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TNF-α induces expression of the circadian clock gene *Bmal1* via dual calcium-dependent pathways in rheumatoid synovial cells

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ABSTRACT

Tumor necrosis factor (TNF)-α is responsible for expressions of several clock genes and affects joint symptoms of rheumatoid arthritis (RA) with diurnal fluctuation. We tried to determine the mechanism involved in over-expression of *Bmal1*, induced by TNF-α, in primary cultured rheumatoid synovial cells. Cells were incubated with intra-cellular Ca^{2+} chelator BAPTA-AM, calcineurin inhibitor FK506 and p300/CBP (CREB binding protein) inhibitor C646, respectively, or transfected with p300 and CBP small interfering RNA (siRNA) before stimulation with TNF-α. Oscillation phase and amplitude of *Bmal1*, transcriptional activator *Rorα*, transcriptional repressor *Rev-erbα*, and histone acetyltransferases (*p300* and *Cbp*) were evaluated by quantitative real-time PCR. As results, TNF-α did not influence the oscillation phase of *Rev-erbα*, while enhanced those of *Rorα*, resulting in over-expression of *Bmal1*. When Ca^{2+} influx was inhibited by BAPTA-AM, TNF-α-mediated up-regulation of *Rorα* was cancelled, however, that of *Bmal1* was still apparent. When we further explored another pathway between TNF-α and *Bmal1*, TNF-α suppressed the expression of *Rev-erbα* in the absence of Ca^{2+} influx, as well as those of *p300* and *Cbp* genes. Finally, actions of TNF-α, in increasing *Bmal1*/*Rorα* and decreasing *Rev-erbα*, were cancelled by C646 treatment or silencing of both *p300* and *Cbp*. In conclusion, we determined a novel role of TNF-α in inducing *Bmal1* via dual calcium dependent pathways; *Rorα* was up-regulated in the presence of Ca^{2+} influx and *Rev-erbα* was down-regulated in the absence of that. Results proposed that inhibition of p300/CBP could be new therapeutic targets for RA.

1. Introduction

Most living organisms on earth possess an internal pacemaker, called a biological clock, and circadian rhythm represents a basic feature of life and disease. The rhythm signals propagated from hypothalamic suprachiasmatic nucleus (SCN), the controlling center for rhythm oscillation in mammals, are subject to a feedback loop provided by core clock genes including *Clock* (circadian locomotor output cycles kaput), *Bmal1* (brain and muscle ARNT like-1), *Per* (period) and *Cry* (cryptochrome), and orphan nuclear hormone receptors *Rev-erbα* and *Rorα*. The circadian expression of these genes is regulated through E-box, REV-ERB/ROR response element (RRE), and DBP/E4BP4 binding element (D-box) in their promoter regions [1]. This core clock machinery regulates circadian time of most of human cells, and oversees thousands of clock-controlled genes.

Rheumatoid arthritis (RA) is a chronic polyarthritis of unknown etiology that is characterized by a 'tumor-like' synovial over growth. Another feature of RA is the circadian manifestation of disease symptoms, such as morning stiffness; one of the most characteristic features of patients with RA [2]. This circadian rhythmicity is also observed in the production of pro-inflammatory

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cytokines such as tumor necrosis factor (TNF)-α and Interleukin (IL)-6 in patients with RA [3]. Indeed, clock genes directly or indirectly regulate production of inflammatory cytokines/chemokines/adipokines, and demonstrate anti-inflammatory effects in host [4], thereby the loss of circadian clock gene aggravates arthritis, and the converse, arthritis significantly disturbs circadian rhythmicity [5]. Thus, the biological clock and arthritis have a close impact on each other.

Recent studies showed that TNF-α affected expression of circadian clock genes *in vitro* and *in vivo* [6,7]. We also have shown that TNF-α induces over-expression of *Bmal1*, while suppressed *Per2* [8]. In primary cultured rheumatoid synovial cells, transcription of Per2 was regulated by D-box binding proteins while the precise manner how TNF-α activates *Bmal1* expression remains unclear. Since recent study has shown that the calcium signaling is associated with clock gene expression [9], in the current study, we have examined the relation between calcium signaling and TNF-α.

