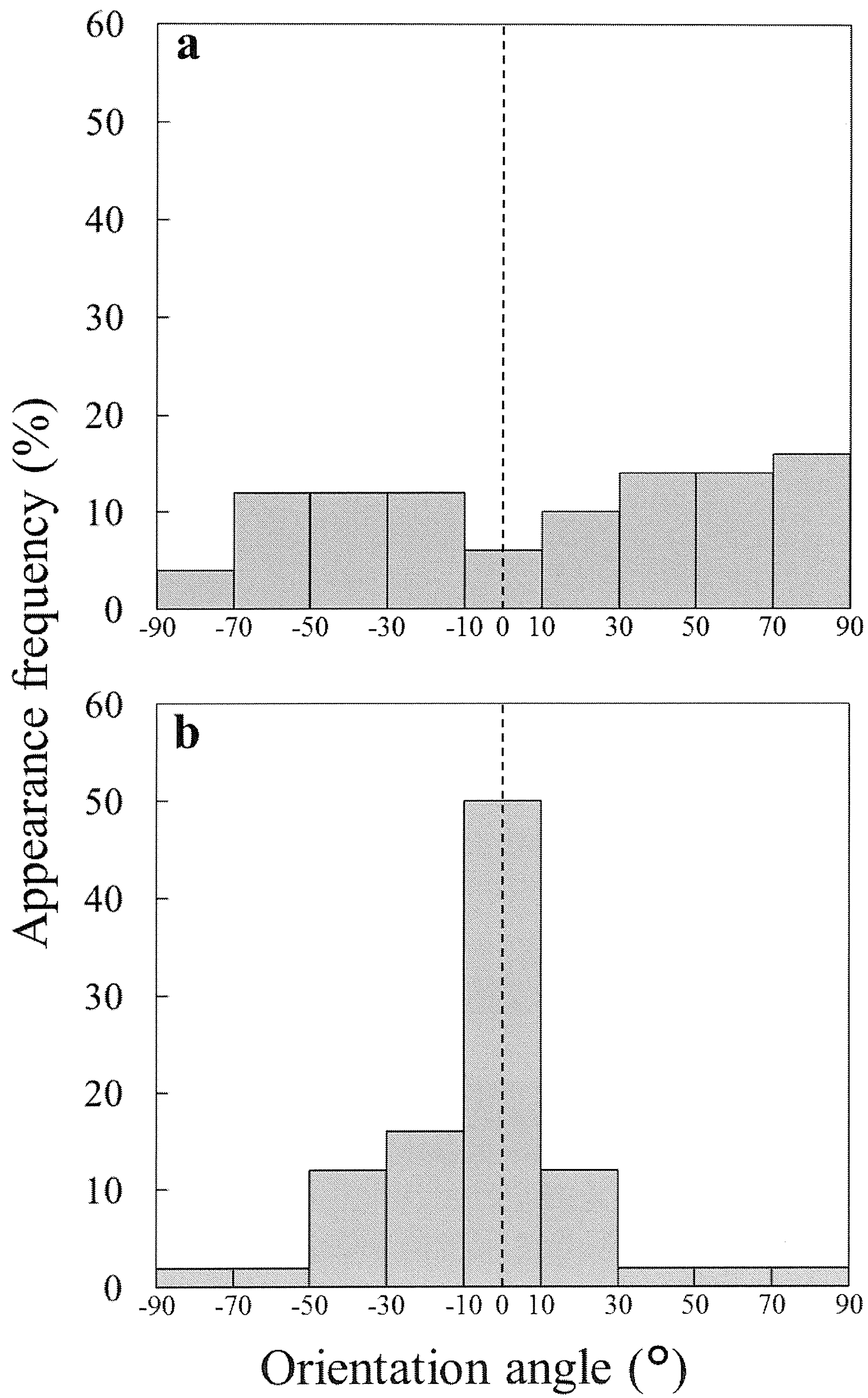


Figure10



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# Prolonged matrix metalloproteinase-3 high expression after cyclic compressive load on human synovial cells in three-dimensional cultured tissue

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*Y. Akamine, K. Kakudo, M. Kondo, K. Ota, Y. Muroi, H. Yoshikawa, K. Nakata: Prolonged matrix metalloproteinase-3 high expression after cyclic compressive load on human synovial cells in three-dimensional cultured tissue. Int. J. Oral Maxillofac. Surg. 2012; 41: 874–881.* © 2011 International Association of Oral and Maxillofacial Surgeons. Published by Elsevier Ltd. All rights reserved.

