Table 1 Mean ± standard deviation of T1ρ values (ms) in the articular cartilage

	Femoral condyle				Tibia plateau	
	Distal area		Posterior area		Superficial	Deep
	Superficial	Deep	Superficial	Deep		
Medial compartment						
PCL injury	48.9 ± 3.2*	43.5 ± 4.3	48.8 ± 4.7	52.2 ± 5.5	51.2 ± 3.4*	41.7 ± 3.4*
Normal	43.5 ± 1.9	37.9 ± 2.7	43.8 ± 2.5	46.5 ± 2.6	43.9 ± 2.8	36.9 ± 2.7
Lateral compartment						
PCL injury	51.1 ± 2.8*	44.3 ± 4.1	49.6 ± 3.4*	50.4 ± 2.9*	46.7 ± 3.3*	38.4 ± 3.4*
Normal	44.3 ± 2.9	39.2 ± 4.5	42.4 ± 4.1	41.2 ± 3.2	39.3 ± 2.6	32.0 ± 3.5

$* p < 0.05$ compared with the normal control

time, which is increased in affected subjects compared with that in normal control subjects. However, T1ρ has a larger range and higher effect size than T2, suggesting the superior sensitivity of T1ρ mapping in the detection of early cartilage degeneration [13, 21]. In fact, the loss of proteoglycans is considered to be an initiating event in early OA, while neither the content nor the type of collagen is altered in early OA [5]. The reliability of T1ρ mapping has been reported by showing the correlation between T1ρ values and the histological staining of proteoglycans in the corresponding area [23]. This is the first report to assess early cartilage degeneration in athletes with chronic PCL deficiency. Several studies investigated cartilage degeneration associated with ACL injury and ACL reconstruction [1, 9, 22]. Young et al. [25] reported a case of PCL injury, which was examined by dGEMRIC within 6 months of injury. This report focused on the effects of bone collision at the first injury or subsequent inflammation rather than on the effects on the cartilage of repetitive mechanical stress resulting from abnormal kinematics of the knee during sports activities.

The current study indicated that the T1ρ values in PCL-deficient patients increased up to 50 ms. Tsushima et al. [23] reported the T1ρ values and histology of articular cartilage in advanced OA patients who underwent total knee arthroplasty. They macroscopically rated the severity of cartilage degeneration on a 5-grade scale (from 0 to 4): Grade 0, normal–smooth surface; Grade 1, swelling and softening; Grade 2, superficial fibrillation; Grade 3, deep fibrillation—(coarse fissuring of the cartilage surface); and Grade 4, subchondral bone exposure. According to this study, a T1ρ value of 50 ms corresponds to Grade 2. There was an apparent loss of proteoglycan staining in the area that showed a T1ρ value of 50 ms. The T1ρ values of well-preserved areas (Grade 0) were rarely as high as 50 ms, even though these values were routinely obtained in severe OA patients who underwent arthroplasty.

A number of studies have reported the long-term clinical results of non-operative treatment for PCL injury [2, 10, 17, 19, 20]. Several studies reported relatively favourable long-term results. Shelbourne et al. [19] reported the radiographic results of 68 out of 133 consecutive patients treated non-surgically after an average follow-up of 5.4 years. Fifteen per cent of patients had degenerative arthritic changes in the involved knee only. Patel et al. [17] also reported that 17 % of 58 consecutive cases showed degenerative changes in the medial compartment at an average follow-up of 6.9 years. In both studies, the posterior laxity measured with a KT-1000 arthrometer was approximately 3–9 mm, and the grade of laxity was not correlated with either radiographic OA change or clinical scores such as the Lysholm and Noyes scores. In contrast, Boynton et al. [2] reported relatively poorer results in 38 untreated patients at an average follow-up of 13.4 years. Radiographic evidence of articular degeneration was seen in the medial tibiofemoral compartment in 53 %, in the lateral tibiofemoral compartment in 20 %, and in the patellofemoral compartment in 13 % of cases. As time from injury increased, articular degeneration seen on radiographs increased. In addition, the incidence of radiographic degeneration was higher in patients with greater instability than in those with lower instability. Keller et al. [10] also reviewed the records of 40 patients with an average follow-up period of 6 years and showed that the longer the interval between injury and evaluation, the greater the incidence of radiographic degeneration. As these studies suggest, the risk of articular degeneration in the PCL-deficient knee is controversial. This is probably because articular degeneration is influenced by many factors such as level and type of sports activity, degree of instability, body weight, muscle strength, time elapsed after the injury, and age. In this study, a very high T1ρ value was observed in patients with the greatest knee laxity and highest level of sports activity. Smaller increases in T1ρ values were seen in patients with less laxity and/or lower levels of sports activity even though the interval between the injury and evaluation was large. These results suggest that the influence on cartilage damage varies among the factors, and thus, some cases do not show any radiographic evidence of degeneration in long-term clinical studies.

Many clinical studies reported the tendency for radiographic degeneration to occur in the medial tibiofemoral compartment [2, 17, 19, 24]. Most previous clinical studies adopted the 45° flexion weight-bearing posterior-anterior radiograph. In the current study, T1ρ values increased in both the medial and lateral tibiofemoral compartments. Both medial and lateral tibial plateau showed significant increases in the T1ρ values. Squatting during sports activities can generate a shear stress on the articular cartilage around the posterior part of the femur, and the force applied to the tibia posteriorly in the squatting position might increase as knee flexion increases. Therefore, the finding of an effect on the posterior part of the femoral condyle in this study is reasonable and cannot be evaluated on plain radiographs. In fact, another advantage of an MRI study is that it allows a three-dimensional analysis. However, the patellofemoral joint, which is known as a predilection site of cartilage degeneration in PCL deficiency, was not examined in this study.

There were some limitations to this study. Since this was a cross-sectional study with small numbers of subjects, it could not be concluded that the cartilage degeneration detected by T1ρ MRI mapping would cause symptomatic OA in the near future. None of the cases showed an apparent radiographic OA finding even though the interval between injury and evaluation was long in two cases. It is unclear whether the findings observed in T1ρ MRI mapping had deteriorated over time since the injury. Even so, it could not be concluded that the MRI findings observed in this study were meaningless. Patients were enrolled because they did not show OA findings. As mentioned above, many factors influence the risk of OA. A longitudinal large-scale study could disclose significant risk factors for the development of OA. Nevertheless, since many clinical studies have proved that some patients actually develop OA, the detection of cartilage degeneration by T1ρ MRI mapping would help evaluate the present status of the injured knee. A bone scintigraph could be an alternative method to evaluate osteoarthritic change as it has been reported to be associated with the severity of OA [12]. The utilisation of single photon emission computed tomography would allow the three-dimensional localisation of cartilage damage. However, T1ρ MRI directly detects the degenerative changes in the cartilage, while bone scintigraphy detects the subchondral bone metabolisms; therefore, the latter might be less sensitive during the early stage of cartilage changes than T1ρ MRI.

The characteristics of patients are heterogeneous. The type and level of sports activity, age, level of joint laxity, and time elapsed after injury are all different. This makes it difficult to come to a definitive conclusion concerning risk factors. Nevertheless, all the patients showed similar increases in T1ρ values at the indicated area in this study.

These results suggest that subclinical degeneration of the cartilage matrix is common in athletes with PCL deficiency.

Since this study did not include a group of patients who underwent PCL reconstruction, it is unclear whether surgical reconstruction would avert these cartilage damages.

Conclusion

This study suggests that T1ρ MRI mapping can detect unexpected cartilage degeneration in the well-functioning PCL-deficient knees of young athletes. One should be alert to the possibility of subclinical cartilage degeneration even when patients appear asymptomatic and have no degenerative findings on plain radiographs or conventional MRIs. This method is useful for the evaluation of lurking cartilage degeneration and the investigation of risk factors for the development of secondary OA.

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CCAAT/enhancer-binding protein β promotes receptor activator of nuclear factor-kappa-B ligand (RANKL) expression and osteoclast formation in the synovium in rheumatoid arthritis

Hidetoshi Tsushima, Ken Okazaki*, Kohei Ishihara, Takahiro Ushijima and Yukihide Iwamoto

Abstract

Introduction: CCAAT/enhancer-binding protein β (C/EBPβ) is a transcription factor that is activated in the synovium in rheumatoid arthritis (RA) and promotes expression of various matrix metalloproteinases. In this study, we examined whether C/EBPβ mediates the expression of receptor activator of nuclear factor-kappa-B ligand (RANKL) and drives osteoclast formation in primary fibroblast-like synoviocytes (FLS) from RA patients. The cooperation of C/EBPβ and activation transcription factor-4 (ATF4) in the regulation of the RANKL promoter was also investigated.

Methods: Immunofluorescence staining was performed for C/EBPβ, RANKL, and ATF4 in synovium from RA patients. Adenovirus expression vectors for two major isoforms, C/EBPβ-liver-enriched activator protein (LAP) and - liver-enriched inhibitory protein (LIP), or small interfering RNA for C/EBPβ, were used to manipulate C/EBPβ expression in RA-FLS. RA-FLS over-expressing C/EBPβ were co-cultured with peripheral blood mononuclear cells (PBMCs) to test osteoclast formation by tartrate-resistant acid phosphatase (TRAP) staining. A promoter assay for RANKL, a chromatin immunoprecipitation (ChIP) assay and an immunoprecipitation (IP) assay were also performed.

Results: Immunofluorescence staining showed colocalization of C/EBPβ, ATF4 and RANKL in RA synovium. Western blotting revealed the expression of C/EBPβ-LAP and -LIP in RA-FLS. Over-expression of either C/EBPβ-LAP or -LIP significantly increased the expression of RANKL mRNA, while C/EBPβ-LIP down-regulated osteoprotegerin (OPG) mRNA. The RANKL/OPG mRNA ratio was significantly increased by C/EBPβ-LIP over-expression. Knockdown of C/EBPβ with siRNA decreased the expression of RANKL mRNA. The number of TRAP-positive multinucleated cells was increased in co-cultures of PBMCs and FLS over-expressing either C/EBPβ-LAP or -LIP, but was more significant with LIP. C/EBPβ-LIP does not have a transactivation domain. However, promoter assays showed that C/EBPβ-LIP and ATF4 synergistically transactivate the RANKL promoter. ChIP and IP assays revealed the cooperative binding of C/EBPβ and ATF4 on the RANKL promoter.