2. Materials and methods

2.1. Synovial cell culture

Twelve synovial tissues were obtained during joint surgery from different patients with RA whose diagnosis fulfilled the American College of Rheumatology (ACR) classification criteria [2] (Table 1). The study was approved by the ethics committee of Kobe University (approval number #579) and Kobe Kaisei Hospital (approval number #0072), and written informed consent was obtained from each patient before study enrolment. Primary cultured synovial cell lines were established from the synovial tissue and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) as previously described [8]. Cells from passages 3–6 were used in the experiment.

2.2. Synchronization of rheumatoid synovial cells by serum shock and stimulation with TNF-α

Synovial cells were synchronized by incubation with 50% horse serum for 2 h and then treated with 25 μg/ml BAPTA-AM, the same dose of FK506, and 25 μM C646 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After treatment with these compounds, cells were stimulated with or without 10 ng/mL TNF-α (R&D Systems, Minneapolis, MN, USA) in serum-free DMEM every 8 h for a 72 h culture period. Each compound was dissolved with DMSO, while the control medium contained the same concentration of DMSO (0.2%).

2.3. RNA interference

Silencer® Select Negative Control siRNA (catalog no. 4390843), Cbp siRNA (s3495), and p300 siRNA (s4696) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). These siRNA were introduced into synovial cells for 48 h using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies). After transfection with these siRNA, cells were synchronized and then stimulated with or without TNF-α.

2.4. Quantitative polymerase chain reaction (qPCR)

Quantitative PCR was performed with TaqMan probes and analyzed on a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The TaqMan probes were: Hs00154147_m1 for *Bmal1*, Hs00536545_m1 for *Rorα*, Hs00253876_m1 for *Rev-erbα*, Hs00914223_m1 for *p300*, Hs00231733_m1 for *Cbp* and Hs00427621_m1 for TATA box binding protein (*Tbp*). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with random hexamer primers (TOYOBO Life Science, Osaka, Japan).

2.5. Statistical analysis

We assessed statistical significance using Student *t*-test or Dunnett's test, as appropriate, and considered a probability of <5% ($P < 0.05$) to be statistically significant.

3. Results

3.1. TNF-α modulates the expression of Bmal1, Rorα and Rev-erbα in synovial cells

We first examined whether TNF-α affects the expression of clock genes including *Bmal1*, its transcriptional activator *Rorα* and repressor *Rev-erbα* in rheumatoid synovial cells. The mRNA expression of *Bmal1* showed circadian oscillation that reached the peak at 8 and 32 h after serum incubation. By stimulation with TNF-α, the *Bmal1* expression was significantly increased between 16 and 32 h ($P < 0.01$, Fig. 1A, left) as previously described [8]. As well as

Table 1
RA patient profile of joint tissues used in this study.

ID	Age	Sex	Tissue	Disease Duration (year)	DAS28 -ESR	CRP (mg/dL)	RF (IU/mL)	ACPA (U/mL)	MTX (mg/week)	PSL (mg/day)	Other DMARDs
S-2	75	F	Knee	28	1.1	0.28	42	43	8	5	None
T-2	45	F	Hip	11	4.9	0.08	86	117	12	5	TCZ
Z-2	80	F	Knee	36	1.1	0.22	46	51	6	5	Tac (1 mg/day)
C-3	66	F	Knee	31	3.7	1.65	56	105	8	0	ETN
F-3	56	F	Knee	17	2.1	0.80	47	87	6	3	Tac (1 mg/day), ADA
K-3	67	F	Hip	21	3.9	2.60	66	167	10	5	IFX
J-3	75	F	Knee	28	1.7	0.35	102	0	8	10	None
L-3	75	F	Knee	40	2.1	1.22	38	61	0	5	Tac (2 mg/day), ETN
N-3	60	F	Knee	16	3.1	0.98	125	221	12	3	ADA
R-3	52	M	Knee	10	1.8	0.14	23	48	8	0	ADA
U-3	41	F	Knee	15	1.6	0.02	54	119	12	0	TCZ
A-4	58	F	Knee	22	1.1	0.05	34	13	8	0	ABT

RA, rheumatoid arthritis; DAS28-ESR, Disease Activity Score in 28 joints - erythrocyte sedimentation rate; CRP, C-reactive protein; RF, Rheumatoid factor; ACPA, anti-citrullinated peptide antibody; MTX, Methotrexate; PSL, methylprednisolone; DMARDs, disease-modifying anti-rheumatic drugs; TCZ, Tocilizumab; Tac, tacrolimus; ETN, Etanercept; IFX, Infliximab; ADA, Adalimumab; ABT, Abatacept.

