

**Fig. 2** Pre-operative anteroposterior (a) and lateral (b) radiographs of the left elbow of a 56-year-old woman (patient no. 9) who had rheumatoid arthritis, with Larsen's grade IV joint destruction.

Anteroposterior (c) and lateral radiographs (d) of the left elbow 268 months after surgery using a stemmed Kyocera type I prosthesis with cement fixation. The Darrach procedure was added simultaneously to the left distal radio-ulnar joint



**Table 1** Features of 54 elbows

Number of elbows (patients)	54 (39)
Age at time of surgery (years)	59.0 ± 8.7 (43–72)
Gender (female/male)	37/2
Side (right/left)	28/26
Follow-up period (months)	150.6 ± 59.4 (61–269)
Number of patient deaths during follow-up	12 (18 elbows)

originally recorded using the Japan Orthopaedic Association (JOA) elbow evaluation score [13], and it was difficult to convert our patients' records to the Mayo Elbow Performance Score (MEPS) [9]. Therefore, the clinical

condition of each elbow before and after surgery was also assessed according to the modified MEPS for pain (0–60 points), range of motion (ROM; 0–30 points), and stability of the joint (0–10 points) [14]. On the basis of this system, the results were defined as good (75–100), fair (50–74), and poor (<50). Functional impairment was assessed separately using the JOA elbow evaluation score; activities assessed comprised washing the face, eating, fastening buttons (underwear), pouring a glass of water, self-hygiene, and putting on and taking off socks. Each activity was worth a potential 2 points (2: easy, 1: difficult, and 0: unable) giving a total of 12 points. The anteroposterior and lateral radiographs were reviewed carefully, and evaluated for the

position of the implant, the cement mantle, radiolucent lines, bone resorption, and osteolysis. Loosening was defined as a progressive radiolucent line  $>1$  mm that was completely circumferential around the prosthesis [5].

### Surgical procedure

The patient was placed in the full lateral position with the upper arm horizontal and the forearm hanging vertically. A posterolateral [15] or posterior surgical approach [16] was used. The ulnar nerve was identified and meticulous dissection was carried out proximally and distally to the first motor branch of the flexor carpi ulnaris. The posterior aponeurosis of the triceps tendon was reflected distally, and the deep part of the triceps was divided along the reflection of the musculotendinous expansion from the olecranon. After resection of the radial head, the elbow joint was further flexed and a synovectomy was performed. Resection of the tip of the olecranon gave broader access to the joint. The humeral end was trimmed and intramedullary reaming was performed along the central guide pin. The guide instrument was set and a square bone cut was made to remove the damaged trochlea. The joint was then dislocated without sacrificing the medial collateral ligament (MCL). If the bony spur expanding along the MCL disturbed the dislocation, the bony spur was resected piece-by-piece from the inside of the joint. After the ulnar surface had been cut away with a bone saw, thus making the thickness of the remaining olecranon approximately 10 mm, reaming was performed with a reamer, and a circular rasp was used to create a circular bed for the ulnar component. The humeral and ulnar trial prostheses were positioned to check the stability and mobility of the joint. After irrigation, the humeral and ulnar components were fixed with bone cement. Before closing the wound, the triceps tendon was firmly sutured to the olecranon by use of two drill holes at the olecranon. An ulnar nerve translocation was typically not needed.

### Post-operative care

A splint was applied with the elbow at  $90^\circ$  of flexion; this remained in place until post-operative day 14. Passive range of motion (ROM) exercise was started around post-operative day 7, followed by active ROM exercise after post-operative day 14. By post-operative day 21–28, the active range of motion was expected to improve from  $30^\circ$ – $125^\circ$  in extension and flexion. A full range of daily activities was permitted after 6 weeks [17].

### Statistical analysis

All continuous measurements are given as mean  $\pm$  SD. Statistical analysis was performed by one-way analysis of

variance. Survival of the prosthesis was analyzed by the Kaplan–Meier method with loosening, and revision with removal of the prosthesis with or without reinsertion of a new implant defined as the end point. All analysis was conducted by use of Prism software (version 5.0a; Graph-Pad Software, San Diego, CA, USA) with a  $P < 0.05$  regarded as significant.