*Abstract.* Excessive mechanical stress is thought to be a factor in the development of joint disorders through the expression of matrix metalloproteinases (MMPs) and related cytokines. Although studies revealed that mechanical stress on the synovium induces MMP expression, it is still not known which MMPs prolonged high level expression. The authors focused on MMP-3, which is one of the major factors in joint disorders such as rheumatism and temporomandibular joint disorders. They examined mRNA and protein levels of MMP-3, other MMPs and related cytokines after loading stress. Human synovial cells were seeded onto a collagen scaffold and different magnitudes of cyclic compressive load were applied for 1 h. Time-dependent mRNA and protein levels for catabolic genes were examined after loading. mRNA expressions of MMP-1, MMP-3, MMP-9, IL-6, IL-8 and IL-1β increased after excessive compression. In particular, only mRNA of MMP-3 was up-regulated and maintained at a high level for 24 h after excessive loading. The concentrations of MMP-3, IL-6 and IL-8 in culture media after loading increased with excessive compression. These results may account for the pathomechanism of MMP-3 induced by cyclic load on synovial cells in joint disorders.

Key words: cyclic compressive stimulation; catabolism; temporomandibular joint synovial cells; collagen scaffold.

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Excessive mechanical stress on the articular joint is thought to erode the condylar cartilage and predispose the joint to osteoarthritis, and greatly contributes to the development of joint disorders[5,7,16]. This pathomechanism is the same as for temporomandibular joint disorders (TMJD). In particular, in anterior disc displacement cases, the posterior part of the synovium in the temporomandibular joint (TMJ) is continuously subjected to direct tensile, compressive and shear

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mechanical stress during various functions, such as chewing and bruxism (grinding), resulting in inflammatory changes of synovitis, such as pain, discomfort and functional disorder[17,20].

There have been many reports on the relation between the expression of matrix metalloproteinases (MMPs) and the onset of joint disorders[12,14]; but this biomechanical and biological pathomechanism has not been clarified sufficiently. Previously, the authors established an *in vitro* three-dimensional (3D) culture system with cyclic compressive load. The results showed that the expressions of mRNAs and proteins for MMP-1, MMP-2, and MMP-3 increased with the application of cyclic compressive loads for 1 h per day for 5 and 15 consecutive days[13,14]; but it remains unknown how the expressions of these genes change after cyclic compression for 1 h. The authors hypothesized that MMP-3, which is one of the major factors in TMJD, prolonged high level expression after mechanical stress.

To test this hypothesis, the authors examined mRNA expression levels and the protein production of MMP-3, other MMPs and related cytokines after 1 h cyclic compressive loads.

## Materials and methods

### Human synovial cell culture

Surgical specimens of human synovial membranes were obtained during arthroscopic knee surgeries. Patients gave informed consent for the use of surgical specimens for experiments. The authors repeated the experiment three times. Experiments 1 and 2 were performed using cells from a female donor aged 21 years. In the third experiment, the cells from a male donor aged 40 years were used. There were no differences in the morphological characteristics and biological responses of mechanical stimulation between donors. The cell culture method was the same as in a previous study[14]. Briefly, an excised synovium was digested with 0.2% collagenase in Dulbecco's modified Eagle's medium (DMEM). The liberated cells were resuspended in medium supplemented with 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were then cultured in a monolayer at 37 °C and in 5% $CO_2$ atmosphere. Cells from the fifth to seventh passage were used for experiments.