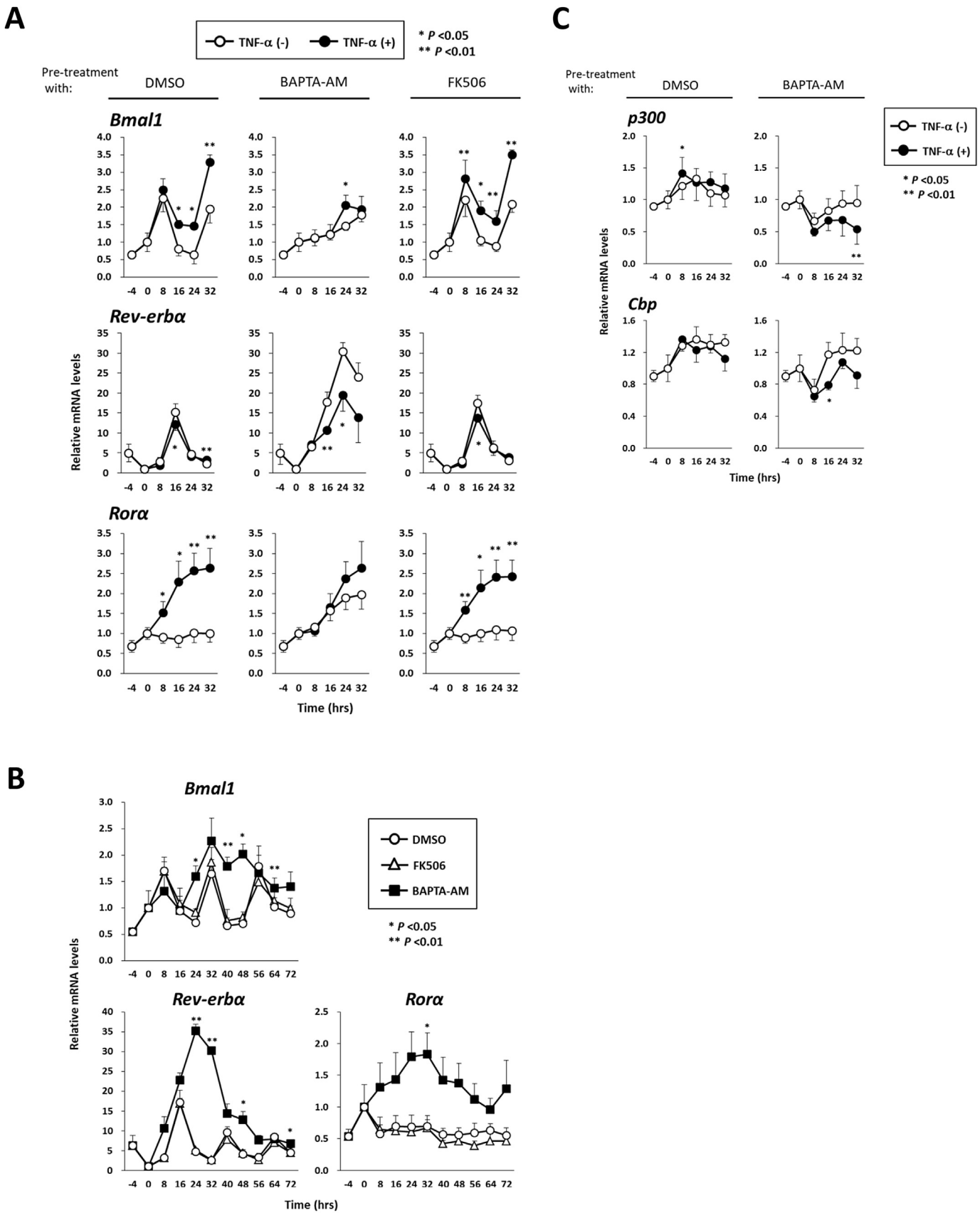

Fig. 1. Role of calcium influx in TNF-α-induced modulation of *Bmal1*, *Rorα* and *Rev-erbα* transcription. (A) The mRNA expression of *Bmal1* and its transcriptional activator *Rorα* were significantly increased, while those of transcriptional repressor *Rev-erbα* was decreased and the oscillation-phase did not differ upon incubation with TNF-α in rheumatoid synovial cells. When cells (S-2, T-2, C-3, J-3, L-3) were treated with an intra-cellular Ca^{2+} chelator BAPTA-AM after synchronization, TNF-α-mediated up-regulation of *Bmal1* and *Rorα* were

Bmal1, expression of *Rev-erbα* mRNA also showed the oscillation that reached the peak at 16 h, while by TNF-α, it was slightly decreased at 16 h ($P < 0.05$) and increased at 32 h thereafter ($P < 0.01$). Interestingly, TNF-α did not affect the oscillation-phase of both *Bmal1* and *Rev-erbα* until 32 hrs' culture period. By contrast, *Rorα* showed no circadian oscillation at all, but expressions of those were significantly increased in the presence of TNF-α ($P < 0.01$, Fig. 1A, left).

3.2. BAPTA-AM regulates the expression of Bmal1, Rorα and Rev-erbα

We next examined the effect of calcium signaling in expression of *Bmal1*, *Rorα* and *Rev-erbα* in synovial cells. When cells were treated with an intra-cellular Ca^{2+} chelator BAPTA-AM, but not a calcineurin inhibitor FK506, circadian oscillation of *Rev-erbα* gene was lost and its expression was significantly increased as compared with DMSO control (Fig. 1B). As well as *Rev-erbα*, the mRNA expression of *Rorα* was constantly increased by treatment with BAPTA-AM. Accordingly, circadian oscillation of *Bmal1* gene, controlled by these