## Results

### Clinical assessment (Supplemental table)

Pain scores and range of motion (ROM) were markedly improved by surgery. Resection of distal ulnar end (Darrach procedure) was combined in 8 elbows (nos. 8, 11, 13, 19, 39, 44, 45, 46) to improve pain at the distal radio-ulnar joint, and forearm rotation. The average post-operative modified MEPS and JOA scores both improved significantly from  $39.7 \pm 14.3$  and  $44.7 \pm 9.4$ , respectively, pre-operatively, to  $89.7 \pm 15.4$  and  $83.1 \pm 12.8$ , respectively, post-operatively ( $P < 0.0001$ ; Tables 2, 3). Functional assessment by use of the JOA score revealed significant improvement from  $4.9 \pm 2.8$  pre-operatively to  $8.5 \pm 3.3$  points post-operatively ( $P < 0.0001$ ; Table 4). No clinical data were available for one patient who had suffered a humeral fracture (elbow no. 40) at final follow-up; this was, therefore, rated as a poor result. Of the 54 elbows, 2 were judged to be good, 20 were fair, and 32 were poor before surgery. At the final follow-up visit, 46 elbows were judged to be good, 5 were fair, and 3 were poor (Table 2).

### Complications (Table 5)

Sixteen of 54 elbows had minor to major complications, so the incidence of complications was 29.6%. One patient had persistent post-operative paresthesia in the distribution of the ulnar nerve (elbow no. 14). Intra-operative fractures of the humerus occurred in 2 elbows in 2 patients (elbow nos. 17, 53). A humeral epicondylar fracture (elbow no. 17) was treated by simultaneous Kirschner wire fixation. A fissure fracture occurred at the humeral shaft of elbow no. 53, but the bone fragment was stable after cement fixation of the humeral component and did not require internal fixation. In both elbows, bone union was seen within 3 months after surgery.

Post-operative fractures occurred as a result of trauma for 6 elbows of 6 patients (elbow nos. 2, 3, 4, 26, 40, 53). Four fractures occurred at the ulna and two at the humerus. For one elbow with an olecranon fracture resulting from a fall (elbow no. 2), the ulnar component was lost from the joint. Because the pain at the elbow joint was mild and the patient did not want re-implantation of the prosthesis, the

**Table 2** Pre and post-operative clinical assessment by use of the modified version of the Mayo Elbow Performance Score

Criteria	Score (points)	Pre-operative	Post-operative
Pain (max., 60 points)		Number of elbows (%)	
None	60	1 (2)	42 (79)
Mild to occasional, no medication	40	2 (4)	8 (15)
Moderate to occasional, activity limited, medication	20	26 (48)	2 (4)
Severe to incapacitating	0	25 (46)	1 (2)
Motion (max., 30 points)		Number of elbows (%)	
Arc of extension/flexion			
>90	30	30 (56)	48 (91)
60–89	20	14 (26)	5 (9)
30–59	10	7 (13)	0
<30	0	3 (6)	0
Stability (max., 10 points)		Number of elbows (%)	
Effect on function of the elbow			
None or mild (does not limit activity)	10	11 (20)	26 (49)
Moderate (impairs certain functions)	5	25 (46)	15 (28)
Severe (markedly limits activity)	0	18 (33)	12 (23)
Total score (max., 100 points)		39.7	89.7
Overall results (good/fair/poor)		2/20/32	46/5/3

Post-operative clinical data were not available for elbow no. 40 because of humeral fracture

**Table 3** Pre and post-operative clinical assessment by use of the Japanese Orthopaedic Association (JOA) Elbow score

Criteria	Pre-operative	Post-operative
Pain (max., 30 points)	8.8	27.8
Function (max., 20 points)	10.0	15.8
Range of motion (max., 30 points)	15.8	25.4
Flexion (°)	118.5	141.6
Extension (°)	−35.6	−18.4
Arc of flexion/extension (°)	82.9	123.2
Pronation (°)	49.6	78.8
Supination (°)	55.6	84.4
Arc of forearm rotation (°)	105.2	163.2
Instability (max., 10 points)	4.4	6.3
Deformity (max., 10 points)	5.8	7.8
Total JOA score (max., 100 points)	44.7	83.1

ulnar component was simply removed, with the cement fragments. This resulted in a fair result at the elbow joint with gross instability that required orthosis. Open reduction and internal fixation was performed for two elbows, one

**Table 4** Pre and post-operative function of the elbow

Function (points)	Pre-operative (n = 44)		Post-operative (n = 51)		P value
	Average	SD	Average	SD	
Washing face (2)	0.7	0.7	1.7	0.7	<0.0001
Eating (2)	0.9	0.6	1.8	0.5	<0.0001
Fastening buttons (underwear) (2)	0.8	0.6	1.4	0.8	<0.0001
Pouring a glass of water (2)	1.0	0.6	1.5	0.8	<0.0001
Self-hygiene care (2)	0.8	0.7	1.4	0.8	0.0003
Putting on and taking off socks(2)	0.8	0.6	0.9	0.8	0.28
Total (12)	4.9	2.8	8.5	3.3	<0.0001