Conclusions: We demonstrated that C/EBPβ, especially C/EBPβ-LIP in cooperation with ATF4, is involved in osteoclast formation by regulating RANKL expression in RA-FLS. These findings suggest that C/EBPβ plays a crucial role in bone destruction in RA joints.

* Correspondence: okazaki@med.kyushu-u.ac.jp
Department of Orthopaedic Surgery, Graduate School of Medical Sciences,
Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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Introduction

Cartilage degeneration and bone destruction are the main features of rheumatoid arthritis (RA) [1]. Inflammation pathways are involved in the catabolic processes of articular cartilage and bone degeneration in RA. Inflammatory cytokines such as IL-1β, TNF-α, IL-6, and IL-17 play significant roles in mediating inflammation and joint destruction. These cytokines are expressed in arthritic joints in RA and induce expression of receptor activator of nuclear factor kappa B ligand (RANKL) in the synovium [2]. RANKL is an essential factor for osteoclast differentiation [3,4]. Osteoprotegerin (OPG) is a decoy receptor that inhibits RANKL activation of osteoclastogenesis and reduces bone resorption [5]. RA synovium-induced RANKL stimulates osteoclast differentiation at sites where bone and RA synovial membranes contact each other.

Inflammatory cytokines in RA joints activate numerous transcription factors including nuclear factor-kappa-B (NF-κB), activator protein-1 (AP-1), janus kinase-signal transducer and activator of transcription (JAK-STAT) and the CCAAT/enhancer-binding protein (C/EBP) family. The C/EBP family consists of six members: C/EBPα, β, δ, ε, γ, and ζ [6]. C/EBPβ is an intron-less gene and has three major isoforms: 38 kD (liver-enriched activator protein Star (LAP*)), 36 kD (LAP) and 20 kD (liver-enriched inhibitory protein (LIP)) [7]. The isoforms, LAP* and LAP, each contain an N-terminal transactivation domain (TAD) and a chromatin remodeling domain. The LIP isoform lacks the TAD, although it retains DNA binding capability, and is generally recognized to be a dominant negative isoform.

Recent studies indicated that C/EBPβ is involved in differentiation of osteoblasts and osteoclasts both physiologically and pathologically. C/EBPβ activates osteocalcin gene transcription and promotes osteoblast differentiation [8-10]. For osteoclast differentiation, the C/EBPβ isoform ratio in mononuclear cells regulates osteoclastogenesis through V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MafB) [11]. C/EBPβ and RANKL are upregulated in GCT. C/EBPβ induces RANKL promoter activity in GCT stromal cells, which causes osteolysis [12]. In inflammatory chronic diseases such as RA, C/EBPβ is strongly induced in response to inflammatory stimulation. C/EBPβ is expressed in synovial tissues and chondrocytes of RA [13,14]. C/EBPβ plays a crucial role in cartilage degradation along with proteolytic enzymes such as matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-13, and aggrecanase-2 (a disintegrin and metalloproteinase with thrombospondin motifs-5: ADAMTS-5) in inflammatory arthritis. Hence, we hypothesized that an imbalance of C/EBPβ isoforms may upset skeletal integrity in RA by being involved in both cartilage and bone destruction.

In this paper, we investigated whether C/EBPβ mediates the expression of RANKL in RA synovium and consequently, whether it induces osteoclast formation. In addition, we analyzed the mechanism of RANKL and OPG expression by the C/EBPβ isoforms, C/EBPβ-LAP and -LIP, and by cooperation with activation transcription factor-4 (ATF4). Determining the mechanisms related to the regulation of RANKL expression and bone resorption by C/EBPβ may provide new insights into the development of potential therapies for RA patients.

Methods

Clinical samples

Tissue samples of synovium were obtained from patients with RA at the time of total knee arthroplasty (TKA) or synovectomy. Patients signed informed consent for providing tissue samples for this study. Subjects included seven RA patients (mean age, 60.3 ± 11.3 years), who fulfilled the 2010 American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) diagnostic criteria for RA [15]. All studies were performed under the approval of the Institutional Ethics Board of Kyushu University (approval number: 22–99) and in accordance with the tenets of the Declaration of Helsinki.

Isolation of human fibroblast-like synoviocytes

Human fibroblast-like synoviocytes were isolated from the synovium of RA patients (RA-FLS). Synovial tissues were minced into small pieces and digested with 2 mg/ml collagenase L (Wako, Osaka, Japan) for 90 minutes at 37°C. The collected cells were resuspended in DMEM supplemented with 10% FBS (Gibco, Gaithersburg, MD, USA). Adherent cells were used after three to five passages.

As a control for RA-FLS, human fibroblast-like synoviocytes (HFLS, Cell Applications, San Diego, California, USA), which is a cell line derived from normal synovial tissue, were also cultured in DMEM supplemented with 10% FBS.

Immunofluorescence staining

Specimens were incubated overnight at 4°C with primary rabbit polyclonal anti-C/EBPβ antibodies (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100, mouse monoclonal anti-RANKL antibodies (ab45039; Abcam, Cambridge, England) diluted 1:50, gout polyclonal anti-OPG antibodies (sc-8468; Santa Cruz Biotechnology) diluted 1:100, rabbit polyclonal anti-ATF4 antibodies (sc-200; Santa Cruz Biotechnology) or normal rabbit IgG (sc-2027; Santa Cruz Biotechnology) diluted 1:100, respectively. RA-FLS plated on glass coverslips were transfected with adenovirus expression vectors for C/EBPβ-LAP, -LIP or LacZ control [16] for 24 hours and then replaced with fresh medium. After 48 hours, immunofluorescence staining was performed.

Treatment of cells with cytokines

Confluent cultures of RA-FLS were subjected to serum-free medium for 24 hours. This medium was replaced with fresh medium containing cytokines as follows: IL-1β (R&D Systems, Minneapolis, MN, USA) at a concentration of 2 ng/ml, TNF-α (Sigma-Aldrich, St Louis, MO, USA) at 10 ng/ml, IL-6 (R&D Systems) at 10 ng/ml, and IL-17 (R&D Systems) at 100 ng/ml. Cells were cultured for a further 48 hours. Concentrations of cytokines were determined based on previous literature [17-19].

Western blotting

Nuclear proteins were isolated using Nuclear and Cytoplasmic Extraction Reagent (NE-PER; Pierce, Rockford, IL, USA). Protein samples were transferred onto nitrocellulose membranes and were treated overnight at 4°C with primary antibodies.

RNA extraction and real-time reverse transcription (RT)-PCR

Quantitative RT-PCR was performed with the LightCycler 2.0 system (Roche, Basel, Switzerland) using SYBR Premix Ex Taq (Takara Bio, Ohtsu, Japan). The primers were as follows: for C/EBPβ, 5'-AGTACAAGATCCGGCGCGAG-3' (sense) and 5'-TGCTTGAACAAGTTCCGCAG-3' (antisense); for RANKL, 5'-ATGAACTCCTTCTCCAC AAGCG-3' (sense) and 5'-CTCCTTTCTCAGGGCTG AG-3' (antisense; purchased from Takara Bio; oligo name HA137381F and R); for OPG, 5'-GCTTGAAACATA GGAGCTG-3' (sense) and 5'-GTTTACTTT GGT GCCAGG-3' (antisense); for ATF4, 5'-TCAAACCTCAT GGGTTCTCC-3' (sense) and 5'-GTGTCATCCAACGT GGTCAG-3' (antisense); and for GAPDH, 5'-GGTGAA GGTCGGAGTCAACGGA-3' (sense) and 5'-GAGGGAT CTCGCTCCTGGAAGA-3' (antisense). Data were normalized to the expression of GAPDH.

Osteoclast formation in a peripheral blood mononuclear cell (PBMC) and RA-FLS co-culture system

Peripheral blood was obtained from healthy donors. Isolated PBMCs $(2 \times 10^5$ cells/well) were resuspended in α-minimum essential medium (α-MEM) containing 10% FBS and 50 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems) and then seeded in 96-well tissue culture plates. Three days later, adherent cells were used for the co-culture system.

Isolated FLS were transfected with adenovirus expression vectors for 24 hours and then fresh medium containing 10% FBS was added. After 48 hours, FLS were added into the 96-well plate with cultured PBMCs in α-MEM containing 10% FBS and 50 ng/ml M-CSF. After 72 hours of co-culture, wells were stained for tartrate-resistant acid phosphatase (TRAP) (Primary Cell Co, Hokkaido, Japan). Osteoclasts were identified as TRAP-positive multinucleated cells that contained more than three nuclei.

Gene knockdown in RA-FLS

Predesigned small interference RNA (siRNA) for C/EBPβ (C/EBPβ siRNA-1 target sequence, 5'-CCCACGUGUAA CUGUCAGCtt-3' (sense) and 5'-GCUGACAGUUACAC GUGGGtt-3' (antisense)) or negative-control siRNA was purchased (Ambion, Austin, TX, USA). Transfection mixes were prepared using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). RA-FLS cells were cultured for 24 hours after transfection and then treated with 10 ng/ml IL-1β for 72 hours.