transcriptional factors, was lost and up-regulated by BAPTA-AM treatment. Since these effects were observed in a dose-dependent manner (Supplementary Fig. 1), our results indicated that Ca^{2+} influx, but not calcineurin-nuclear factor of activated T cells (NFAT) pathway, affected both circadian oscillation and gene expression of *Bmal1* through *Rorα* and *Rev-erbα* transcription in rheumatoid synovial cells.

3.3. Roles of Ca^{2+} influx for the disruption of Bmal1, Rorα and Rev-erbα, induced by TNF-α

To further examine the roles of Ca^{2+} influx in TNF-α-induced activation of the *Bmal1* gene, synovial cells were incubated in the presence of TNF-α after treatment with BAPTA-AM or FK506. As results, up-regulation of *Rorα* were cancelled by treatment with BAPTA-AM although that of *Bmal1* was still apparent at 24 h. Of the three treatments, BAPTA-AM demonstrated the strongest inhibition of *Rev-erbα* as compared with DMSO control or FK 506 (Fig. 1A).

3.4. TNF-α affects on histone acetyltransferases p300 and Cbp

Since it has been reported that a histone acetyltransferase including p300 and CREB-binding protein (CBP) are partly regulated by calcium signaling [10], we then investigated the effect of TNF-α on mRNA expression of these histone acetyltransferases in synovial cells. Upon incubation with TNF-α, expression of *p300* mRNA was significantly increased at 8 h ($P < 0.05$), while those of *Cbp* was not (Fig. 1C, left). By contrast, when cells were pre-treated with BAPTA-AM, mRNA expression of both *p300* and *Cbp* genes, as well as *Rev-erbα*, were significantly decreased by stimulation of TNF-α at 32 h ($P < 0.01$) and 16 h ($P < 0.05$), respectively (Fig. 1C, right).

