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Research article

Short cytoplasmic isoform of IL1R1/CD121a mediates IL1β induced proliferation of synovium-derived mesenchymal stem/stromal cells through ERK1/2 pathway

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ABSTRACT

Objectives: IL1β enhances proliferation of synovial mesenchymal stem/stromal cells (synMSCs) although they don't express its receptor, IL1R1/CD121a, on the cell surface. This study was aimed to elucidate the underlying mechanisms of IL1β-mediated growth promotion.

Methods: Human synMSCs were isolated from the suprapatellar synovial membrane. Cell proliferation was measured by MTT. Flowcytometric analyses were performed for surface antigen expression. Intracellular signaling pathway was analyzed by western blotting, immunocytochemistry and Q-PCR.

Results: IL1β enhanced proliferation through IL1R1/CD121a because IL1 receptor antagonist (IL1Ra) completely inhibited it. Expression analyses indicated that a short isoform of IL1R1/CD121a is expressed in synMSCs. Immunocytochemistry indicated that IL1R1/CD121a was majorly localized to the cytoplasm. Western blotting indicated that IL1β induced delayed timing of the ERK1/2 phosphorylation and IκBα degradation in synMSCs. Q-PCR analyses for IL1β-target genes indicated that cyclin D was specifically downregulated by a MAPK/ERK inhibitor, U0126, but not by a NFκB inhibitor, TPCA-1. In contrast, the expression of inflammatory cytokines such as IL1α and IL6 are significantly decreased by TPCA-1 but less effectively decreased by U0126.

Conclusion: Our data indicated that the cytoplasmic IL1R1/CD121a transduced IL1β signal in synMSCs. And the growth-promoting effect of IL1β can be separated from its inflammatory cytokine-inducing function in synMSCs.

1. Introduction

Synovial tissues include slow-cycling cells under the physiological conditions those start to proliferate after joint injury [1]. Since they are shown to migrate to the injury site and contribute to the process of cartilaginous tissue regeneration, these cells are considered to be tissue-residing mesenchymal stromal/stem cells (MSCs) in the knee joint [1, 2, 3, 4]. We have shown that fibroblastic cells isolated from the synovial membrane selectively form a relatively homogeneous cell population that meets the minimum criteria to define MSCs, as proposed by the International Society for Cell Transplantation (ISCT), which are plastic-adherent when maintained in standard culture conditions, positive for MSC-related surface antigens, such as CD73, CD90, and CD105, and ability to differentiate into osteoblastic, chondrocytic, and adipocytic-cells in vitro [5, 6]. We have reported that synovial MSCs (synMSCs) have higher proliferative and chondrogenic potentials than those from other tissues, such as bone marrow, muscle, and subcutaneous fat tissues in vitro [6]. Preclinical experiments indicated that transplantation of synMSCs into the knee joint enhanced the process of cartilaginous tissue repair in various animal models [3, 7]. Based on these previous studies, we started clinical trials in which autologous synMSCs were arthroscopically transplanted into the knee joint with articular cartilage and/or meniscal defects. The 3-year follow-up studies indicated good clinical outcomes of autologous

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synMSCs transplantation in all patients subjected to trials [8]. However, we sometimes found it difficult to have sufficient numbers of MSCs for transplantation in clinical settings, possibly due to the patient's individual differences. Since animal experiments revealed that transplantation of higher numbers of MSCs (10^6 to 10^7 cells) results in better clinical outcomes than lower numbers (10^5 cells) [9, 10], we believe that it is quite important to optimize the in vitro cell expansion protocols to stably obtain a sufficient number of autologous synMSCs in addition to controlling the quality and viability of cells. We believe that the use of growth-promoting factors without affecting the stemness of synMSCs should be one approach to achieve this issue.

We have previously reported that an inflammatory cytokine, IL1β, acts as a potent growth factor for synMSCs in vitro [11]. Although there are some individual differences between the patients, in all cases tested, 100 pg/ml to 10 ng/ml IL1β significantly enhanced the proliferation of synMSCs without affecting their stem cell characteristics in vitro [11], suggesting that IL1β might be used to stably expand MSCs in vitro. However, by flow cytometric analysis, we found that only 10% of synMSCs expressed IL1R1/CD121a, a ligand-binding subunit of the IL1β receptor complex, by flowcytometric analyses [12].

The purpose of this study was to elucidate the molecular mechanisms underlying the enhancement of the proliferation of synMSCs by IL1β. Here, we showed that synMSCs express a short isoform of CD121a, which is majorly located in the cytosolic compartment, and induces delayed ERK1/2 phosphorylation in synMSCs. We also showed that the growth-promoting effect of IL1β is mediated by ERK pathways, whereas IL1β-induced cytokine expression is mainly mediated by the NFκB signaling pathway. These data suggest that by using specific intracellular signaling pathway inhibitors, we can control the balance between anabolic and catabolic features of IL1β in vitro.

2. Materials and methods

2.1. Ethics

This study was approved by the Ethical Committee of Medical Hospital, Tokyo Medical and Dental University, and written informed consent was obtained from all study subjects (approval number: M2017-142).