Human RANKL promoter reporter constructs

Promoter constructs for human RANKL were sub-cloned into the pGL-4.10 (luc2) vector (Promega, Madison, WI, USA). The 5'-upstream region (−1591 bp) of the human RANKL gene was prepared using human genomic DNA as a template (p-full). There are four putative binding sites for C/EBPβ between −1591 bp and +12 bp. A 2-bp mutation (AA to CC) was made at one site on the p-full construct using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

Plasmid transfection and luciferase assay

HeLa cells seeded in 12-well plates were co-transfected with 0.5 μg/well RANKL promoter constructs and various concentrations of pCMV-LAP, an expression vector of rat C/EBPβ-LAP directed by a cytomegalovirus promoter [20], or pCI-neo-LIP, an expression vector of rat C/EBPβ-LIP [21], or pCMV6-AC-GFP-tagged ATF4 (OriGene, Rockville, MD, USA), an expression vector of human ATF4, using Lipofectamine LTX (Invitrogen). pRL-SV40 (Promega) was used as an internal control. Luciferase activity was then assayed using the Dual-luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation (ChIP) assay

RA-FLS cells were transfected with the adenovirus vector C/EBPβ-LIP and incubated for 72 hours. A ChIP assay was performed with a ChIP Assay kit (Upstate Biotechnology, Lake Placid, NY, USA). The primers used in the PCR for RANKL promoter sequences were as follows: 5'-GAGGGCGAAAG GAAGGAAGGGGAG-3' (sense) and 5'-GGCGTTGGA GAGCCCTGGCCTCGG -3' (antisense), which amplified between −125 bp and +26 bp. For a negative control, sequence between −1727 bp and −1487 bp was used. The PCR products were amplified for 33 cycles.

Immunoprecipitation (IP)

Nuclear proteins were isolated from RA-FLS transfected with adenovirus vector C/EBPβ-LIP for 72 hours. The IP protocol used Dynabeads Protein A (Invitrogen). Anti-C/EBPβ antibodies, anti-ATF4 antibodies or normal rabbit IgG and Dynabeads-complex, respectively, were added to antigen-containing lysates. Proteins were separated by SDS-PAGE and immunoblotted using specific antibodies.

Statistical analyses

For *in vitro* investigations, nonparametric comparisons were performed using the Mann-Whitney U-test. P-values less than 0.05 were considered significant.

Results

Co-localization of C/EBPβ and RANKL in the synovium from RA patients

We initially examined C/EBPβ and RANKL expression by immunofluorescence staining in erosive areas of synovial tissue from RA patients. C/EBPβ and RANKL were expressed in RA synovial tissue (Figure 1A). The distribution patterns of C/EBPβ and RANKL were similar and both were strongly expressed in the synovial lining layer rather than in the sub-lining layer. The co-localization of C/EBPβ and RANKL in RA synovium suggests that C/EBPβ is involved in the regulation of RANKL expression.

Expression of C/EBPβ in RA-FLS after treatment with pro-inflammatory cytokines

Primary cultures of FLS were established and C/EBPβ expression was examined by western blotting. C/EBPβ-LAP* (38 kDa), –LAP (36 kDa) and -LIP (20 kDa) were detected with LIP showing dominant expression. There was a varying degree of C/EBPβ expression (Figure 1B). The difference in expression levels of C/EBPβ may depend on the history of the patients such as degree of inflammation at the time of sample collection, disease duration, or therapies. Human FLS from normal articular joints lacks C/EBPβ protein expression.

Next, we set out to determine whether pro-inflammatory cytokines could promote C/EBPβ protein in FLS. Western blots revealed that stimulation with IL-1β (2 ng/ml), TNF-α (10 ng/ml), IL-6 (5 ng/ml), or IL-17 (100 ng/ml) increased the expression of both LAP and LIP isoforms in nuclear extracts, whereas the samples without any treatment did not show expression of C/EBPβ protein (Figure 1C). Interestingly, the expression of LIP was higher than that of LAP as shown in experiments of primary cultured RA-FLS.

Overexpression of C/EBPβ regulates expression of RANKL and OPG in RA-FLS

RA-FLS cells were transfected with adenovirus expression vectors expressing C/EBPβ-LAP, –LIP or LacZ control. Western blots confirmed the exogenous overexpression of LAP or LIP in whole protein extracts isolated from transfected cells (Figure 2A). RANKL mRNA expression was examined by quantitative RT-PCR. The overexpression of LAP induced RANKL mRNA expression up to 80-fold compared to the LacZ control in a time-dependent manner. In RA-FLS transfected with the LIP vector, RANKL mRNA expression was increased approximately 6-fold (Figure 2A). We also investigated the expression of OPG.

Expression of OPG mRNA was upregulated by LAP in RA-FLS, whereas LIP significantly reduced OPG mRNA. Consequently, the RANKL-OPG ratio was highly upregulated in RA-FLS transfected with LIP (Figure 2B).

In addition, we examined whether C/EBPβ induced RANKL expression at the protein level by cell fluorescent immunostaining in a time-course experiment. The stimulated expression of C/EBPβ was observed in the nucleus of RA-FLS at 24 hours (Figure 2C). RANKL protein was localized in the cell cytoplasm of FLS over-expressing LAP or LIP at 72 hours. Similarly, the expression of OPG was also examined in a different series of experiments. The expression of OPG was stimulated by C/EBPβ-LAP, but not by C/EBPβ-LIP in RA-FLS at 72 hours.

C/EBPβ knockdown by siRNA reduced RANKL expression in RA-FLS

We assessed the effect of C/EBPβ knockdown on RANKL mRNA expression using siRNAs targeting C/EBPβ mRNA. Transfected cells were cultured with IL-1β. C/EBPβ knockdown significantly reduced RANKL mRNA expression by 50% after IL-1β treatment in RA-FLS (Figure 2D).

C/EBPβ induced osteoclast formation through RANKL expression in RA-FLS

To investigate whether C/EBPβ-induced RANKL expression stimulated osteoclast formation, we co-cultured RA-FLS, which were transfected with adenovirus vectors expressing C/EBPβ-LAP, –LIP or LacZ control, and PBMCs, which were isolated and stimulated with M-CSF. After three days of co-culture, formation of TRAP-positive multinucleated cells was observed in co-cultures of RA-FLS over-expressing LAP or LIP, but not in control cells. Interestingly, more multinucleated cells were induced in the co-cultures of RA-FLS over-expressing LIP than with LAP transfected cells (Figure 3A). As a negative control, we performed monotype cell cultures of RA-FLS over-expressing C/EBPβ, or PBMC over-expressing C/EBPβ. These cells did not form osteoclasts (Figure 3B).