3.5. p300 and CBP are responsible for the transcription of Bmal1, Rorα, and Rev-erbα

By using siRNA and C646 inhibitor, we examined the role of *p300* and *Cbp* for TNF-α-mediated modulation in clock genes' transcription. First, we transfected either p300 siRNA and/or Cbp siRNA to synovial cells, and then confirmed the inhibition of their gene expression, respectively, as compared with negative control siRNA (Fig. 2A, upper). When cells were transfected with control siRNA, TNF-α did not influence the oscillation-phase of *Rev-erbα* while enhanced expression of *Rorα*, resulting in over-expression of *Bmal1* (Fig. 2A, lower), as the same manner shown in Fig. 1A, left. Interestingly, we could not detect any effect even if solely silencing *p300* or *Cbp* (Supplementary Fig. 2), however, effects of TNF-α on clock genes were completely cancelled when *p300* and *Cbp* were silenced at the same time (Fig. 2A, lower). Finally, C646, the dual inhibitor of *p300* and *Cbp*, also exhibited the same effects on TNF-α (Fig. 2B).

4. Discussion

In this study we have shown that TNF-α enhanced expression of *Rorα* gene, but did not affect the phase of oscillation in *Rev-erbα* gene. Accordingly, TNF-α also did not affect the oscillation-phase, but enhanced expression of *Bmal1* gene in rheumatoid synovial cells. ROR activates *Bmal1* but is dispensable for its rhythmicity, whereas REV-ERBα and β are essential for keeping rhythmic expressions of Bmal1 *in vitro* and *in vivo* [11,12]. In these points of view, our results indicate that TNF-α modulates the transcription, but not circadian oscillation, of *Bmal1* by up-regulating *Rorα*.

It has been reported that periodic Ca^{2+} influx is a critical process for the function of circadian pacemaker such as *Per1/2* genes in rodent [9]. Therefore, we assumed that calcium signaling also regulated *Bmal1* gene and its transcriptional regulator *Rev-erbα* and *Rorα* in rheumatoid synovial cells, and showed that the inhibition of Ca^{2+} influx disrupted the phase and expression of *Bmal1*, by using an intracellular Ca^{2+} chelator BAPTA-AM. Although RORα transactivates expression of *Bmal*1 and REV-ERBα represses that [13], we show that expressions of both *Rev-erbα* and *Rorα* mRNA are simultaneously up-regulated by BAPTA-AM. Our results suggest that expression of *Bmal1* is strongly affected by *Rorα* but not *Rev-erbα* in the absence of Ca^{2+} influx, however, further studies are required for elucidating precise regulations.

We also showed that BAPTA-AM cancelled up-regulation of both *Bmal1* and *Rorα* induced by TNF-α. These results are coincide with a previous study that TNF-α-induced expressions of clock genes such as *Per1* and *Dbp* are cancelled by inhibition of Ca^{2+} influx in NIH3T3 mouse fibroblast cells [14]. Indeed, it has been reported that TNF-α enhanced not only the concentration of intracellular Ca^{2+} in human airway myocytes [15] but also the oscillation of Ca^{2+} influx in human macrophages cells [16]. Taken together, our and previous results indicated that TNF-α could modulate expressions of *Bmal1* and *Rorα* by association with Ca^{2+} influx. Interestingly, we also found that BAPTA-AM was highly effective on suppressing *Rev-erbα* as compared with DMSO control under stimulation with TNF-α, suggesting that *Rev-erbα* also could regulate expression of *Bmal1* in absence of Ca^{2+} influx.

It has been reported that intracellular Ca^{2+} stimulates the phosphorylation of cyclic AMP-responsive element-binding protein (CREB) through calcium- and calmodulin-dependent kinases, and in turn, phosphorylated CREB is activated by the association with