Each function was evaluated and rated as: easy; 2 points, difficult; 1 point, and unable; 0 points, according to the Japanese Orthopaedic Association (JOA) Elbow score. Data were obtained from the records of 44 patients pre-operatively and 51 patients post-operatively

SD standard deviation

P value <0.05 was regarded as significant

**Table 5** Complications recorded for 54 elbows

Complications (number of elbows)	Elbow no. in supplemental table
Ulnar neuropathy(1)	14
Intra-op. fracture (2)	17, 53
Post-op. fracture (6)	2, 3, 4, 26, 40, 53
Post-op. implant fracture (1)	11
Dislocation or subdislocation (6)	9,18, 27, 35, 40, 52
Infection (1)	52
Aseptic loosening (4)	12, 19, 40, 53
Revision surgery (3)	3,11, 53
Implant removal (2)	2, 52

with an olecranon fracture (elbow no. 3) and another with a bi-condylar humeral fracture after loosening (elbow no. 53); both were treated by implant re-implantation with cement fixation. The remaining 3 elbows were treated conservatively. As a result, the outcomes were rated good in 3 cases, fair in one, and poor in 2 at final follow-up.

In this case series, 6 dislocations were noted among 6 patients (elbow nos. 9, 18, 27, 35, 40, 52). Three elbows had severe pre-operative joint destruction rated as Larsen's grade V (elbow nos. 18, 27, 52), one elbow was dislocated with humeral fracture (elbow no. 40), and the causes of dislocation of the remaining two elbows (elbow nos. 9, 35) were unknown. The time of dislocation varied among 4 elbows (1 month, 11 months, 6 years, and 6 years), and was not clear for 2 elbows. MCL repair was performed for 2 elbows (elbow nos. 9, 18), but the remaining 4 patients

declined the surgery, and were treated conservatively by orthosis. Of these 4 elbows, deep infection was noted for one elbow of one patient (elbow no. 52) 95 months after surgery, caused by repeated aspiration of synovial fluid for sub-cutaneous bursitis involving the lateral epicondyle of the humerus after dislocation. Both humeral and ulnar implants were removed and replaced by antibiotic-containing cement beads but the infection could not be controlled. Finally, the elbow was treated by intensive debridement and continuous irrigation. This floppy elbow was then further treated by orthosis but the clinical result was poor.

Radiographic loosening occurred for 4 elbows in 4 patients. One patient (elbow no. 40) had been lost to follow-up until 254 months post-TEA, when she visited her local hospital with humeral fracture, massive loosening around the humeral component, and joint dislocation, and was treated conservatively. For the other 2 elbows (elbow nos 12, 19), a radiolucent line was visible around the ulnar stem but the loosening did not cause elbow joint instability during the follow-up period.

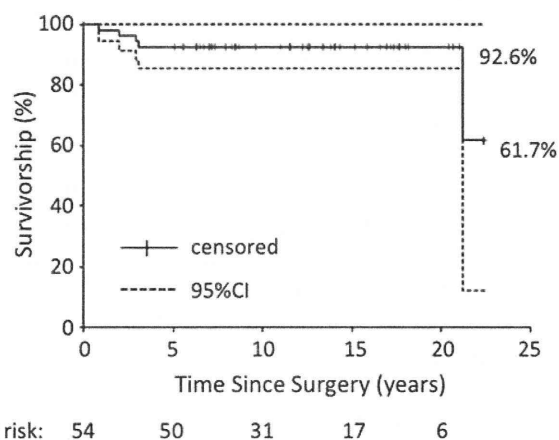
Revision surgery was performed for 3 patients (3 elbows) because of humeral fracture of one elbow (elbow no. 53) and ulnar fracture of one elbow (elbow no. 3) as described above, and ulnar component fracture of one elbow (elbow no. 11). The ulnar component fracture of elbow no. 11 was observed at the screw hole connecting the high-density polyethylene and the ceramic stem, and the elbow was treated by removal of the broken ulnar component, and re-implantation of a new ulnar component with cement fixation.