C/EBPβ functions as an activator of the human RANKL promoter

We further analyzed the *in vitro* promoter activity of human RANKL using HeLa cells. A luciferase reporter gene construct containing –1591 bp of the RANKL promoter was co-transfected with the expression vectors for C/EBPβ-LAP (pCMV-LAP) or C/EBPβ-LIP (pCI-neo-LIP) into HeLa cells. RANKL promoter activity was upregulated in a dose-dependent manner with either LAP or LIP (Figure 4A). To identify the C/EBPβ responsive element in the RANKL promoter, mutation analysis was performed using site-directed mutagenesis. We created four single mutation constructs in the RANKL promoter: single mutation 1 (mut-1), mutation 2 (mut-2), mutation 3 (mut-3),
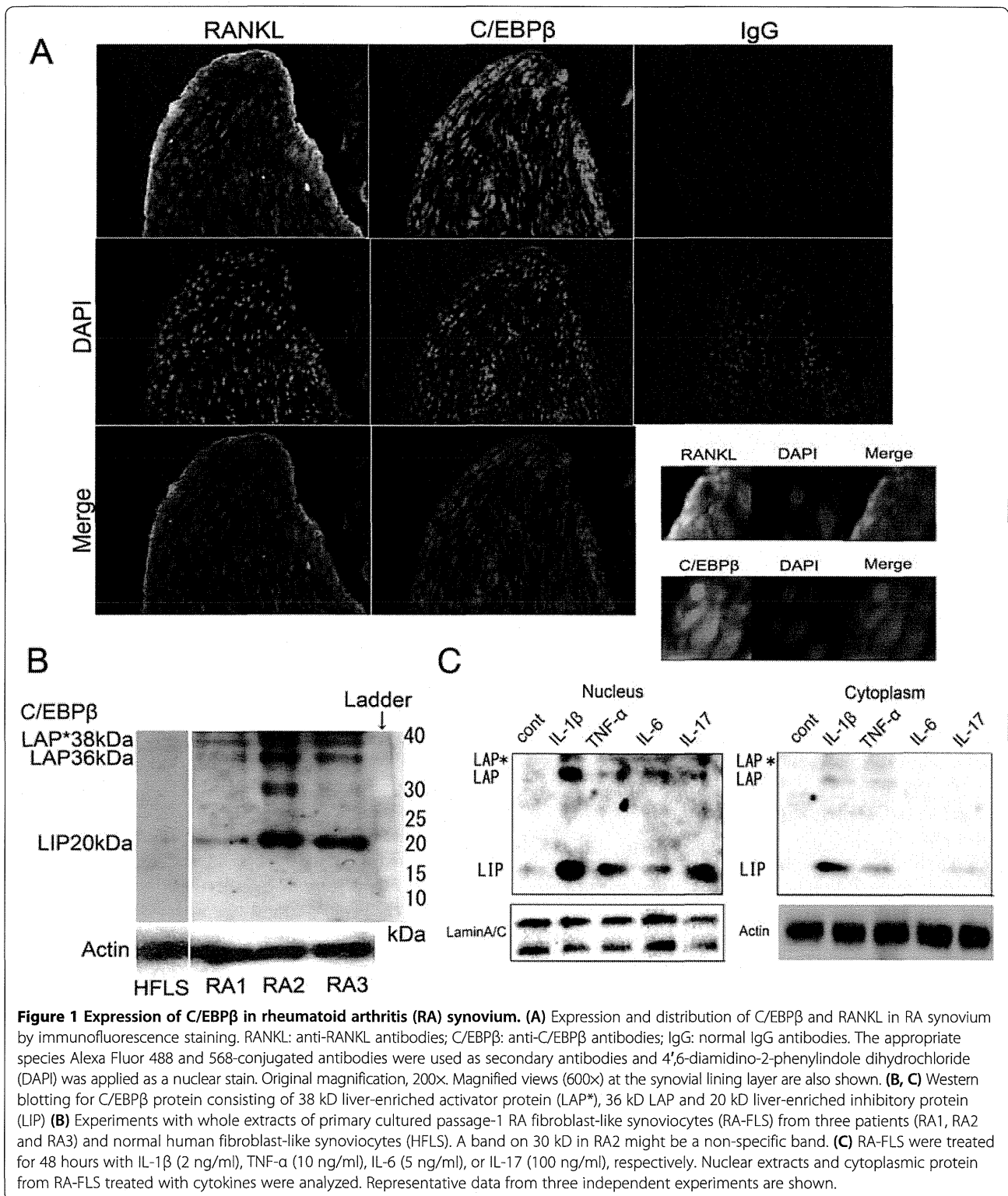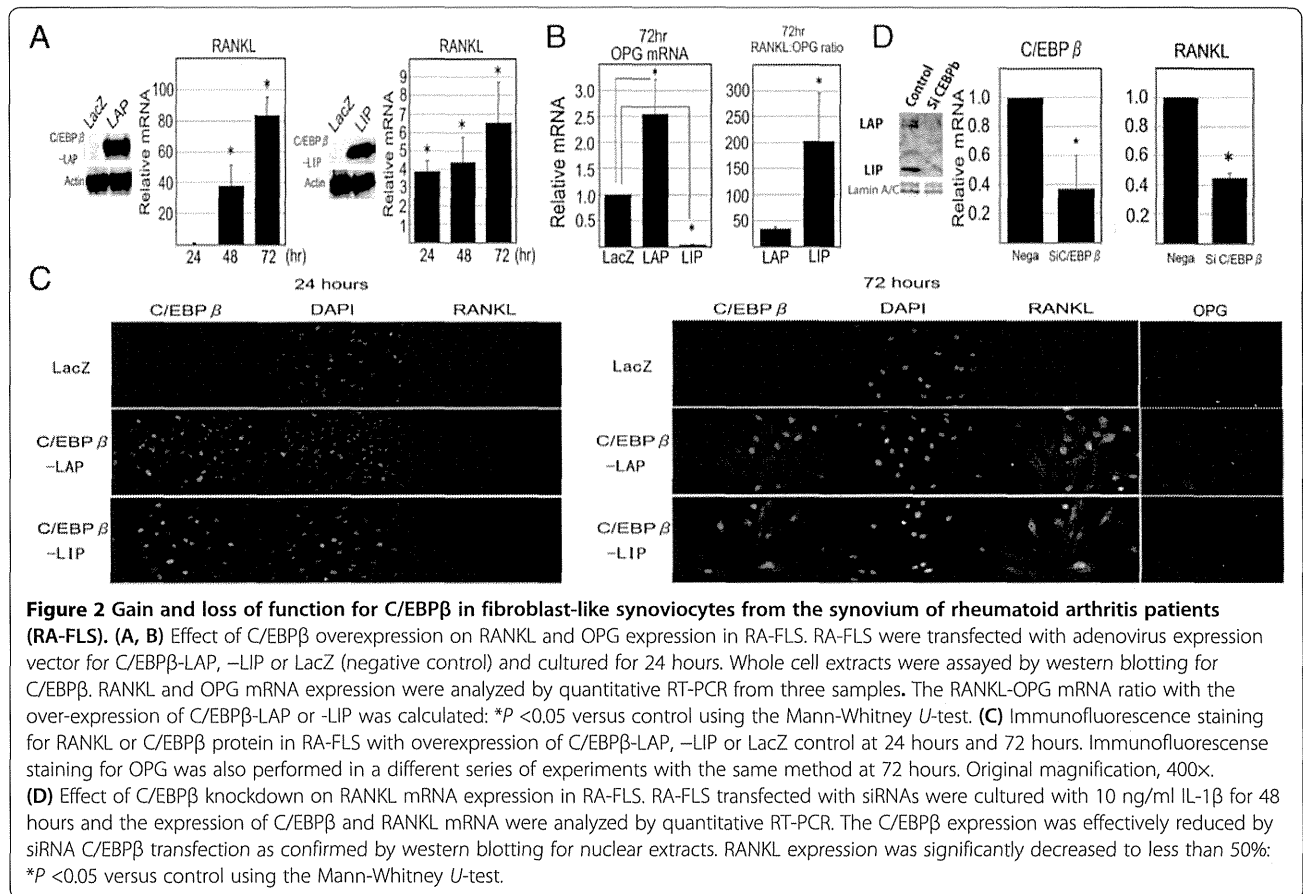
Figure 1 Expression of C/EBPβ in rheumatoid arthritis (RA) synovium. (A) Expression and distribution of C/EBPβ and RANKL in RA synovium by immunofluorescence staining. RANKL: anti-RANKL antibodies; C/EBPβ: anti-C/EBPβ antibodies; IgG: normal IgG antibodies. The appropriate species Alexa Fluor 488 and 568-conjugated antibodies were used as secondary antibodies and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was applied as a nuclear stain. Original magnification, 200×. Magnified views (600×) at the synovial lining layer are also shown. **(B, C)** Western blotting for C/EBPβ protein consisting of 38 kD liver-enriched activator protein (LAP*), 36 kD LAP and 20 kD liver-enriched inhibitory protein (LIP) **(B)** Experiments with whole extracts of primary cultured passage-1 RA fibroblast-like synoviocytes (RA-FLS) from three patients (RA1, RA2 and RA3) and normal human fibroblast-like synoviocytes (HFLS). A band on 30 kD in RA2 might be a non-specific band. **(C)** RA-FLS were treated for 48 hours with IL-1β (2 ng/ml), TNF-α (10 ng/ml), IL-6 (5 ng/ml), or IL-17 (100 ng/ml), respectively. Nuclear extracts and cytoplasmic protein from RA-FLS treated with cytokines were analyzed. Representative data from three independent experiments are shown.

and mutation 4 (mut-4). Luciferase activities of mut-1, mut-2, and mut-3 reporter constructs were equally increased with pCMV-LAP, while mut-4 reporter construct decreased luciferase activity by 30% (Figure 4B). Similarly, using the C/EBPβ-LIP expression vector, mut-2 and mut-3

did not show difference of activity from full reporter construct, while mut-4 decreased to 25%. These results showed that the putative C/EBPβ binding site is located between −59 bp and −52 bp in the RANKL promoter. The rationale for the increased activity of mut-1 with C/EBPβ-

Figure 2 Gain and loss of function for C/EBPβ in fibroblast-like synoviocytes from the synovium of rheumatoid arthritis patients (RA-FLS). (A, B) Effect of C/EBPβ overexpression on RANKL and OPG expression in RA-FLS. RA-FLS were transfected with adenovirus expression vector for C/EBPβ-LAP, –LIP or LacZ (negative control) and cultured for 24 hours. Whole cell extracts were assayed by western blotting for C/EBPβ. RANKL and OPG mRNA expression were analyzed by quantitative RT-PCR from three samples. The RANKL-OPG mRNA ratio with the over-expression of C/EBPβ-LAP or -LIP was calculated: *P <0.05 versus control using the Mann-Whitney U-test. **(C)** Immunofluorescence staining for RANKL or C/EBPβ protein in RA-FLS with overexpression of C/EBPβ-LAP, –LIP or LacZ control at 24 hours and 72 hours. Immunofluorescense staining for OPG was also performed in a different series of experiments with the same method at 72 hours. Original magnification, 400×. **(D)** Effect of C/EBPβ knockdown on RANKL mRNA expression in RA-FLS. RA-FLS transfected with siRNAs were cultured with 10 ng/ml IL-1β for 48 hours and the expression of C/EBPβ and RANKL mRNA were analyzed by quantitative RT-PCR. The C/EBPβ expression was effectively reduced by siRNA C/EBPβ transfection as confirmed by western blotting for nuclear extracts. RANKL expression was significantly decreased to less than 50%: *P <0.05 versus control using the Mann-Whitney U-test.

LIP expression vector has not been discovered yet as we failed to show the direct binding of C/EBPβ on this site.

C/EBPβ-LIP and ATF4 synergistically stimulate RANKL expression

C/EBPβ-LIP does not have an activation domain and is considered to be a dominant negative isoform. However, our results suggest that LIP is involved in RANKL expression in RA-FLS and plays a role in induction of osteoclast formation. We hypothesized that some transcriptional co-factors may cooperate with C/EBPβ-LIP to activate transcription of the RANKL promoter. ATF4 is known to stimulate RANKL expression in osteoblasts [22]. Additionally, ATF4 has been shown previously to interact with C/EBPβ, which activates various downstream factors such as osteocalcin and discoidin domain receptor tyrosine kinase (DDR2) [23]. Thus, we considered that a similar mechanism might exist for the regulation of RANKL gene expression. A luciferase assay showed that ATF4 slightly activated the RANKL promoter (Figure 4C). RANKL promoter activity was significantly enhanced following co-transfection of LIP and ATF4. Mut-4 abrogated the responsiveness of the RANKL promoter to the combination of LIP and ATF4. A ChIP assay was performed using RA-FLS over-expressing LIP and primers

constructed from the human RANKL promoter sequence, which amplify sites including the C/EBPβ consensus site-4. This analysis indicated that LIP binds to the RANKL promoter region containing CS-4 and that ATF4 also binds in the same region (Figure 4D). IP and immunoblotting demonstrated that ATF4 bound to over-expressed LIP in RA-FLS (Figure 4E). Collectively, these results suggest that C/EBPβ-LIP cooperates with ATF4 in activating RANKL gene expression.