cancelled, while down-regulation of *Rev-erbα* was predominantly expressed as compared with DMSO control and a calcineurin inhibitor FK506. (B) The effect of BAPTA-AM and FK506 compounds on the mRNA expression of *Bmal1*, *Rorα* and *Rev-erbα*. Cells (L-3, J-3, N-3) were treated with BAPTA-AM or FK506 after synchronization. The mRNA expression of *Bmal1*, *Rorα* and *Rev-erbα* genes were significantly increased, in particular, the phase of circadian oscillation in *Bmal1* and *Rev-erbα* were lost by treatment with BAPTA-AM, but not FK506. (C) The effect of TNF-α on mRNA expression of the histone acetyltransferases *p300* and *Cbp* in the presence or absence of Ca^{2+} influx. Synovial cells (S-2, T-2, C-3, J-3, L-3) were treated with 25 μg/ml BAPTA-AM for 1 h after synchronization, and then cells were stimulated with TNF-α. Each of synovial cells from at least 3 different patients was tested independently, and values shown are means ± standard error of the mean (SEM). Expression levels were normalized to *Tbp* and are represented relative to those of cells after synchronization (0 h). $^{*}P < 0.05$, $^{**}P < 0.01$.

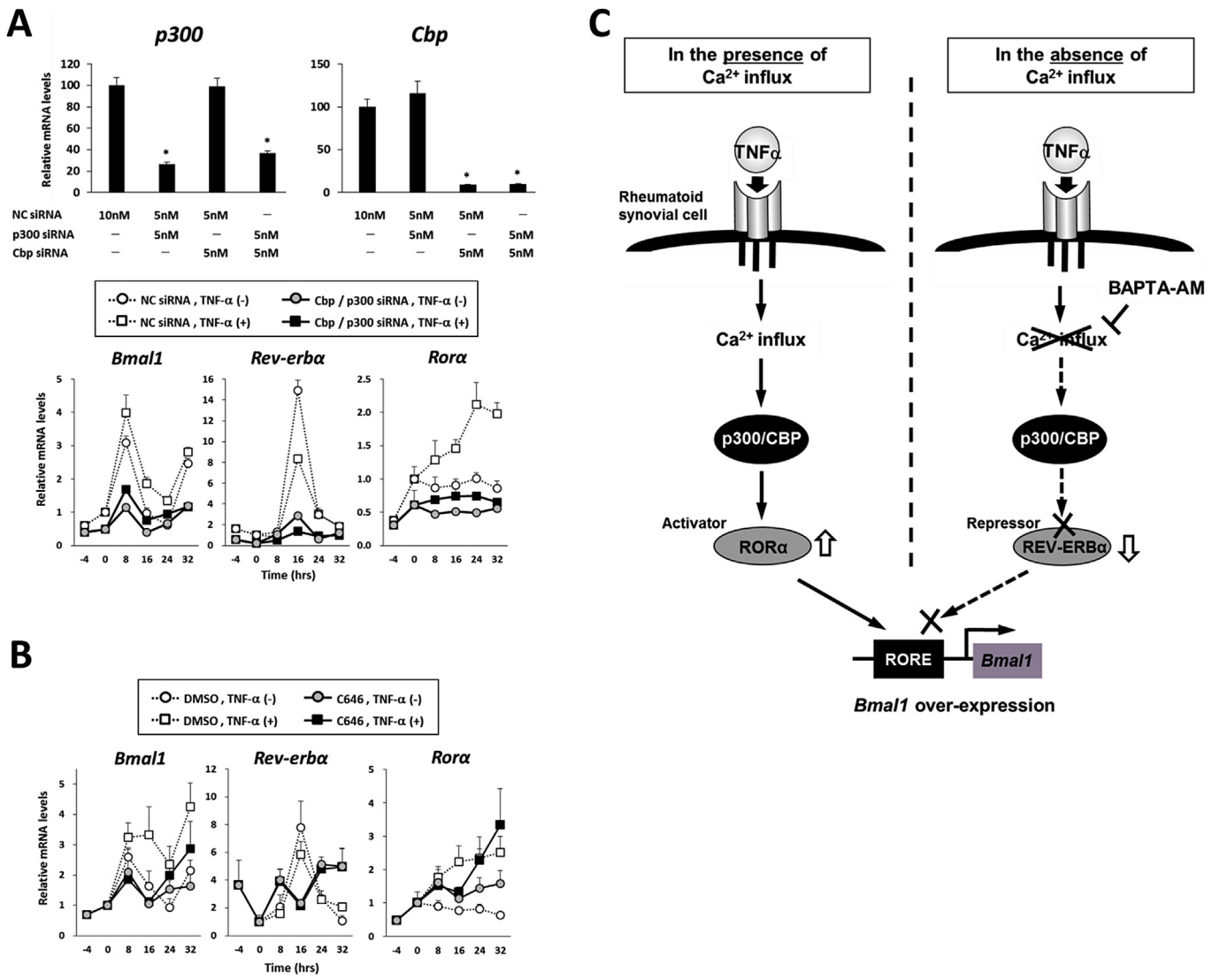

Fig. 2. Role of p300/CBP in TNF-α-induced modulation of *Bmal1*, *Rorα* and *Rev-erbα* transcription. (A, upper) Synovial cells (Z-2, U-3, A-4) were transfected with each siRNA (total 10 nM) for 48 h, and mRNA expression levels were normalized to *Tbp* and are represented relative to those of cells after transfection with negative control (NC) siRNA. Transfection with p300 and Cbp siRNA significantly reduced their target genes expression, respectively. (A, lower) Synovial cells (Z-2, U-3, A-4) were transfected with 10 nM NC siRNA (dashed line) or both 5 nM p300 siRNA and the same dose of Cbp siRNA (solid line) for 48 h, and cells were synchronized and then stimulated with TNF-α. Expression levels were normalized to *Tbp* and are represented relative to those of cells after transfection with NC siRNA (0 h). (B) Cells (T-2, K-3, R-3) were treated with DMSO control (dashed line) or 25 μM C646, a p300/CBP inhibitor (solid line), for 1 h after synchronization, and then cells were stimulated with TNF-α. Expression levels were normalized to *Tbp* and are represented relative to those of cells after synchronization (0 h). Each of synovial cells from 3 different patients was tested independently, and values shown are means ± SEM. (C) Predicted dual calcium dependent pathways of TNF-α-mediated *Bmal1* over-expression in rheumatoid synovial cells.