Of the 37 elbows in 32 patients with <5-years of follow-up, 1 peri-operative humeral fracture was recorded for 1 elbow, and post-operative fractures were recorded for 2 elbows. An implant fracture at the polyethylene–ceramic stem interface of the ulnar component was recorded for one elbow and required revision surgery. Aseptic loosening was noted for four elbows in 3 patients.

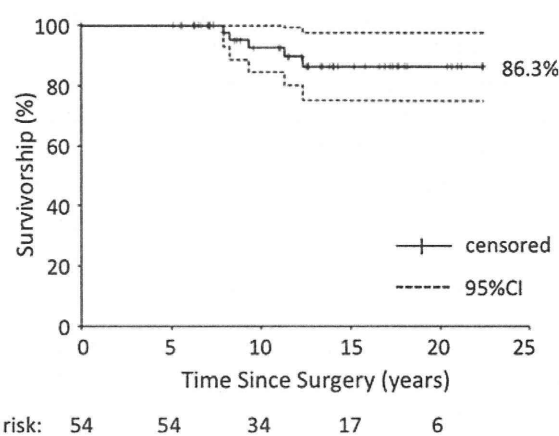
### Survival analysis

Survival of implants was analyzed by the Kaplan–Meier method. Using loosening as end point, survival of prostheses used for 54 elbows was 92.6 % (95 % confidence interval (CI) 85.6–100.0) for up to 20 years. The first occurrence of loosening was observed at 0.8 years. For loosening as end point, survival did not decrease after reaching 92.6 % at 3.1 years (Fig. 3). However, the late report of loosening at 254 months (elbow no. 40) reduced survival to 61.7 %.

Using implant revision with removal as the definition of end point, survival of prostheses in 54 elbows was 86.3 % (95 % CI 75.0–97.6) for up to 20 years. The first occurrence of implant revision was observed at 7.9 years. For implant revision as end point, survival did not decrease after reaching 86.3 % at 12.3 years (Fig. 4).



**Fig. 3** Kaplan–Meier survival curve for loosening as the endpoint for 54 stemmed Kyocera type I prostheses. The broken lines indicate 95 % confidence intervals



**Fig. 4** Kaplan–Meier survival curve for revision with removal of the implant with or without re-insertion of a new implant as the endpoint for 54 stemmed Kyocera type I prostheses. The broken lines indicate 95 % confidence intervals

### Discussion

Clinical outcomes of pharmaceutical treatment for RA have changed dramatically over the past decade, with new treatment strategies and the introduction of biologic agents [18]. Even for patients with long-standing disease, synovial inflammation has been reduced, and soft tissue disorganization has been much improved among patients who have a good response to treatment. Destructive bone resorption that was seen in the natural course of the disease has been replaced by osteoarthritis-like joint damage among some of the patient population after effective treatment with methotrexate and biological agents [19]. In this regard, a TEA with anatomic design might be indicated more widely than ever before, and preservation of the remaining soft tissue structure would be realistic for longer survival of the implant among younger patients. In this retrospective case series we report, for the first time, mid to long-term follow-

up results for cemented alumina ceramic TEA with an SKC-I prosthesis for reconstruction of RA elbows.

High-density polyethylene articulating against a metallic femoral head or distal femoral mold has been the commonly used bearing couple in total joint replacement, but the biological response to polyethylene particulate debris from bearing surfaces is regarded as one cause of periprosthetic osteolysis and aseptic loosening of the prosthesis [20]. Alumina ceramics are not soluble and do not break down in the body; they are also relatively inert biologically and are therefore biocompatible materials without risk of ion release. Biomechanical characteristics of low friction are suitable for artificial joint surfaces when combined with an HDP surface. This is the first study to describe the long-term clinical results and longevity of an unlinked alumina ceramic TEA. The results reveal long-term outcomes are satisfactory for the SKC-I prosthesis without ceramic failure. However, an ulnar component fracture at the screw hole connection between the HDP and ceramic stem was seen for one elbow (elbow no. 11). To solve this problem, a new model of implant after the SKC-I, developed and introduced in 1999, used an all-polyethylene ulnar component.

There are several literature reports analyzing the survival of TEAs with long-term follow-up extending beyond 10–15 years. Among unlinked implants, survival was 90 % at 16 years for Kudo type-3 prosthesis [7], 87 % at 12 years [5] and 77.4 % at 10 years [21] for the Souter–Strathclyde prosthesis with revision or implant removal as the end point, and 89 % at 81 months for the Kudo type-5 prosthesis with loosening as the end point [22]. Among linked implants, survival was 88–94 % for the GSBIII prosthesis [10, 23], and 92.4 % for the Coonrad–Morrey prosthesis at a minimum of 10 years [24]. The results of our study are indicative of favorable survival of the SKC-I prosthesis over 10 years, and are comparable with those for established implants. In this study, survival for “loosening” as end point was higher than for “revision” as end point. This might be partly because radiographic loosening determined in this study did not always lead to implant displacement or clinical impairment.