ATF4 constitutively exists in RA synovium

We then examined the localization of ATF4 in RA synovium. ATF4 was observed in erosive areas of RA synovium by immunofluorescence staining (Figure 5A). Western blotting showed that ATF4 was expressed in whole cell extracts of RA-FLS (Figure 5B). Next, we examined whether ATF4 expression was affected by C/EBPβ in RA-FLS transfected with adenovirus expression vectors in time-course experiments. ATF4 mRNA expression was not significantly changed by C/EBPβ (Figure 5C). In addition, we performed organ cultures using RA synovium tissue. In RA synovium transfected with adenovirus LacZ control, ATF4 mainly localized in the cytoplasm of cells. Interestingly, in RA synovium over-expressing LIP, ATF4 was mainly located in the nucleus (Figure 5D). The results suggest that ATF4
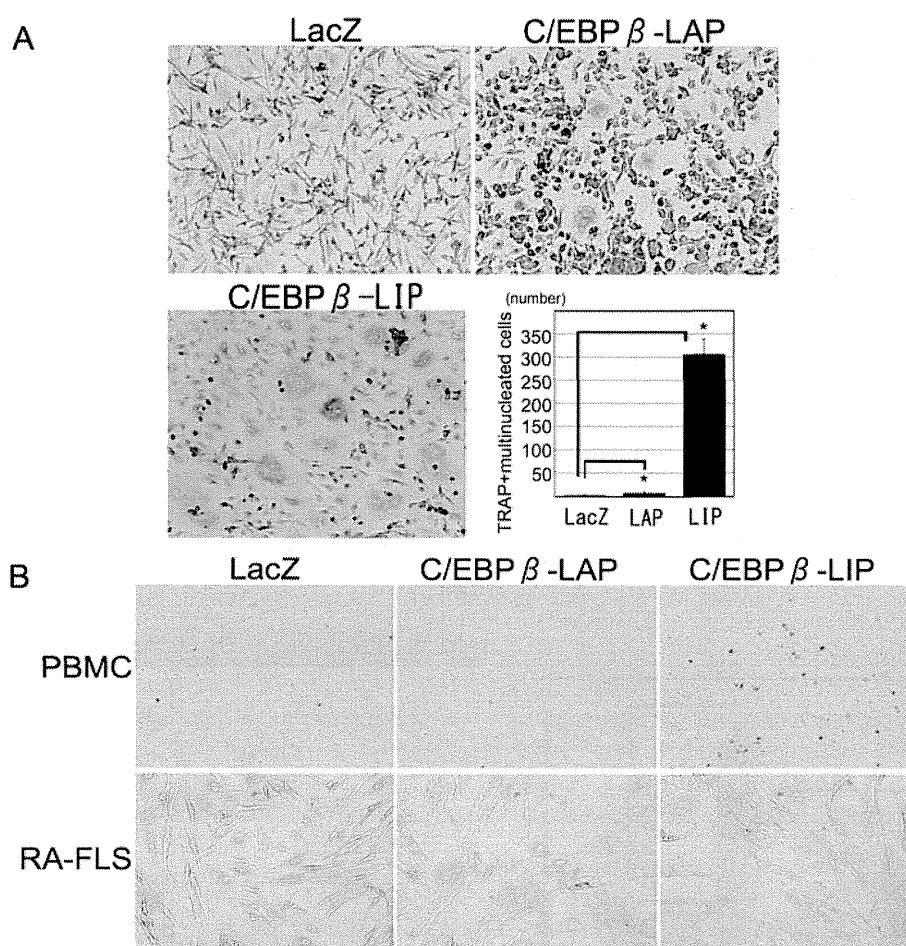
Figure 3 Osteoclast formation from peripheral blood mononuclear cells (PBMC) is promoted by the enhanced expression of C/EBPβ in fibroblast-like synoviocytes from the synovium of rheumatoid arthritis patients (RA-FLS). (A) Co-cultures of PBMCs and RA-FLS transfected with adenovirus vectors expressing C/EBPβ-LAP, −LIP or LacZ control for 72 hours. Number of TRAP-positive multinucleated cells recognized in the co-culture system. Osteoclasts were identified as TRAP-positive multinucleated cells that contained more than three nuclei. Original magnification, 100×. *P <0.05 versus control using the Mann-Whitney U-test. **(B)** Negative control for the co-culture experiments. PBMC or RA-FLS over-expressing C/EBPβ-LAP, −LIP or LacZ control, respectively, were cultured for 72 hours. No TRAP-positive multinucleated cells were observed.
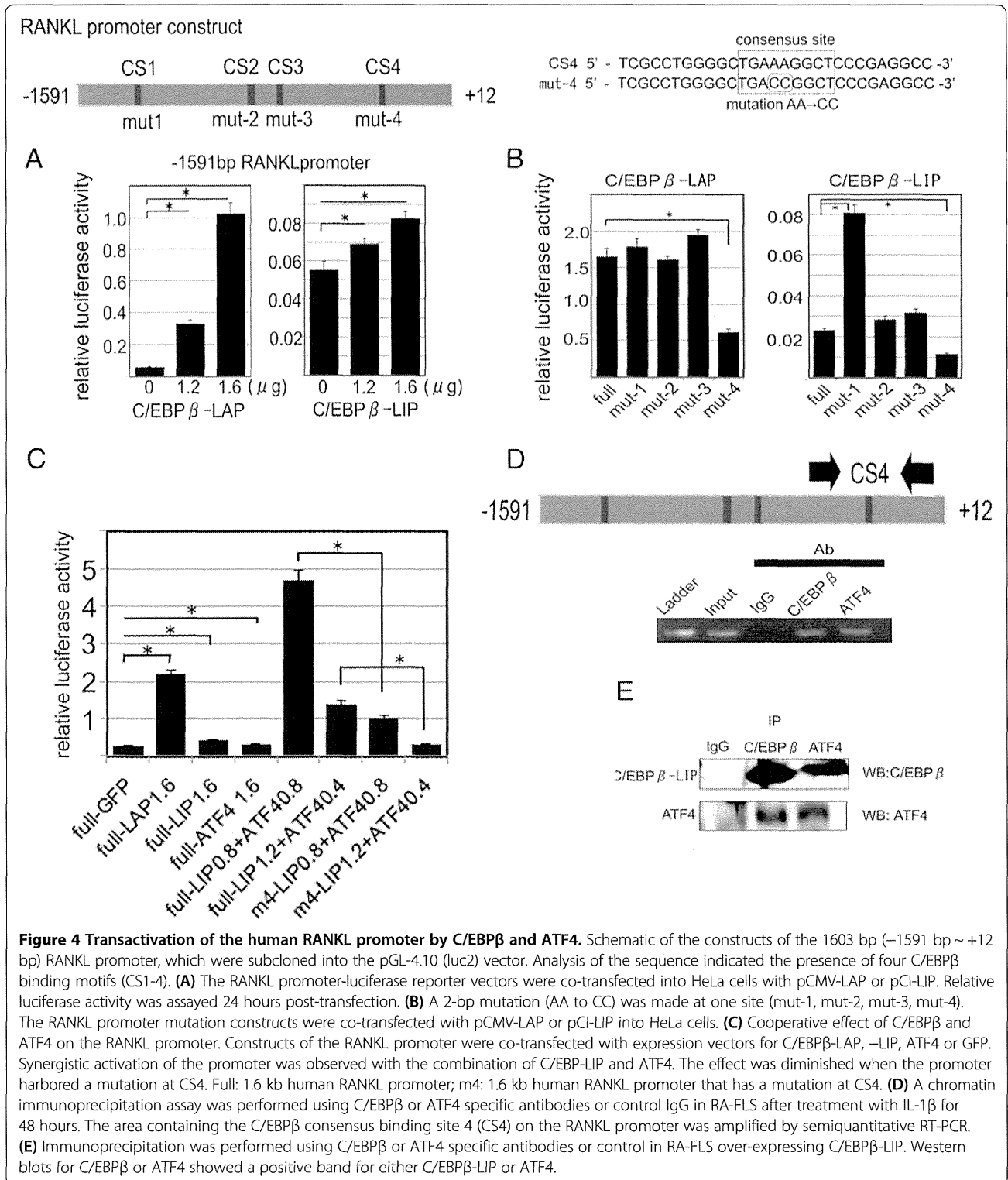
is translocated from the cytoplasm into the nucleus in the RA synovium overexpressing C/EBPβ-LIP although the mechanisms are not clear.

Discussion

In this study, we have shown that the transcription factor C/EBPβ promotes the expression of RANKL in RA synovium. C/EBPβ-induced RANKL in synovium could induce the formation of osteoclasts. This paper demonstrates that RA-FLS expressed the C/EBPβ-LIP isoform more dominantly than the C/EBP-LAP isoform in response to pro-inflammatory cytokines. C/EBPβ-LIP stimulated RANKL expression even though C/EBPβ-LIP lacks the transactivating domain. Recruitment of ATF4, which is constitutively expressed in the cytoplasm of RA-FLS, to the RANKL promoter might be the mechanism by which
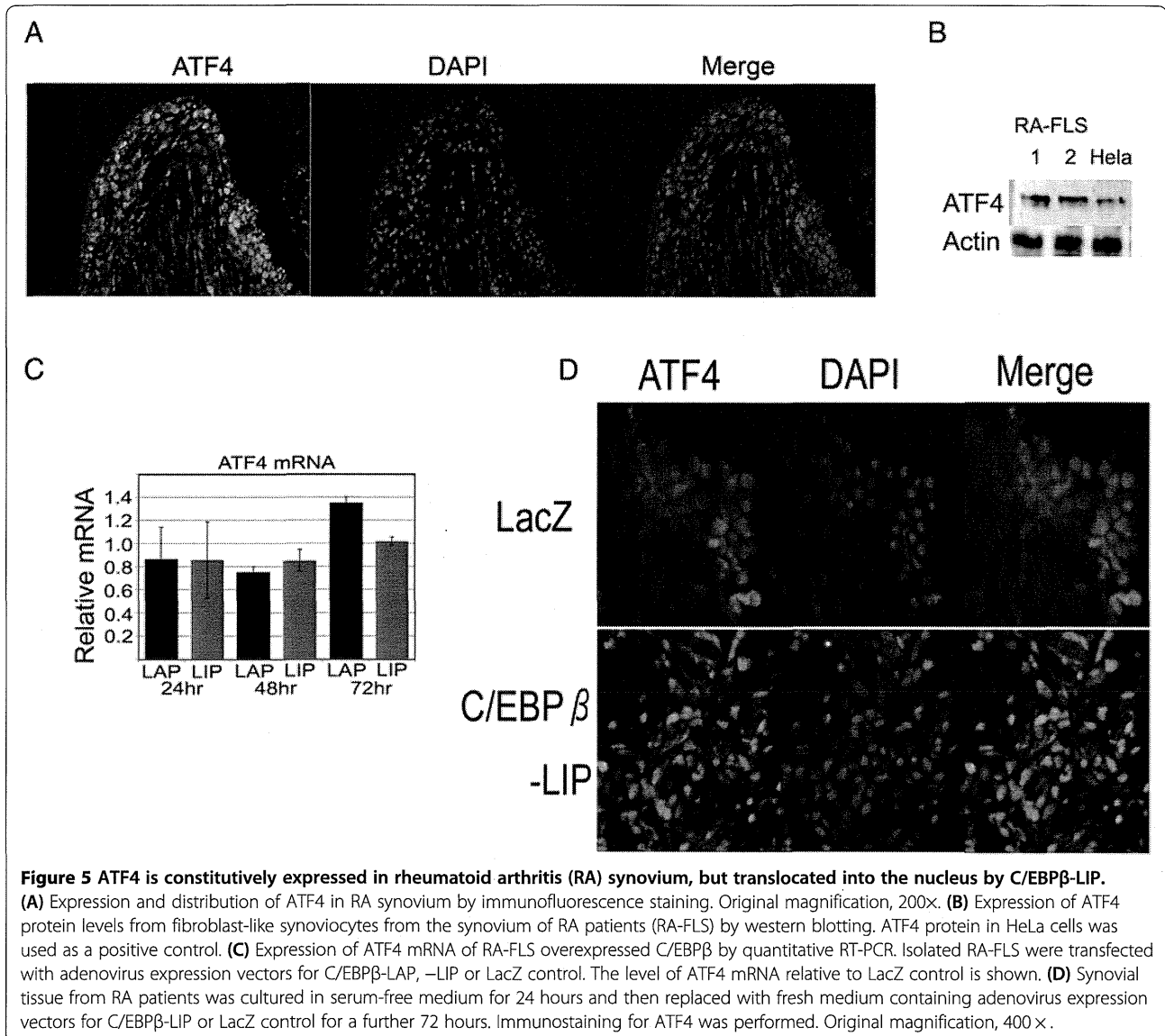
C/EBPβ-LIP activates the promoter. More interestingly, the effect of C/EBPβ-LIP in osteoclast formation is stronger than that of C/EBPβ-LAP. The lower expression of OPG might be involved in this mechanism. These results suggest that C/EBPβ-LIP is one of the key regulators of inflammation-induced osteoclast formation. As C/EBPβ is also involved in cartilage degradation [19], C/EBPβ may play a crucial role in joint destruction in RA.