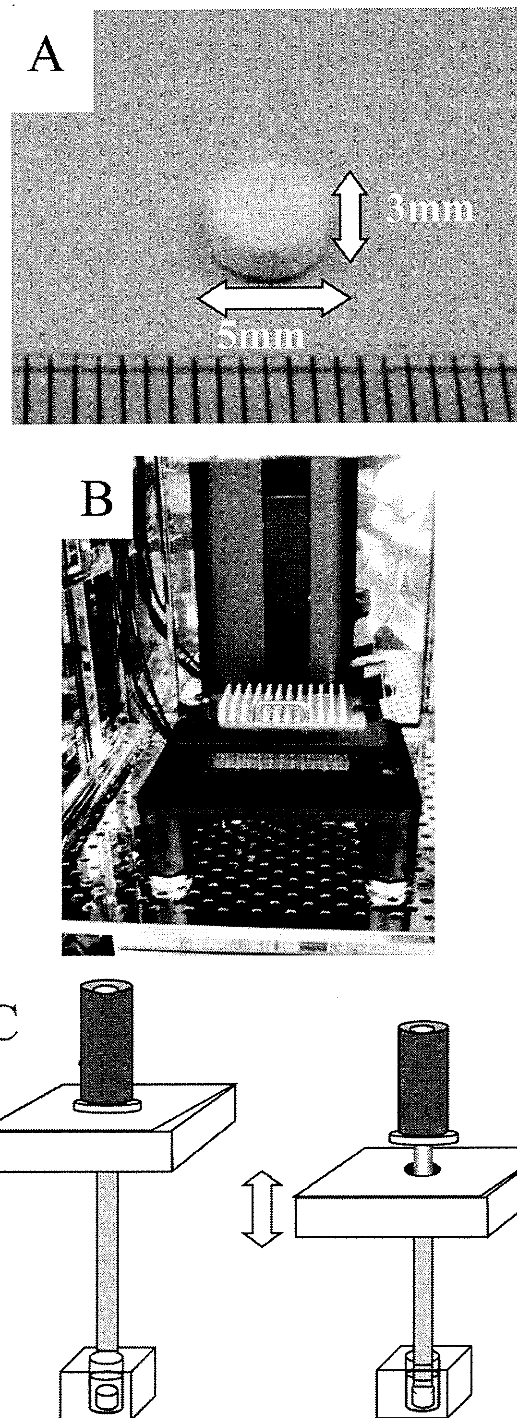


*Fig. 1.* Cyclic compressive loading experimental system. (A) Porous collagen sponge was produced as described by Nakata et al.[14] Briefly, type I collagen was extracted from bovine skin removal of telopeptide. Atelocollagen gel® was freeze-dried, cross-linked and sterilized with formaldehyde and γ-ray (10 kGy). (B) Custom-made apparatus, cyclic loading bioreactor, produces cyclic compressive loading under the culture conditions in an ordinary $CO_2$ incubator. (C) Cyclic load bioreactor, consists of cylindrical loading pistons connected to weights, a moving stage that raises and drops the loading pistons onto the 3D cultured tissue, and a linear actuator that controls the motion of the moving stage.

### Collagen scaffold preparation

A porous collagen sponge was produced as described previously[15]. Briefly, Atelocollagen® (KOKEN, Tokyo, Japan) gel was freeze-dried, and then cross-linked and sterilized with formaldehyde and γ-ray (10 kGy) to produce a porous collagen sponge (AteloCell® Atelocollagen sponge, MIGHTY; KOKEN). The pore size was designed to be 30–200 μm, and the pores were inter-connected. In this study, the

*Table 1.* Primer sequences.

| Gene name | Primer set | Sequences | Size (bp) |
|---|---|---|---|
| GAPDH | Forward | 5-tctctgctcctcctgttcgac-3 | 101 |
| | Reverse | 5-gttgactccgaccttcaccttc-3 | |
| MMP-1 | Forward | 5-cccaaaagcgtgtgacagtaag-3 | 113 |
| | Reverse | 5-cttccgggtagaagggatttg-3 | |
| MMP-3 | Forward | 5-cgtgaggaaaatcgatgcag-3 | 134 |
| | Reverse | 5-cttcagctatttgcttgggaaag-3 | |
| MMP-9 | Forward | 5-agtccacccttgtgctcttc-3 | 122 |
| | Reverse | 5-tttcgactctccacgcatc-3 | |
| MMP-13 | Forward | 5-cttcccaaccgtattgatgc-3 | 149 |
| | Reverse | 5-acttcttttggaagacccagttc-3 | |
| IL-6 | Forward | 5-gtgtgaaagcagcaaagagg-3 | 140 |
| | Reverse | 5-cctcaaactccaaaagaccag-3 | |
| IL-8 | Forward | 5-tggcagccttcctgatttc-3 | 109 |
| | Reverse | 5-gggtggaaaggtttggagtatg-3 | |
| IL-1β | Forward | 5-tccagggacaggatatggag-3 | 133 |
| | Reverse | 5-tctttcaacacgcaggacag-3 | |
| TIMP-1 | Forward | 5-gcatcctgttgttgctgtgg-3 | 141 |
| | Reverse | 5-aggtggtctggttgacttctgg-3 | |

porous collagen sponge, whose compressive modulus is close to that of native cartilage was 5 mm diameter and 3 mm thick to fit a 96-well cell culture plate (Fig. 1A).

### Cell seeding in collagen scaffold and 3D culture of cell-scaffold constructs

Collected cells ($5.0 \times 10^5$/scaffold) were resuspended in 1 × DMEM containing 10% FBS, 1% antibiotics, and 0.5% collagen solution (Atelocollagen® gel, KOKEN). The cell–collagen solution mixture was then seeded onto a collagen scaffold in a 96-well cell culture plate by centrifugal force for 5 min at 500 × *g* and incubated at 37 °C for 1 h to produce a 3D cell-scaffold construct. After gelation, 3D cultured tissue was incubated for 3 days before cyclic compressive load.