The complication that distinguishes an unlinked prosthesis from linked devices is the incidence of dislocation [25]. The rate of dislocation of an unlinked TEA varies from 3 to 15 % in the literature [5, 26–28]. The Kudo elbow prosthesis is an established elbow prosthesis that has relatively strong intrinsic stability and reproduces the soft tissue tension by use of a spacer effect. A common practice during implantation of the Kudo prosthesis involves sacrificing the MCL during surgery for better access to the joint and easier exposure of the bones for proper setting of the prosthesis, and to facilitate treatment of flexion contractures. Therefore, the joint line tends to shift further distally, and improvement of extension is often not

expected. Tanaka et al. [29] stressed the importance of correct orientation of the components and restoration of length, without preservation of the anterior oblique component of the MCL, for preventing dislocation.

Unlike the Kudo or Souter–Strathclyde prostheses, intrinsic stability of the SKC-I prosthesis is minimal, and the SKC-I prosthesis largely depends on the soft tissue for stability. Preservation of the MCL makes intra-operative soft tissue balancing easier, resulting in prevention of internal and/or external rotational malposition of the ulnar component relative to the trochlea, and minimizes mal-tracking of the joint. Thus, pre-operative inflammatory tears or intra-operative injury to the MCL or bony destruction of the humeral and ulnar insertions of the MCL are risk factors for post-operative dislocation and/or sub-dislocation when SKC-I prostheses are used. A report by Weiland et al. [30] described their experience of 20 % malarticulation and 5 % dislocation with capitellocondylar TEAs. In a comparative study, Little et al. [31] reported dislocation in 1 of 33 Souter–Strathclyde prostheses and 2 of 33 Kudo implants. In our series, there were 6 cases of dislocation or subdislocation (11.1 %). We believe the initial learning curve involved in becoming familiar with the surgical technique needed for this new prosthesis in the 1980s, and the early challenge of the relatively broad indication of this unlinked prosthesis with relatively weak intrinsic constraints to Larsen grade V elbows resulted in relatively high dislocation and/or subdislocation. Caution should be exercised when deciding whether or not the elbow has sufficient bone stock, capsular and ligamentous integrity, and muscle strength at the elbow joint to justify use of an unlinked prosthesis for reconstruction of a rheumatoid elbow [32].

In our earlier experience, TEAs using SKC-I prostheses without cement fixation failed because of loosening of the humeral component [11], which showed early proximal subsidence and simultaneously tilted with the proximal end of the humeral stem displaced anteriorly and the ceramic trochlea posteriorly. Because the solid ceramic trochlea does not resist the forces at the joint, the intramedullary stem is the only structure that stabilizes the humeral component, as occurs with the Souter–Strathclyde prosthesis. In contrast, the Kudo prosthesis, with a saddle-shaped humeral surface, provides reliable uncemented fixation for the humeral component because it holds the cortical bone of the trochlea. As a result, occurrence of loosening with the Kudo prosthesis is high around the ulnar component rather than the humeral component [33]. Potter et al. [22] also pointed out that the valgus tilt of the ulnar component might be related to loosening of the Kudo prosthesis. Interestingly, for elbows fitted with an SKC-I prosthesis with or without cement fixation, most loosening occurs around the humeral component rather than the ulnar component; this might be partly explained by the different

intrinsic stability of the Kudo and the SKC-I prostheses. The results of our study, showing that the incidence of aseptic loosening is lower among patients with >5 years of follow-up than among those with <5 years of follow-up, might be indicative of improvement of the cementing technique during the initial learning process; however, post-operative fractures increased with time. Olecranon fractures occurred as a result of direct trauma for 4 elbows in 3 patients, and for two elbows in patients with severe osteolysis around the humeral component. Osteolysis related to accumulation of polyethylene wear particles seems to be a concern in the long term.

This study had some limitations. First, one surgeon performed all the surgery, and as one of the designers of the prosthesis was well-informed of its advantages and disadvantages. As previously pointed out by Potter et al. [22], results from the center where a prosthesis was designed are usually good, and evidence from other centers to confirm the reproducibility of clinical outcomes are needed. Second, the last observation carried forward method used in this study might cause bias in interpreting the results by over-reporting beneficial outcomes or under-reporting the incidence of late complications of the surgery; however, in our local district most information about severe complications in TEAs that require treatment would be reported to our center. The deaths of 27 patients (39 elbows) were revealed during this review, which is a fate of this type of long-term follow-up study.