Understanding the mechanisms that mediate RANKL gene expression may aid development of new therapies for reducing bone resorption in RA. We showed that LAP and LIP directly bind a site located between −59 bp and −52 bp of the RANKL promoter. LIP is increased in the RA synovium and forms a complex with constitutively expressed ATF4. This complex may activate transcription of RANKL by binding to the C/EBPβ binding

Figure 4 Transactivation of the human RANKL promoter by C/EBPβ and ATF4. Schematic of the constructs of the 1603 bp (−1591 bp ~ +12 bp) RANKL promoter, which were subcloned into the pGL-4.10 (luc2) vector. Analysis of the sequence indicated the presence of four C/EBPβ binding motifs (CS1-4). **(A)** The RANKL promoter-luciferase reporter vectors were co-transfected into HeLa cells with pCMV-LAP or pCI-LIP. Relative luciferase activity was assayed 24 hours post-transfection. **(B)** A 2-bp mutation (AA to CC) was made at one site (mut-1, mut-2, mut-3, mut-4). The RANKL promoter mutation constructs were co-transfected with pCMV-LAP or pCI-LIP into HeLa cells. **(C)** Cooperative effect of C/EBPβ and ATF4 on the RANKL promoter. Constructs of the RANKL promoter were co-transfected with expression vectors for C/EBPβ-LAP, −LIP, ATF4 or GFP. Synergistic activation of the promoter was observed with the combination of C/EBP-LIP and ATF4. The effect was diminished when the promoter harbored a mutation at CS4. Full: 1.6 kb human RANKL promoter; m4: 1.6 kb human RANKL promoter that has a mutation at CS4. **(D)** A chromatin immunoprecipitation assay was performed using C/EBPβ or ATF4 specific antibodies or control IgG in RA-FLS after treatment with IL-1β for 48 hours. The area containing the C/EBPβ consensus binding site 4 (CS4) on the RANKL promoter was amplified by semiquantitative RT-PCR. **(E)** Immunoprecipitation was performed using C/EBPβ or ATF4 specific antibodies or control in RA-FLS over-expressing C/EBPβ-LIP. Western blots for C/EBPβ or ATF4 showed a positive band for either C/EBPβ-LIP or ATF4.

motif of the RANKL promoter. ATF4 belongs to the ATF/cAMP responsive element binding protein (CREB) family, which contains a basic leucine zipper region, and is one of the major regulators of osteoblast differentiation [24]. Moreover, ATF4 regulates RANKL expression [22].

This study showed that ATF4 is expressed in RA synovium and is involved in RANKL expression. Interestingly, in *ex vivo* experiments (Figure 5D), ATF4 was mainly expressed in the cytoplasm of FLS transfected with the LacZ adenovirus vector (control), while ATF4 tended to

Figure 5 ATF4 is constitutively expressed in rheumatoid arthritis (RA) synovium, but translocated into the nucleus by C/EBPβ-LIP.
(A) Expression and distribution of ATF4 in RA synovium by immunofluorescence staining. Original magnification, 200×. **(B)** Expression of ATF4 protein levels from fibroblast-like synoviocytes from the synovium of RA patients (RA-FLS) by western blotting. ATF4 protein in HeLa cells was used as a positive control. **(C)** Expression of ATF4 mRNA of RA-FLS overexpressed C/EBPβ by quantitative RT-PCR. Isolated RA-FLS were transfected with adenovirus expression vectors for C/EBPβ-LAP, -LIP or LacZ control. The level of ATF4 mRNA relative to LacZ control is shown. **(D)** Synovial tissue from RA patients was cultured in serum-free medium for 24 hours and then replaced with fresh medium containing adenovirus expression vectors for C/EBPβ-LIP or LacZ control for a further 72 hours. Immunostaining for ATF4 was performed. Original magnification, 400 ×.

be located in the nucleus of FLS that overexpressed LIP. ATF4 in cooperation with C/EBPβ might be a crucial regulator of RANKL expression in mediating synovium-induced bone resorption in RA. Other transcription factors, such as NF-κB, AP-1, STAT3 and Runt-related transcription factor-2 (Runx2), may also interface with C/EBPβ. Runx2 and C/EBPβ cooperatively promote the expression of Indian Hedgehog in hypertrophic chondrocytes [25]. STAT3 is induced by IL-1β, TNF-α, and IL-6 and increases the expression of IL-6 and RANKL. A positive feedback loop, via IL-6 and STAT3, enhanced RANKL expression and osteoclastogenesis in inflammatory arthritis [26]. C/EBPβ was previously known as nuclear factor for IL-6 expression (NF-IL6) [27]. IL-6 induced by C/EBPβ regulates C/EBPβ gene transcription

with recruitment of STAT3 to the promoter of the C/EBPβ gene, especially in hepatocytes [28]. Therefore, we consider that positive feedback loops involving pro-inflammatory cytokines, IL-6, STAT3 and C/EBPβ, might strongly increase RANKL expression in joints with RA. Indeed, STAT3 is essential for stimulation of RANKL and its binding element is located at −82 bp of the RANKL promoter, which is next to the C/EBPβ responsive motifs. C/EBPβ-LIP may exist as an anchor to form a complex with other transcription factors mediated by inflammatory pathways.

The C/EBPβ isoform ratio can alter in response to cellular processes [7,29,30]. Of note, the LAP-LIP ratio is significant for osteoclastogenesis in PBMC through the mTOR pathway [11]. These papers indicate that an

appropriate LAP-LIP ratio results in higher transcriptional activation of the target gene, which is very important in proliferation and differentiation. We showed that LIP protein is more highly expressed in RA-FLS than LAP. This imbalance of the LAP-LIP ratio caused a concomitant change in OPG mRNA expression. OPG is an endogenous inhibitor of RANKL-RANK interaction and is produced in synovial cells of patients with RA. The balance between levels of RANKL and OPG (RANKL-OPG ratio) is correlated with the extent of bone resorption in RA joints [31]. The current study demonstrates that LIP drastically increased the RANKL-OPG ratio in RA-FLS, which subsequently induced significant osteoclast formation.

Previous studies showed that C/EBPβ-LAP is a key regulator of cartilage degradation in inflammatory arthritis. C/EBPβ-LAP plays a crucial role in cartilage degradation along with proteolytic enzymes such as MMP-1, MMP-3, MMP-13, and aggrecanase-2 (ADAMTS-5) in chondrocytes and FLS in inflammatory arthritis [19,32,33]. The role of LIP is not well investigated in inflammatory arthritis. Our unpublished data revealed that overexpression of LIP in FLS increased MMP-1, MMP-3, MMP-9, MMP-13, and ADAMTS-4 mRNA similar to the overexpression of LAP. The data presented here suggest that LAP and LIP coordinate in enhancing expression of RANKL, MMPs, and ADAMTSs, which may result in cartilage degradation and bone destruction of RA joints. C/EBPβ may be a common regulator, which can be stimulated in response to pro-inflammatory cytokines and upregulated in RA synovium. Therefore, selective blockage of C/EBPβ expression may be one potential strategy for preventing inflammation and bone resorption in arthritis.

This study has several limitations. First, double staining for C/EBPβ and RANKL did not work well on the RA synovium sections, although the reason was unclear. Therefore, we stained C/EBPβ and RANKL separately. However, the distribution of these molecules overlapped each other, suggesting that C/EBPβ and RANKL are co-expressed. Next, the promoter assays were performed in HeLa cells. We could not obtain reliable results of the promoter assay in RA FLS probably because of low transfection efficiency. However, the purpose of these experiments was to see the effect of various transcription factors that were exogenously introduced by expression vectors. Therefore, the influence of cell characteristics on the results is considered to be limited. Thirdly, the promoter that harbors a mutation in CS1 showed increased activity with C/EBPβ-LIP (Figure 4B). This result suggests that LIP act as a repressor in CS1. However, ChIP assay for CS1 sequences did not show binding of C/EBPβ on CS1 (data not shown). Therefore, the function of CS1 remains unclear at the moment.