2.2. Synovial MSCs

Human synMSCs were prepared from the suprapatellar synovial membrane of patients with knee osteoarthritis during total knee arthroplasty, as described previously [13]. Cells less than 6 passages were used in this study. Patient demographic data and sample information for each experiment are summarized in Supplemental Table 1.

2.3. Cell growth analyses

Fifty-thousand cells were seeded on 60 cm^2 dishes and cultured for 9 days in reduced (0.1% FBS) or normal growth medium (MEM-alpha#12561 supplemented with 10% FBS#10437, and antibiotics#15240, Gibco, New York, NY, USA). The IL1β group contained 10 ng/mL of human recombinant proteins (R&D Biosystems#201-LB/CF, Minneapolis, MN, USA). At days 0,5,7, and 9, cells were dispersed by trypsin (Gibco#25200) and counted using a hemocytometer as we described before [11].

MTT (3-(4,5-Dimethylthial-2-yl)-2,5-Diphenyltetrazalium Bromide) Assay.

Cells were seeded at 7.5×10^2 cells/well in 96-well dishes (Corning#3595, New York, NY, USA). The next day, the culture medium was replaced with fresh medium supplemented with 10% FBS, IL1β and/or inhibitors (U0126: 20μM, Cell Signal Technology#9903S, Danvers, MA, USA; TPCA-1: 1μM, Sigma#T1452, St. Louis, MO, USA; and IL1Ra: 60 ng/mL, PeproTech#200-01RA, East Windsor, NJ, USA). Each group used 8 wells. The cell proliferation rate was determined by the MTT assay (Thermo Fisher#M6494, Waltham, MA, USA), as described previously [14].

2.4. Flow cytometry

Cells were detached by trypsin and stained with PE-conjugated human IL-1R1/CD121a antibody (R&D biosystems#FAB269P) for 30 min on ice. The surface antigen-positive cell fraction was measured using a FACSVerse flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA) as described previously [12].

2.5. Western blotting analyses

SynMSCs were seeded on 6 cm-diameter dishes at 5×10^5 cells and incubated in normal growth medium for 1 day. Cells were starved in MEM-alpha supplemented with 0.1% FBS and antibiotics for 24 h to reduce phosphorylation of tyrosine residues, and then treated with serum and/or rhIL1β (10 ng/mL). Total cell lysates were prepared using a cell lysis buffer (Cell Signaling Technology#9803, Danvers, MA, USA). Western blotting was performed as described previously [15, 16]. Antibodies against phospho-p44/42(#9101), p44/42(#9102), and IκBα(#9242) were purchased from Cell Signaling Technology. Antibody against CD121a, which recognizes intracellular (TIR) domain of CD121a(#sc-393998), was purchased from Santa Cruz.

2.6. Gene expression analyses

Total RNA was extracted from synMSCs using the High Pure RNA Isolation Kit (Roche#11828665001, Basel, Switzerland). First-strand cDNA was prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche#04897030001). Relative RNA expression levels were evaluated using the FastStart TaqMan Probe Master (Roche#04673433001) and Universal ProbeLibrary Set (Roche#78018100, #78018200) kit and Lightcycler480 (Roche) as described previously [12]. Primer sequences and the corresponding TaqMan probe numbers are listed in Supplemental Table 2.

2.7. Fluorescence immunocytochemistry

Immunocytochemical analyses were performed as described previously [17]. Briefly, synMSCs were seeded at 5000–10000 cells on 8-well chamber slides (Corning#354118, New York, NY, USA) and cultured for 5 days. Cells were fixed in 4% paraformaldehyde (Wako#162-16065, Tokyo, Japan) and permeabilized in methanol (Wako#137-01823, Tokyo, Japan). Cells were incubated with CD121a antibodies (Santa Cruz#sc-393998, 1:100) overnight at 4 °C and anti-mouse IgG antibodies (Thermo Fisher#A11001, 1:250) at 20–25 °C for 1 h. To determine the localization of the cell membrane, antibodies against Na^+/K^+ ATPases (Abcam#ab70620, 1:500) were co-stained with the CD121a antibody. Images were obtained using a BX63 automated fluorescence microscope (Olympus, Tokyo, Japan). Analysis was performed using ImageJ software (NIH, USA).

2.8. Statistical analysis

One-way or two-way ANOVA followed by the Tukey–Kramer method was performed using GraphPad Prism (version7, GraphPad Software, LLC. San Diego, CA). Differences were considered statistically significant at P values less than 0.05.

3. Results

3.1. The growth-promoting effect of IL1β on synMSCs is through type I IL1β receptor, IL1R1/CD121a, although it is not presented on the cell membrane

Since CD121a is a prerequisite for the IL1β receptor complex, we hypothesized that the number of CD121a-positive MSCs should selectively increase during culture if the IL1β receptor complex is involved in the process of MSC proliferation. Flow cytometric analyses indicated the average basal population of CD121a-positive cells was 1%–2%, which was significantly lower than that of HEK293FT and HeLa cells (Figure 1A). Treatment with IL1β did not increase the number of cells positive for CD121a during the in vitro culture, whereas the total number of cells was increased (Figure 1B). However, IL1β-dependent growth-promotion of MSCs seemed to require IL1β-CD121a interaction as IL1Ra, which is a direct inhibitor of IL1β-CD121a interaction, almost completely prevented the growth-promoting effects of IL1β (Figure 1C).