In conclusion, we demonstrated quite favorable long-term results with the SKC-I prosthesis, an anatomically-designed alumina ceramic elbow prosthesis for reconstruction of damaged rheumatoid elbows with reasonable pain relief and improved ROM. Survival was comparable with that reported for other implants. Loosening related to osteolysis over the long term remains a problem. The risk of dislocation can be minimized by using proper indications for this implant and careful surgical technique.

**Conflict of interest** The authors declare that they have no conflict of interest. In the Department of Orthopaedic Surgery (Head of department, Professor Toshifumi Ozaki), Okayama University, there is a small department named "Department of Medical Materials for Musculoskeletal Reconstruction" donated by the KYOCERA Medical Corporation.

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## IL-6 negatively regulates osteoblast differentiation through the SHP2/MEK2 and SHP2/Akt2 pathways in vitro

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**Abstract** It has been suggested that interleukin-6 (IL-6) plays a key role in the pathogenesis of rheumatoid arthritis (RA), including osteoporosis not only in inflamed joints but also in the whole body. However, previous in vitro studies regarding the effects of IL-6 on osteoblast differentiation are inconsistent. The aim of this study was to examine the effects and signal transduction of IL-6 on osteoblast differentiation in MC3T3-E1 cells and primary murine calvarial osteoblasts. IL-6 and its soluble receptor significantly reduced alkaline phosphatase (ALP) activity, the expression of osteoblastic genes (Runx2, osterix, and osteocalcin), and mineralization in a dose-dependent manner, which indicates negative effects of IL-6 on osteoblast differentiation. Signal transduction studies demonstrated that IL-6 activated not only two major signaling pathways, SHP2/MEK/ERK and JAK/STAT3, but also the SHP2/PI3K/Akt2 signaling pathway. The negative

effect of IL-6 on osteoblast differentiation was restored by inhibition of MEK as well as PI3K, while it was enhanced by inhibition of STAT3. Knockdown of MEK2 and Akt2 transfected with siRNA enhanced ALP activity and gene expression of Runx2. These results indicate that IL-6 negatively regulates osteoblast differentiation through SHP2/MEK2/ERK and SHP2/PI3K/Akt2 pathways, while affecting it positively through JAK/STAT3. Inhibition of MEK2 and Akt2 signaling in osteoblasts might be of potential use in the treatment of osteoporosis in RA.

**Keywords** Interleukin-6 · Osteoblast differentiation · MEK2 · Akt2 · Signaling pathway

### Introduction

Inflammation-mediated bone loss is a major feature of various bone diseases, including rheumatoid arthritis (RA). Interleukin-6 (IL-6) contributes to the development of arthritis and is present at high concentrations in the serum and synovial fluid of patients with RA [1–4]. Soluble IL-6 receptor (sIL-6R) is also elevated in the serum and synovial fluid of RA patients [5, 6], and IL-6 exerts its action by binding either to its membrane-bound receptor (mIL-6R) or to sIL-6R. Moreover, IL-6 is closely associated with the expression of receptor activator of NF- $\kappa$ B ligand (RANKL) in osteoblasts [7]. That is to say, IL-6 acts indirectly on osteoclastogenesis by stimulating the release of RANKL by cells within bone tissues such as osteoblasts [8]. It can unquestionably be said that IL-6 plays a major role in the pathogenesis of RA [9–12], including osteoporosis not only in inflamed joints but also in the whole body.

There have been several studies on the effect of IL-6 on bone turnover in animal models. In IL-6 knock-out mice,

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microstructure abnormalities in cortical bones and delayed fracture healing were observed [13, 14], in spite of the evident normal phenotype [15]. Also, bone loss after estrogen depletion was mitigated in IL-6-deficient mice, while a high level of IL-6 and bone loss are seen in wild-type mice [13]. Moreover, IL-6-overexpressed-transgenic mice develop osteopenia and defective ossification, in which the activity of mature osteoblasts is significantly decreased [16]. All these findings, together with studies on human RA patients [17, 18], indicate that IL-6 plays a major role in bone turnover and is an important regulator of bone homeostasis.