Figure 6 Scheme of the involvement of C/EBPβ-LAP and -LIP for cartilage and bone destruction in rheumatoid arthritis (RA). Pro-inflammatory cytokines stimulate expression of C/EBPβ-LAP and -LIP in RA synovium. Synovium over-expressing C/EBPβ, particularly LAP, caused cartilage degeneration through MMPs and ADAMTSs. C/EBPβ-LIP promoted osteoclast formation through stimulation of RANKL and repression of OPG leading to bone destruction. ATF4 cooperates with C/EBPβ-LIP to stimulate RANKL expression.

Conclusions

In conclusion, C/EBPβ increased RANKL expression in RA-FLS and induced osteoclastogenesis (Figure 6). Pro-inflammatory cytokines significantly induced C/EBPβ-LIP, which strongly induced osteoclastogenesis by increasing the RANKL-OPG ratio in RA-FLS. LIP possesses transactivation activity for the RANKL promoter by recruiting ATF4, which constitutively exists in the cytoplasm of RA-FLS, to the C/EBP binding site in the RANKL promoter. In pathological inflammatory arthritis, C/EBPβ is a crucial factor in damaging cartilage and bone in joints.

Authors' contributions
HT participated in all the experiments and drafted the manuscript. KO conceived of the study, and participated in its design and coordination and helped to draft the manuscript. KI participated in the western blotting for RA-FLS and immunofluorescense staining of cells for OPG and helped to revise the manuscript. TU participated in the knockdown experiment with

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細胞だけで立体的な構造体を作製する
Scaffold-free 3D Biofabrication system の開発
A Development of Scaffold-free 3D Biofabrication System for Regenerative Medicine

中山功一

佐賀大学　大学院工学系研究科　先端融合工学専攻
先端融合工学講座　医工学　教授

特集 | バイオファブリケーション～機械で臓器を作る～

細胞だけで立体的な構造体を作製する
Scaffold-free 3D Biofabrication system の開発
A Development of Scaffold-free 3D Biofabrication System for Regenerative Medicine

―――中山功一*

再生医療研究では，立体的な臓器を人工的に作るためは，細胞と足場となる生体材料の組み合わせが世界の常識とされている。それに対し，筆者らは古典的な生物学の知見である細胞凝集と，骨折の外科的治療という，二つの古くからある知見を組み合わせ，全く新しい再生医療の技術を確立した。さらに複数のものづくり系企業と連携し，バイオ3Dプリンター（Biofabrication system）を開発した。稼働してまだ日が浅いが，予備的な実験で良好な成果が出始めている。将来的には自分自身の細胞だけで移植可能な臓器を体外で作製できることが期待されている。

1. はじめに

　臓器移植が本邦でも行われるようになって十年近く経過しているが，慢性的なドナー不足の問題はいまだ解消されていない。特に，移植大国とされる米国でも，一番のドナー発生要因とされていた自動車事故が，エアーバッグに代表される安全性能の飛躍的向上により臓器不足が深刻化していると伝え聞く[1]。このような深刻な臓器不足を背景に再生医療研究の究極の目標として，人工的に移植可能な臓器を生体外で作るというのは多くの再生医療研究者の夢であるといっても過言ではない。

　当時，九州大学整形外科で研修医として4年ほど関連する病院で研修を行った筆者は，2001年に臨床大学院に入り，研究テーマとして関節軟骨の再生を拝命した。ヒポクラテスの時代から一度

損傷した関節軟骨の自然治癒は困難とされていたが，体外で細胞を増殖して移植する方法により再生できるということが徐々に明らかになっていた頃であった。

　すでに欧米では自家培養軟骨細胞移植（ACI）が臨床フェーズに到達しており，中長期的な臨床データが報告され[2]，国内でもJ-TECのアテロコラーゲン包埋軟骨細胞移植が治験を開始しようとする時期であった。当時主流であった自家軟骨細胞移植は，患者さん自身の関節から内視鏡的に軟骨細胞を採取し，拡大培養を行った後に移植するという方法であったため，採取できる正常な軟骨細胞に甚大な限界があると認識されていた。特に軟骨細胞は単層培養で継代を続けると，脱分化して正常な軟骨組織に必須のII型コラーゲンの産生が低下するという問題があり，質の高い軟骨細胞で自家細胞移植を行うには拡大できる細胞数に

*Koichi Nakayama　佐賀大学　大学院工学系研究科　先端融合工学専攻　先端融合工学講座　医工学　教授

限りがあることが問題とされた。

そこで，筆者らは骨髄由来の間葉系幹細胞（Mesenchymal Stem Cell：以下MSC）が軟骨細胞に分化誘導できるという報告[3]に着目し，幹細胞から軟骨再生を行うことを目標にした。当時九大整形外科の医局では誰も細胞培養を中心とした再生医療の実験・研究を実施しておらず，ゼロからの研究立ち上げであった。

まず先行研究にならって，ウサギから採取した骨髄由来MSCを軟骨細胞に分化誘導する実験を開始した。そのプロトコールでは，単層培養で拡大したMSCをトリプシン/EDTAで単離した後，1.5ccの培地に回収して15ccのコニカルチューブで遠心，沈殿した細胞ペレットを崩さないようにチューブごとインキュベーターで培養し，翌日にはチューブを軽く指で弾いてペレットをチューブから浮遊させて数日間培養するという方法であった。

これは一本のチューブに一個のペレットが1.5ccの培地中に形成され，数日に一度は細長いチューブの底のペレットをロストせずに行う培地交換が必要であった。たくさんの誘導軟骨細胞塊を作製するにはその分のチューブと丁寧な培地交換作業が必要であり，当時一台のインキュベーターを複数人とシェアしていた筆者にはチューブ数本をスタンドで並べてインキュベーター内のスペースを占拠するのは色んな意味で大変な作業であった。

2. 細胞凝集塊（スフェロイド）とモールド方式

ペレットを一人で量産しようと悪戦苦闘していた大学院一年目の秋ごろ，ヒト肝癌由来の細胞株であるHepG2の細胞塊（スフェロイド）を効率よく形成できるという96ウェルのマルチプレートの広告を偶然見つけた。これは，接着系の培養を強制的に高密度浮遊培養系に持ち込むと，細胞が自然凝集するという古典的知見[4,5]を応用した製品であり，肝臓系の細胞以外に神経細胞系のニューロスフェアの形成も容易と記載されていた。

さっそくサンプルのプレートを入手し，MSCを播種したところ，見事にきれいな球状のスフェロイドが96個得られ軟骨への分化誘導が確認できた。

この細胞塊（スフェロイド）を眺めていた時に，複数のスフェロイドを同じウェルに入れると，軟骨細胞が吐き出す細胞外マトリックス（ECM）同士が融合し，大きな立体構造体ができるのではないかと思いついた。さっそく，ピペットで4個ほどのスフェロイドを一つのウェルに入れ一晩培養した翌日，予想外の現象がみられた。通常，MSCを分化誘導かけた場合，軟骨細胞に分化するにはおよそ10日程度はかかるとされる。しかし，ウェルに入れられたスフェロイドは翌日には接着・融合している印象であった。さらに翌日には完全に融合し，大きな塊となっていた。この現象に驚いた筆者は，このスフェロイドを大量に使えば，細胞だけで立体的な構造体，将来的には曲面を持った立体的な関節様の構造体が作製できるのではないかと考えた（当時，再生医療の実現にはポリマーなどの足場材料に細胞を付着させて立体的な臓器を作製するティッシューエンジニアリングのコンセプトが主流であった[6]が，培養液中で加水分解されずに長期安定している高分子量の生分解性ポリマーは市販されておらず，入手困難であった）。

そこで巨大なスフェロイドを作るべく1×10^6個の単離された細胞を入れてみた（通常1ウェルあたり1×10^3個未満の細胞を播種している）。すると，数時間後にはウェル内の培地が黄色になり，翌朝には全細胞が浮遊し細胞塊を形成していなかった。今度は，一旦1×10^3個の細胞を用いたスフェロイドを作製した翌日に，1ウェルに96個のスフェロイドを投入したところ，その翌日にはスフェロイド同士が結合していた。

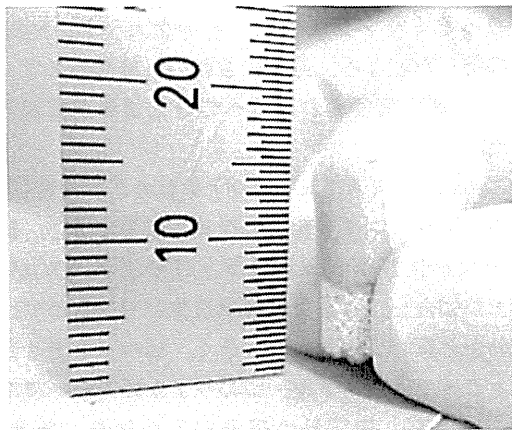この結果が意味するところは，単層培養で増殖していた細胞を一旦スフェロイド化することによって，細胞の栄養要求性が低下するのではないかと考えた。

そこで，クライオチューブの底に穴をあけ滅菌

図1 モールド方式で作製された円柱状の細胞だけ
の構造体

し，そこに手元にあったありったけのスフェロイ
ドを流しこんでみた。すると，翌日にはチューブ
の形状に沿った形で，細胞だけの立体構造体が得
られた（図1）。

当時，細胞だけで円柱状の構造体を作製した類
似の論文が複数発表されていたが，どれも数ヵ月
の培養期間を要し，その結果の厚み（高さ）は約
2mmが上限であった。

筆者らのこの型枠方式（モールド方式と呼んで
いる）では，細胞の条件がよければ厚みが1cm
程度の構造体を作製することが可能になった。

いくつか条件を試行錯誤したのち，家兎の骨髄
由来の間葉系幹細胞で円柱状の構造体を安定して
作製することが可能となった。早速，家兎の膝関
節に自家細胞移植を行ったところ，非常に良好な
関節軟骨の再生が得られた。

他の骨軟骨再生のシステムと異なり，拡大培養
しスフェロイド化した間葉系幹細胞を未分化なま
まで移植しただけで，細胞が適材適所に（骨の層
は骨に，軟骨の層は軟骨に）分化し，関節軟骨に
特徴的な微細な構造まで再生されていた[7, 8]。