### Cyclic loading system and loading protocols

Intermittent dynamic loading was carried out using a custom-made apparatus, a cyclic loading bioreactor (CLS5J-Z®; Technoview, Osaka, Japan), described previously[14]. In this study, 20 kPa and 40 kPa were applied at 0.5 Hz for 1 h. This apparatus, consists of cylindrical loading pistons connected to weights, a moving stage that raises and drops the loading pistons onto the constructs, and a linear actuator that controls the motion of the moving stage. The pistons (and weights) are raised by the moving stage and then allowed to fall onto the 3D constructs without actually being attached to the moving stage, during loading, so stimulation of each sample is subject to constant peak load, due to the weight on each piston. The weights on the top of each piston are exchangeable, so that the cyclic load bioreactor can apply a designated peak load to each 3D tissue in the culture wells. Specimens were cultured in 96-well culture plates for loading of uni-axial unconfined compression (Fig. 1B and C).

### Histology

The constructs after cyclic loading for 1 h at 40 kPa were rinsed with PBS and fixed with 10% formalin. Paraffin sections were prepared for staining with haematoxylin and eosin ($n = 3$). For histological sections, three constructs were used.

### Time course of mRNA expression level

The constructs at pre-loading, and 0, 3, 6, 12, and 24 h post-loading were processed for extraction of total RNA using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Eighteen constructs were used this experiment at each time point by three constructs. After washing with 80% ethanol, RNA was treated with RNase-free DNase (DNase I; TAKARA Bio Inc., Shiga, Japan) to remove genomic DNA, and purified with phenol–chloroform. Single-strand cDNA was synthesized by reverse transcriptase (PrimeScript® RT reagent Kit; TAKARA Bio Inc.). The cDNA was synthesized during 15 min incubation at 37 °C with reverse transcriptase and a random hexamer primer, followed by enzyme inactivation at 85 °C for 5 s. The mRNA expressions of the specimens were quantitatively determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR) by Lightcycler® DX400 (Roche Diagnostics Ltd., Lewes, UK) with associated enzyme and reagents (SYBR® *Premix Ex Taq*™ II; TAKARA Bio Inc.). Primer sequences for MMP-1, MMP-3, MMP-9, MMP-13, interleukin-6 (IL-6), IL-8, IL-1β, and tissue inhibitor of MP-1 (TIMP-1) were designed using Primer3 on the Web (http://frodo.wi.mit.edu/primer3) (Table 1). PCR amplification of the cDNA samples was carried out by initial denaturing for 30 s at 94 °C, denaturing for 10 s at 94 °C, and annealing for 20 s at 60 °C for 40 cycles ($n = 9$).

### Assay for protein production in culture media

Protein concentration in culture media from 24 h after loading was measured in six constructs of each group by enzyme-linked immunosorbent assay (ELISA) or homogeneous time-resolved fluorescence (HTRF®; Cisbio International, Bagnols, France). ELISA was performed using a commercially available kit for MMP-1 (matrix metalloproteinase-1 (MMP-1) Biotrak activity assay system; GE Healthcare Biosciences, Little Chalfont, UK) and MMP-3 (Panaclear® MMP-3, Daiichi Fine Chemical Co., Tokyo, Japan). IL-1β, IL-6 and IL-8 protein concentrations were examined by the Artemis TR-FRET

Microplate Reader for HTRF® (Cosmo Bio Co., Tokyo, Japan) with reagents (HTRF® package insert, Cisbio) according to the manufacturer's instructions ($n = 6$).

### Statistical analysis

Data from the experimental groups were analysed by one-way ANOVA and Tukey's HSD test for multiple comparisons between individual groups. Statistical significance was established at the $p < 0.05$ level. Three independent experiments were performed in triplicate.

## Results

### Histology of 3D construct

The cells in the 3D culture were evenly embedded in the collagen scaffold by haematoxylin and eosin stain. There was neither cell leakage nor tissue destruction of the 3D construct after loading at 40 kPa (Fig. 2A–C).