Recently, several biological agents have been introduced for the treatment of RA and have demonstrated not only potent anti-inflammatory effects but also inhibitory effects on joint destruction. Among these biological agents, tocilizumab, an anti-IL-6 receptor antibody, has been reported to increase serum bone formation markers in RA patients [19], suggesting that IL-6 has a negative effect on osteoblast differentiation. However, previous reports regarding the effects of IL-6 on osteoblast differentiation *in vitro* have been inconsistent [20]. IL-6 has been shown to decrease the expression of differentiation markers in osteoblasts [21, 22] and to inhibit bone formation [23], while it has been shown to induce osteoblast differentiation [24, 25].

Binding of IL-6 with sIL-6R or mIL-6R leads to subsequent homodimerization of the signal-transducing molecule gp130, followed by activation of two major intracellular signaling pathways, Janus protein tyrosine kinase (JAK)/signal transducer and activator of transcription factors (STAT) 3, or Src-homology domain 2 containing protein-tyrosine phosphatase (SHP2)/mitogen-activated protein kinase-extracellular signal-regulated kinase kinase (MEK)/mitogen-activated protein kinase (MAPK), also called extracellular signal-regulated kinase (ERK) [26]. There have been many reports in which the effects of IL-6 on JAK/STAT3 and SHP2/ERK signal transduction pathways have been studied in osteoblasts, though it is still controversial whether differentiation is enhanced by IL-6 [9, 20]. SHP2 can also form a tertiary complex with the scaffolding proteins Gab1/2 and the p85 subunit of phosphatidylinositol-3-kinase (PI3K) [27], which leads to activation of the Akt pathway. Several papers have so far reported that the PI3K/Akt pathway triggered by IL-6 plays important roles in various cells [28–32], but no reports have been published regarding the effect of IL-6 on this pathway in osteoblasts.

The purpose of this study was to clarify the effect of IL-6 on osteoblast differentiation *in vitro*, with consideration of intracellular signaling pathways in murine MC3T3-E1 osteoblastic cells and primary murine calvarial osteoblasts.

## Materials and methods

### Ethics statement

Prior to the study, all experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

### Cell culture

MC3T3-E1 osteoblastic cells were purchased from Riken Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) containing 10 % fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA) and 1 % penicillin and streptomycin at 37 °C in a humidified atmosphere of 5 % CO<sub>2</sub>. All media were purchased from Life Technologies Japan (Tokyo, Japan). Murine primary osteoblasts were isolated from the calvariae of 3-day-old C57BL/6 mice (Charles River Laboratories Japan, Inc, Osaka, Japan) by sequential collagenase digestion as described previously [33].

MC3T3-E1 cells and murine calvarial osteoblasts were seeded at  $1 \times 10^5$  cells per well in 12-well plates. After the cells reached confluence, the medium was replaced to induce osteoblast differentiation. The differentiation medium contained 10 % FBS, 10 mM  $\beta$ -glycerophosphate, and 50  $\mu$ g/ml ascorbic acid in the absence or presence of recombinant mouse (rm) IL-6 (R&D Systems, Inc., Minneapolis, MN, USA) (10, 50 ng/mL), and rm sIL-6R (R&D Systems) (100 ng/mL). The medium and reagents were renewed every 3 days.

To study signal transduction, the following inhibitors or vehicle (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) were added to culture medium at several concentrations; MEK inhibitor (U0126; 1, 2.5, 5  $\mu$ M; Cell Signaling Technology, Danvers, MA, USA), STAT3 inhibitor (V Stattec; 2.5, 5  $\mu$ M; Calbiochem, La Jolla, CA, USA), PI3K inhibitor (LY294002; 1, 2.5, 5  $\mu$ M; Cell Signaling Technology), and SHP2 inhibitor (PHPS1; 5, 20, 40  $\mu$ M; Sigma-Aldrich). These inhibitors were added 1 h before treatment with IL-6/sIL-6R. All inhibitors were maintained until the end of the culture period at the indicated concentrations.

### Alkaline phosphatase (ALP) staining and activity

MC3T3-E1 cells and murine calvarial osteoblasts were treated with or without IL-6/sIL-6R and signal pathway inhibitors after the cells reached confluence and were incubated for 6 days.

For ALP staining, after fixation with 10 % formalin, cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and incubated with ALP substrate solution,

0.1 mg/ml naphthol AS-MX (Sigma-Aldrich), and 0.6 mg/ml fast violet B salt (Sigma-Aldrich) in 0.1 M Tris-HCl (pH 8.5) for 20 min.