このモールド方式の軟骨再生は，現在ヒト幹細
胞指針にそって臨床研究の準備を進めている。お
そらく，関節軟骨は相対的に低酸素な組織である
ため，この培地循環に関してはあまり細胞にとっ
て望ましくないモールド方式でも立体的な細胞だ

けの構造体が作られると考えていた。

ただし，大量のスフェロイドを手作業で準備し
て型枠に流し込むという方法は単純だが単調な作
業が続くため，なんとか自動化できないかと思う
ようになっていた。ちょうど，光硬化型のラピッ
ドプロトタイピングが九大医学部の共同実験室に
設置されていたことを思いだし，ラピッドプロト
タイピングの原理，とくに積層方式を応用して，
スフェロイドを自動で積層する装置が作れないか
と考えた。一個一個のスフェロイドを積み木のよ
うに積み上げて立体化することで，モールド方式
では理論的に困難な中空状の構造体や，複数の種
類の細胞を任意の位置に積みあげて臓器を模した
細胞だけの構造体が作れるのではないかという発
想である。

そこで，機械工学や制御の知識が全くなかった
当時の筆者は，企業に装置の開発をお願いしよう
と考え，地元のロボットメーカーや複数のものづ
くり系の企業に装置のコンセプトを提案し，共同
開発ができないか相談した。しかし，当時大学院
生であった筆者には研究資金に乏しいという致命
的な問題があり，ほとんどの企業にことごとく断
られた。2年ほど複数のものづくり系企業を回っ
た後，最終的に開発に応じてくれた企業は二足歩
行のロボットを使ったアミューズメント系ベン
チャーとクリーンベンチを製造販売する地場のバ
イオ系メーカーであった。これらの企業と一緒に
なってJST育成研究に〝バイオラピッドプロト
タイピング〟プロジェクトとして研究資金を応募
したところ，見事採択され，具体的な開発に着手
できた。このメンバーとの議論の際に，ロボット
ベンチャーの開発者が，スフェロイドを針で刺し
てハンドリングするという突拍子もないアイデア
を持ちかけてきた。当初はそんな細胞を針で刺す
なんて非常識だ，バイオ・細胞に関して無知な工
学系エンジニアの変わった発想だということで，
針で刺す案は却下されていた。

3. 創外固定法

JST採択前の会議で，具体的にスフェロイドを

どのように積層していくのか，単純にモールド方式では複雑な形状を作製するのは困難で何か別の方法が必要だと議論がつづいていたが，具体的なアイデアがなかなか浮かばない日が続いた。そんなある日，整形外科の術後カンファレンスで学生たちが眺めていた教科書のページが目に留まった。それは骨折の治療法が記載されていた章であった。通常，骨折の治療では，ずれた骨片をおおよその位置に整復しギプスなどで固定を行う。できるだけ，非観血的に行うのが望ましく，うまく整復できれば1〜2ヵ月程度固定を行うと骨癒合が得られリハビリを開始する。しかし，骨折の転位（ずれ）が著しい場合などはやむを得ず手術となる。その場合は，骨片を解剖学的に（ある程度）正しい位置に整復し，チタンやステンレス製のプレートやスクリュー，ピンなどで骨片を固定する（内固定）。整形外科医以外の医師には驚かれることが時々あるが，小児の骨折の場合はある程度ずれた位置や少々折れ曲がった状態で骨が融合しても成長と共に骨がまっすぐになり，数年で正常な骨と見分けのつかない骨になる（骨のリモデリングと呼ばれる）。通常の内固定では骨癒合が得られてから，約半年以降に，固定材を抜去・摘出する抜釘と呼ばれる手術を行うのが一般的である。

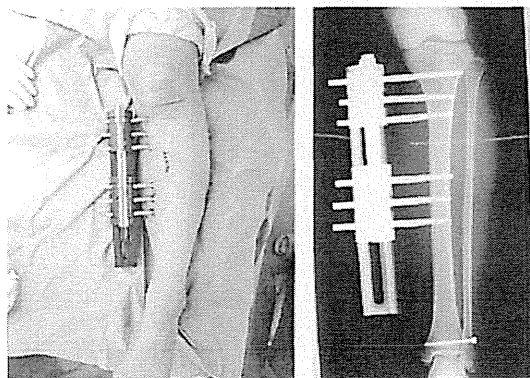　これらの骨折の外科的な治療の一つに，創外固定（External fixation）というテクニックがある

図2　創外固定（External fixation）
九州大学整形外科　中島康晴先生　提供

（図2）[9]。これは，皮膚からピンを骨片に貫通させ，ピン同士を固定器で止めるという方法である。骨折部を直接解放せずに固定でき，通常のプレート固定と比べると皮膚や筋肉を大きく切開せずに治療できることが多いため，骨折のパターンによっては非常に便利で有効なテクニックである。

　これら骨折の治療の章を（学生から取り上げて）眺めているうちに，スフェロイドを仮固定するというアイデアを思いついた。基本的に骨折の外科的治療の根底にある原理は，骨片をある程度の位置に仮固定し，あとは適切な環境（清潔，栄養管理）で維持すると，生体の自己治癒能力に任せて，骨同士が融合するのを待つということである。治癒後に仮固定材を抜去することで，治療が完了し，リハビリを本格的に行う。

　細胞，特にスフェロイドにダメージを与えることなく，仮固定を行えれば，スフェロイド同士が結合・融合するのではないかという仮説を思いついた。半年前にロボットエンジニアから出たスフェロイドを刺すアイデアとリンクした。

　つまり，細い針でスフェロイドを刺して串団子を作り，強制的にスフェロイド同士を隣接させるというアイデアであった。さっそく，手持ちにあったリード線をほぐして，スフェロイドに刺してみると，悪戦苦闘したがとりあえず串刺しに成功した（図3左）。しかし，銅線はイオンが出て細胞毒性があるのではないかと危惧し，様々な材料を探してみた。特に生体に無害な材料ということで，当初着目したのは歯ブラシの毛であった。近くスーパーで極細と記載されている歯ブラシを数種類購入し，毛を一本ずつ切離して，実体顕微鏡下でスフェロイドの刺入を試みた。毛のコシが弱く，たった2個のスフェロイドを刺入するのに2，3時間かかったが，何とかうまく刺入できた（図3右）。この"串団子"を10cmの培養皿に入れた培地に入れ，インキュベーターに一晩おいたところ，翌朝培養皿は真黄色になっていた。コンタミだった。

　やはり抗菌コート云々記載されていた製品でもオートクレーブが必要だろうと認識したが，歯ブ

ラシの毛はほとんどがナイロン製であったため，別の素材を探すことにした。

　特に直径 0.5mm の細胞団子に刺せるほど細い針は，なかなかスーパーや DIY センター，手芸店には置いていなかった。そんなとき，たまたま整形外科の外来に置いてあった医療系の雑誌に，世界で一番細い注射針"ナノパス"（テルモ社製）の開発者である岡野工業の社長の記事が目に入った。この針だと思い，さっそくネットで岡野工業の電話番号を調べ，電話をかけてみるといきなり開発者の岡野社長が出てこられた。単刀直入に生の針だけをくださいとお願いすると，二つ返事でOK と快諾いただいた。後日，加工前の針を販売

元のテルモからいただき，滅菌してスフェロイドを刺入したところ，翌日には見事にスフェロイドの融合が得られた。

　悪戦苦闘して，針を 3×3 本正方形に配置し，実体顕微鏡下でスフェロイドを刺せるようになったのはさらに数ヵ月後のことであった（図 4）。こうして細胞だけである程度任意の形状の立体構造体が作れるようになってきた頃，この技術が整形外科領域，すなわち軟骨細胞や MSC 以外の細胞でも応用できる可能性に気付いた。

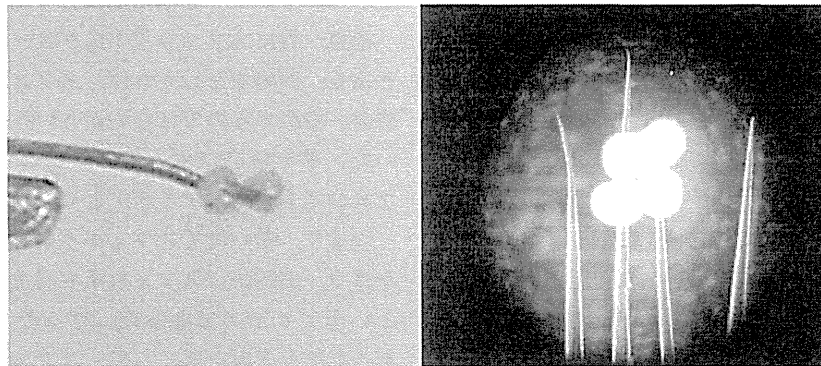　そこで心筋細胞を入手し個別に拍動する心筋細胞スフェロイドを剣山に刺してみたところ，個別に拍動していた心筋細胞のスフェロイドが翌日に
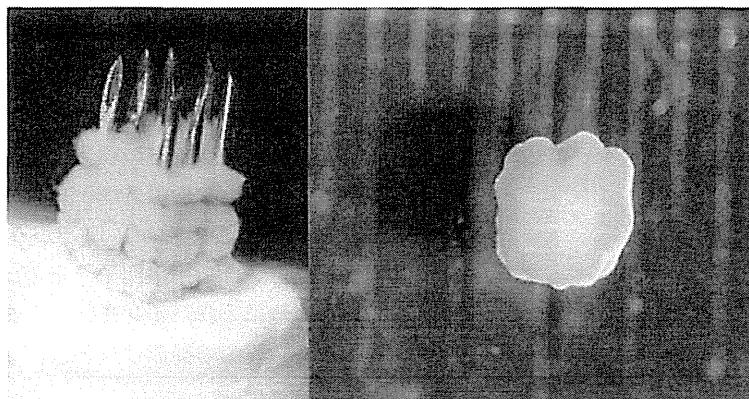

図 3　針で刺入された細胞塊
（左）リード線，（右）ナイロン歯ブラシの毛先

図 4　（左）3×3 の剣山に刺入されたスフェロイド，（右）翌日針から抜くと，
立体的な細胞構造体が得られた