### Quantification of mRNA expression levels by real-time RT-PCR

The expressions of mRNA levels are shown in Fig. 3. The mRNA expression levels of MMP-1, MMP-3, MMP-9, IL-6, IL-8, and IL-1β in the 40 kPa-loaded sample were significantly higher than in the 0 and 20 kPa-loaded samples. MMP-13 and TIMP-1 expressions did not change after cyclic compressive load amongst different loading magnitudes. All but the MMP-3 expression level peaked 3 or 6 h after loading, and then decreased until 24 h after loading. The expression level of MMP-3 with 40 kPa load increased and the high level expression was prolonged for 24 h after the cyclic compressive load.

### Protein production in media

The concentrations of protein production in culture media are shown in Fig. 4. The concentrations of MMP-1 in culture media 24 h after loading were significantly higher in 20 kPa- and 40 kPa-loaded samples than in unloaded samples. The concentrations of MMP-3, IL-6 and IL-8 in culture media 24 h after loading were significantly higher in 40 kPa-loaded samples than in unloaded and 20 kPa-loaded samples. There were no statistically significant differences in the concentrations of IL-1β amongst groups.

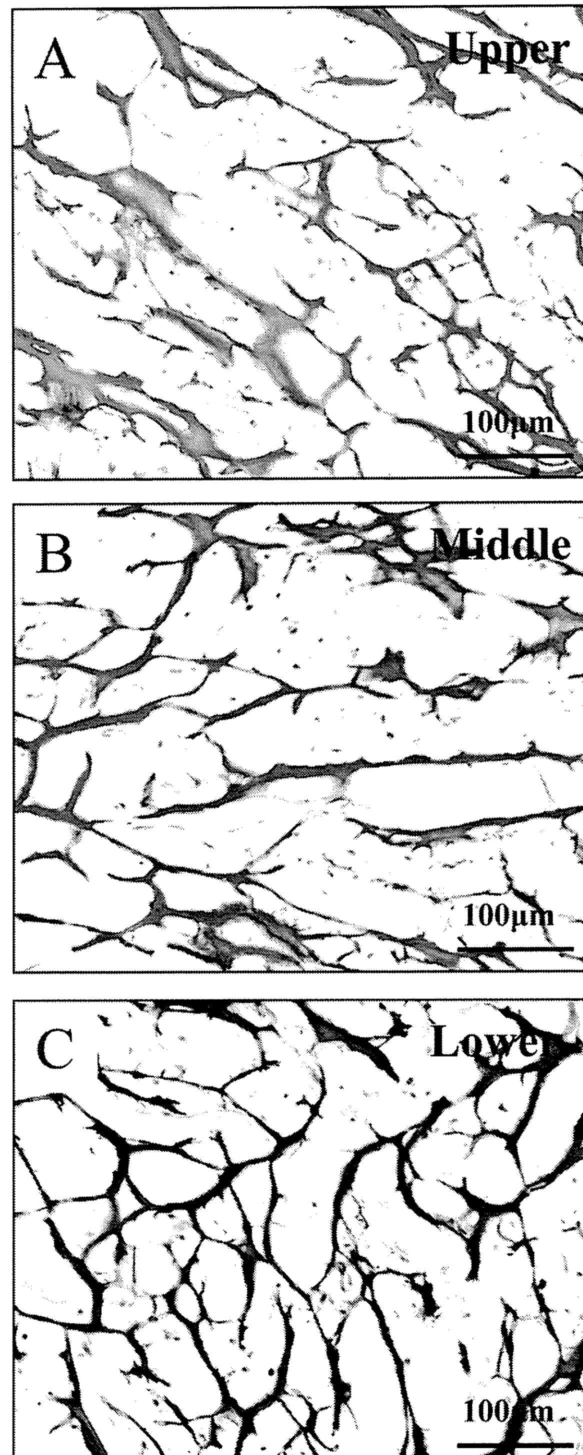


*Fig. 2.* Haematoxylin and eosin stained sections after cell seeding: (A) lower layer of construct; (B) middle layer of construct; (C) upper layer of construct. Bar = 100 μm. The cells in the 3D construct were evenly embedded in the collagen scaffold, with no cell leakage and collagen breakage after cell seeding (100×).