To measure ALP activity, cells were washed twice with PBS and lysed in Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. ALP activity was assayed using *p*-nitrophenylphosphate as a substrate by an Alkaline Phosphatase Test Wako (Wako Pure Chemicals Industries, Ltd., Osaka, Japan), and the protein content was measured using the Bicinchoninic Acid Protein Assay Kit (Pierce).

#### Proliferation assay

MC3T3-E1 cells were cultured in 96-well plates at a concentration of  $2.0 \times 10^4$  cells/cm<sup>2</sup> in  $\alpha$ -MEM containing 10 % FBS. Cells were incubated for 1 day, after which the medium was treated with IL-6/sIL-6R for 3 days. Cell proliferation was assessed using the Premix WST-1 Cell Proliferation Assay System (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. We performed this assay every 24 h.

#### Alizarin red staining

After fixation with 10 % formalin, MC3T3-E1 cells and murine calvarial osteoblasts were washed with distilled water, and stained with alizarin red S solution (Sigma-Aldrich) (pH 6.0) for 10 min, followed by incubation in 100 mM cetylpyridinium chloride for 1 h at room temperature to dissolve and release calcium-bound alizarin red. The absorbance of the released alizarin red was then measured at 570 nm [34]. To measure the value of absorbance for alizarin red, the absorbance data were normalized by total DNA content. Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany).

#### Knockdown of MEK1, MEK2, Akt1 and Akt2 using RNA interference

MC3T3-E1 cells were transfected with small interfering RNAs (siRNA) using Lipofectamine RNAiMAX (Life Technologies Japan) according to the reverse transfection method in the manufacturer's protocol.

The siRNAs for MEK2, Akt1 and Akt2 and that for MEK1 were purchased from Cell Signaling Technology and Qiagen, respectively, with negative controls for each molecule. MC3T3-E1 cells transfected with siRNA were seeded in 24-well plates at a concentration of  $1.0 \times 10^4$  cells/cm<sup>2</sup> for 48 h. The medium was then replaced with differentiation medium with vehicle or with 20 ng/ml IL-6 and 100 ng/ml sIL-6R and the cells were incubated for 3 days prior to use for further experiments.

#### Western blotting

Cells cultured in 6-well plates for 2 days were washed twice with PBS and then homogenized with 100  $\mu$ l of Kaplan buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 % NP40, 10 % glycerol, and 1 tablet per 50 ml buffer of protease inhibitor cocktail and phosphatase inhibitor cocktail). The lysates were centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatants were used for electrophoresis after a protein assay using bovine serum albumin as standard. Western blotting was performed by use of the following antibodies purchased from Cell Signaling Technology, except for phosphate anti-Akt2 antibody from Enogene Biotech (New York, NY, USA): phosphate anti-STAT3 (Tyr705) (1:2000) and anti-STAT3 (1:1000); phosphate anti-Akt (Ser473) (1:2000), phosphate anti-Akt2 (Ser474) (1:1000), anti-Akt1, anti-Akt2, and anti-Akt (1:1000); phosphate anti-ERK (Thr202/Tyr204) (1:2000), anti-MEK1, anti-MEK2 and anti-ERK (1:1000); and phosphate anti-SHP2 (Tyr542) (1:1000). To control for protein loading, blots were additionally stained with anti- $\beta$  actin antibody (1:1000).

#### Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells with an RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized using SuperScript II RNase H-reverse transcriptase (Life Technologies Japan). Then PCR was performed using Ex Taq (Takara Bio) and the following primers:

*Osteocalcin* (forward primer 5'-CTCACTCTGCTGGCC CTG-3'; reverse primer 5'-CCGTAGATGCGTTTGTAGGC-3');

*Osterix* (forward primer 5'-AGGCACAAAGAAGCCATAC-3'; reverse primer 5'-AATGAGTGAGGGAAGGGT-3');

*Runx2* (forward primer 5'-GCTTGATGACTCTAAACCTA-3'; reverse primer 5'-AAAAAGGGCCAGTTCTGAA-3');

*GAPDH* (forward primer 5'-TGAACGGGAAGCTCAC TGG-3'; reverse primer 5'-TCCACCACCCTGTTGCTGTA-3').

#### Quantitative real-time PCR analysis

We obtained cDNA by reverse transcription as mentioned above, and proceeded with real-time PCR using a Light Cycler system (Roche Applied Science, Basel, Switzerland). The SYBR Green assay using a Quantitect SYBR Green PCR Kit (Qiagen), in which each cDNA sample was evaluated in triplicate 20- $\mu$ l reactions, was used for all