histone acetyltransferase paralogues p300 and CBP. Consequently, CREB-p300/CBP complex promotes their target gene transcription [10]. Therefore, we investigated the effect of p300/CBP on TNF-α-induced up-regulation of *Bmal1* and *Rorα*, and down-regulation of *Rev-erbα* in synovial cells. We found, for the first time, that such actions did not differ in cases of suppression of either *p300* or *Cbp*, whereas it was cancelled by silencing of both *p300* and *CBP*. This was consistent with the result in the case of pre-treatment with p300/CBP inhibitor, C646 compound. These results suggest that disruption of *Bmal1*, *Rorα* and *Rev-erbα*, induced by TNF-α, were required for both *p300* and *Cbp*, which these functions could be overlapped and supplied each other. In addition, the inhibition of p300/CBP function affected the oscillation-phase and expression of *Rev-erbα* and *Rorα* gene.

In conclusion, we proposed a novel role of TNF-α in inducing *Bmal1* via dual calcium dependent pathways; *Rorα* was up-regulated in the presence of Ca^{2+} influx and *Rev-erbα* was down-regulated in the absence of that. In addition, these pathways were regulated by p300/CBP histone acetyltransferases in rheumatoid synovial cells (Fig. 2C). Recent studies have been reported that BMAL1 protein is markedly localized in the cytoplasm of RA synovium as compared with osteoarthritis [7], and also contributes to the transcription of a cell cycle regulator p21 and Wee1 kinase [17,18]. Indeed, the proliferation of rheumatoid synovial cell is enhanced through the suppression of p21 and overexpression of the transcription factor c-fos [19], while mitotic activity is inhibited through wee-1 kinase [20], resulting in the characteristic 'tumor-like' synovial overgrowth of RA. Thus, Ca^{2+} influx-p300/CBP pathway may provide a novel therapeutic strategy for RA by regulating *Bmal1* expression.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. KY and AH had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: KY and AH. Acquisition of data: KY, AN, KK, HN, KS, KU, TH, YK, KT, NN, NS, YS and AH. Analysis and interpretation of data: KY, AN and AH.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.12.015.

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