## Discussion

In anterior disc displacement, the posterior part of the TMJ synovium is subject to direct mechanical stress[19]. Excess direct mechanical stress on the posterior part of the TMJ synovium is thought to damage the synovial tissue and cause inflammation, since the mechanical properties of the synovium are too weak to

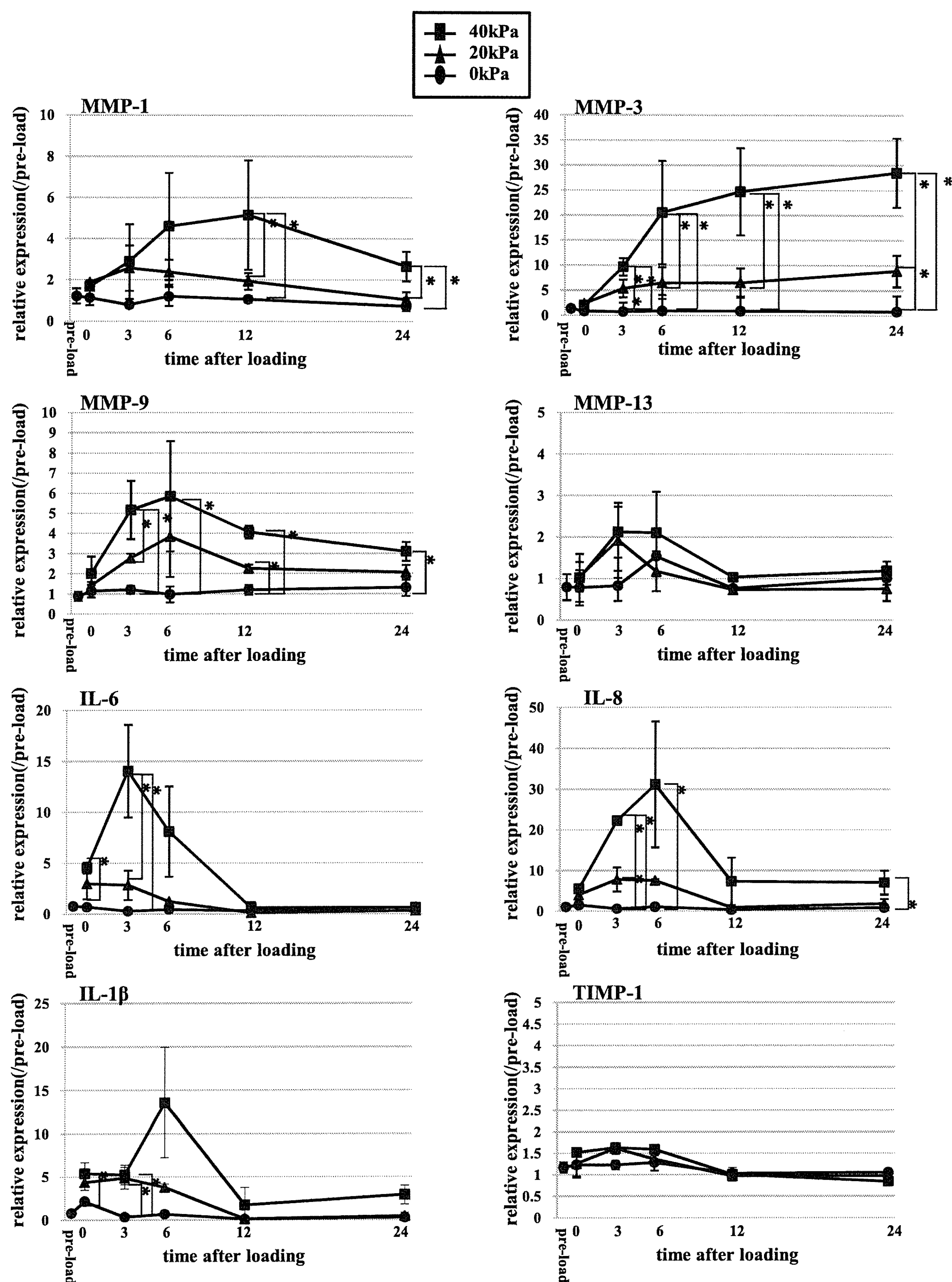


*Fig. 3.* Change in mRNA expression levels of MMP-1, MMP-3, MMP-9, MMP-13, IL-6, IL-8, IL-1β, and TIMP-1 after cyclic loading for 1 of 24 h. Real-time RT-PCR was performed with pre-loading, right after loading, 3, 6, 12, and 24 h after loading the sample. All mRNA expression levels were divided by that of GAPDH and the value at each time point was calculated by the relative quantity value based on pre-loading. Error bars indicate standard deviation ($*p < 0.05$).

bear compressive stress during chewing or bruxism[21]. In osteoarthritis (OA), synovial macrophages, synovial fibroblasts, and chondrocytes may induce the release of proteinases that destroy joint cartilage[11,19]. Known as the main proteinases of the human body, MMPs can degrade extracellular matrix and are expressed in the synovial fluid of TMJD patients[8]. Human TMJ synovial cells have been reported to synthesize MMP-1, MMP-3, and MMP-9 *in vitro*[9]. In the knee joint, it has been reported that synovial fluid of OA contains MMP-3 and MMP-9, and MMP-13[1,4,18].

In particular, MMP-3, stromelysin, is known as a proteoglycan-degrading enzyme and is recognized as an important

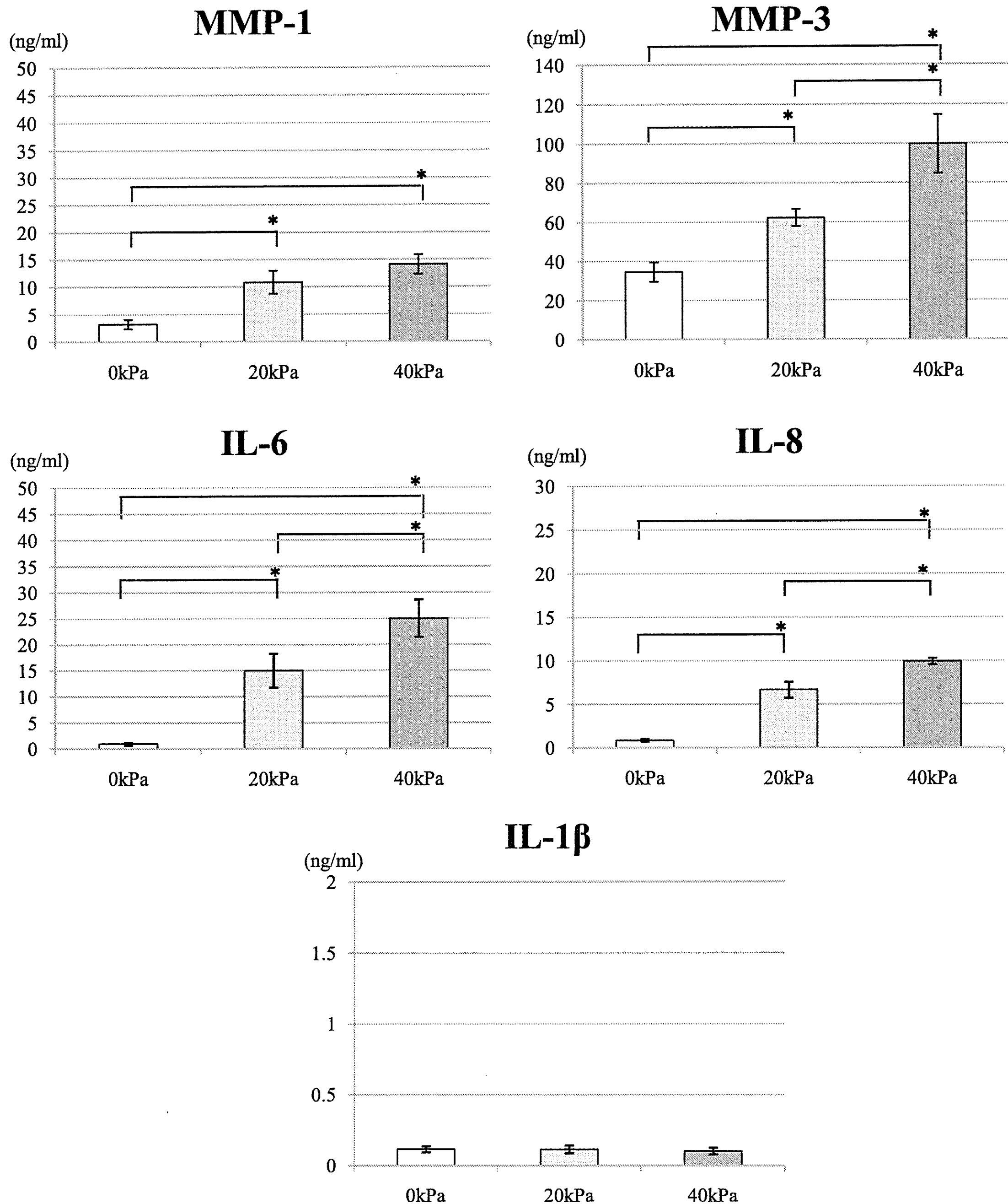


*Fig. 4.* Protein concentrations of MMP-1, MMP-3, IL-6, IL-8, and IL-1β in culture media from 24 h after loading were measured by ELISA or homogeneous time-resolved fluorescence (HTRF®) (*$p < 0.05$).

molecule in joint disease. In OA patients, the MMP-3 protein level of blood samples is often used in clinical diagnosis and indicates progressive joint destruction. Kubota et al. demonstrated that MMP-3 activity in synovial fluid in TMJ was greatly increased in patients with cartilage degradation[8]. They speculated that a high concentration of MMP-3 in synovial fluid is an important indicator of early joint deterioration in TMJ.

Previously, the authors examined the 5- and 15-day effects of cyclic compressive stress applied to human synovial cells using a custom-designed bioreactor to mimic the *in vivo* situation found in the human TMJ. From the previous results, the loading protocol did not affect the DNA content or the number of apoptotic cells[14]. This ensured that the protocol used was suitable for investigation of the biological molecular events of cells.

The authors chose cyclic compressive loading of 20 kPa or 40 kPa as physiological or excessive stress, respectively, because application of 20 kPa or 40 kPa induced approximately 5–8% or 15–20% deformation strain on 3D tissue. A frequency of 0.5 Hz was adopted because this condition was close to the human bruxism rate[10].

In the present study, the authors demonstrated that by the application of cyclic compressive loads at 40 kPa, mRNA expressions of MMP-1, MMP-3, and MMP-9 in 3D cultured human synovial cells tended to increase compared with 20 kPa-loaded and non-loaded groups. Amongst the up-regulated MMPs, only the expression of MMP-3 increased and high level expression was prolonged at 40 kPa for 24 h after compressive loading.

Protein levels of MMP-3 in the culture media 24 h after loading at 40 kPa were significantly higher than non-loaded or 20 kPa-loaded constructs. At 24 h after cyclic load, the level of mRNA expression of MMP-1 and MMP-9 genes fell; that of MMP-3 at 24 h after cyclic load was still high, indicating that MMP-3 mRNA expression was prolonged. It was speculated that a prolonged high level of mRNA expression and protein concentration of MMP-3 after excessive compressive loading accounted for deterioration of the extracellular matrix in TMJ, resulting in OA.

The level of mRNA expressions of MMP-1 and MMP-9 peaked at 6–12 h and decreased at 24 h after loading. The time course of the MMP-3 mRNA level was unique amongst mRNA levels of MMPs and ILs, different from those of MMP-1 and MMP-9. These results implied that transcriptional regulation of the MMP-3 gene is different from that of other MMP genes after excessive compressive loading. Further studies are needed to elucidate the transcriptional regulation of MMP-3 and other MMPs by mechanical loading.

Several pro-inflammatory cytokines, such as IL-6, IL-8 and IL-1β, have been detected in the synovial fluid or synovial tissue[6] obtained from patients with internal derangement (ID) and OA of TMJ[2,3]. From the authors' results, mRNA levels of IL-6 and IL1β, pro-inflammatory cytokines were expressed at a high level in the early phase and decreased after compressive loading at 40 kPa.

Increased production of IL-6 is known to be associated with the disturbance of homeostasis, such as trauma or inflammation, and in primary OA chondrocytes in the early stage of OA[19]. In this regard, the authors' culture system may mimic the *in vivo* gene expression pattern of OA. The protein assay revealed that IL-6 in the culture media of 40 kPa-loaded samples was significantly higher than in other groups. Accumulation of the protein level of IL-6 by compressive stress is thought to affect connective tissue in the articular joint including the TMJ. In the present study, the mRNA expression level of IL-8 in the 40 kPa-loaded group was highest 3 h after loading and gradually decreased; but it was still significantly high 24 h after loading and the protein level in the culture media in the 40 kPa-loaded group was significantly higher than in other groups. IL-8 is an important cytokine for angiogenesis in the inflamed synovium. It was suggested that IL-8 also plays an important role as a cause of synovitis from excessive compressive loading in the TMJ.

The mRNA level of TIMP-1, an inhibitor of MMPs, did not change after cyclic compressive load. Taken together with the results of up-regulation of the MMP mRNA level, these findings account for catabolism by compressive stress.

There are some limitations of this study to extrapolate the results to the pathomechanism of TMJD. The authors used human synovial cells obtained from knee synovium because TMJ synovium was not available in sufficient quantities to perform this study. It is uncertain whether TMJ synovial cells show the same cellular reaction as knee synovial cells.

The results obtained give new insight into the biological pathomechanism of the relation between the expressions of MMPs, especially MMP-3, and the onset of TMJD.

In conclusion, the authors have demonstrated that a high level of mRNA and protein expression of MMP-3 was prolonged after cyclic compressive load on 3D culture tissue containing human synovial cells.

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## Competing interests

None declared.

## Ethical approval

This study approval was obtained from the ethics committee of Osaka University Graduate School of Medicine.

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