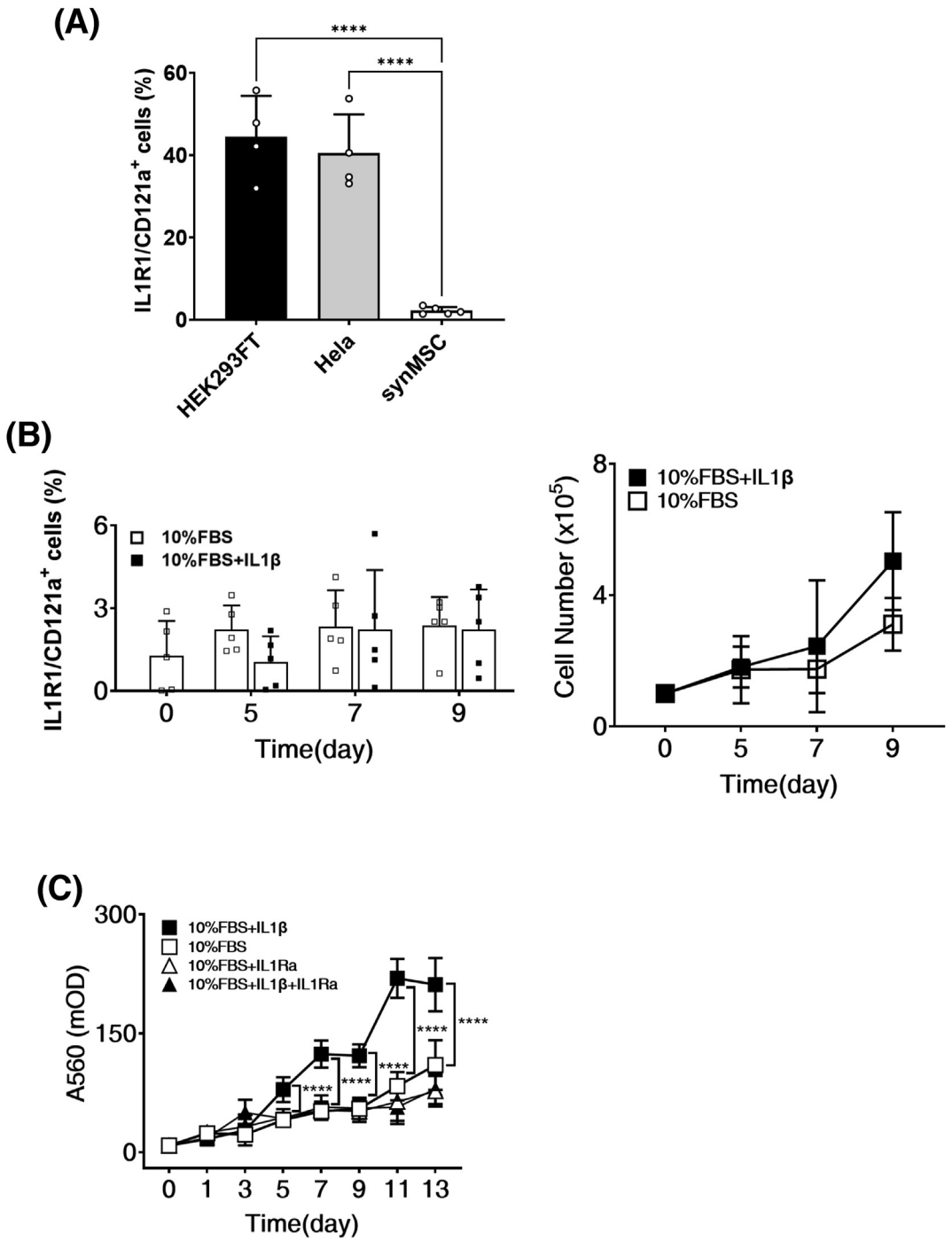

Figure 1. The growth-promoting effect of IL1β on synMSCs is through type I IL1β receptor, IL1R1/CD121a, although it is not presented on the cell membrane. (A) Population of the cells positive for IL1R1/CD121a measured by flow cytometer. Cells were maintained in the normal growth medium for 7days and stained with anti-CD121a antibody. Data are presented as mean ± SD (n = 5). (B) IL1β (10 ng/mL) enhances MSC proliferation without affecting the IL1R1/CD121a-positive population. IL1R1/CD121a-positive cell population was measured by a flowcytometer (Left). Cell number was counted by hemocytometer (Right). Data are presented as mean ± SD (n = 4). (C) MTT assay indicated that IL1Ra (60 ng/mL) completely inhibited the growth-promoting effects of IL1β (10 ng/mL) in synMSCs. Data are presented as mean ± SD (n = 6). **** indicates $p < 0.001$ by One-way ANOVA followed by the Tukey–Kramer method.

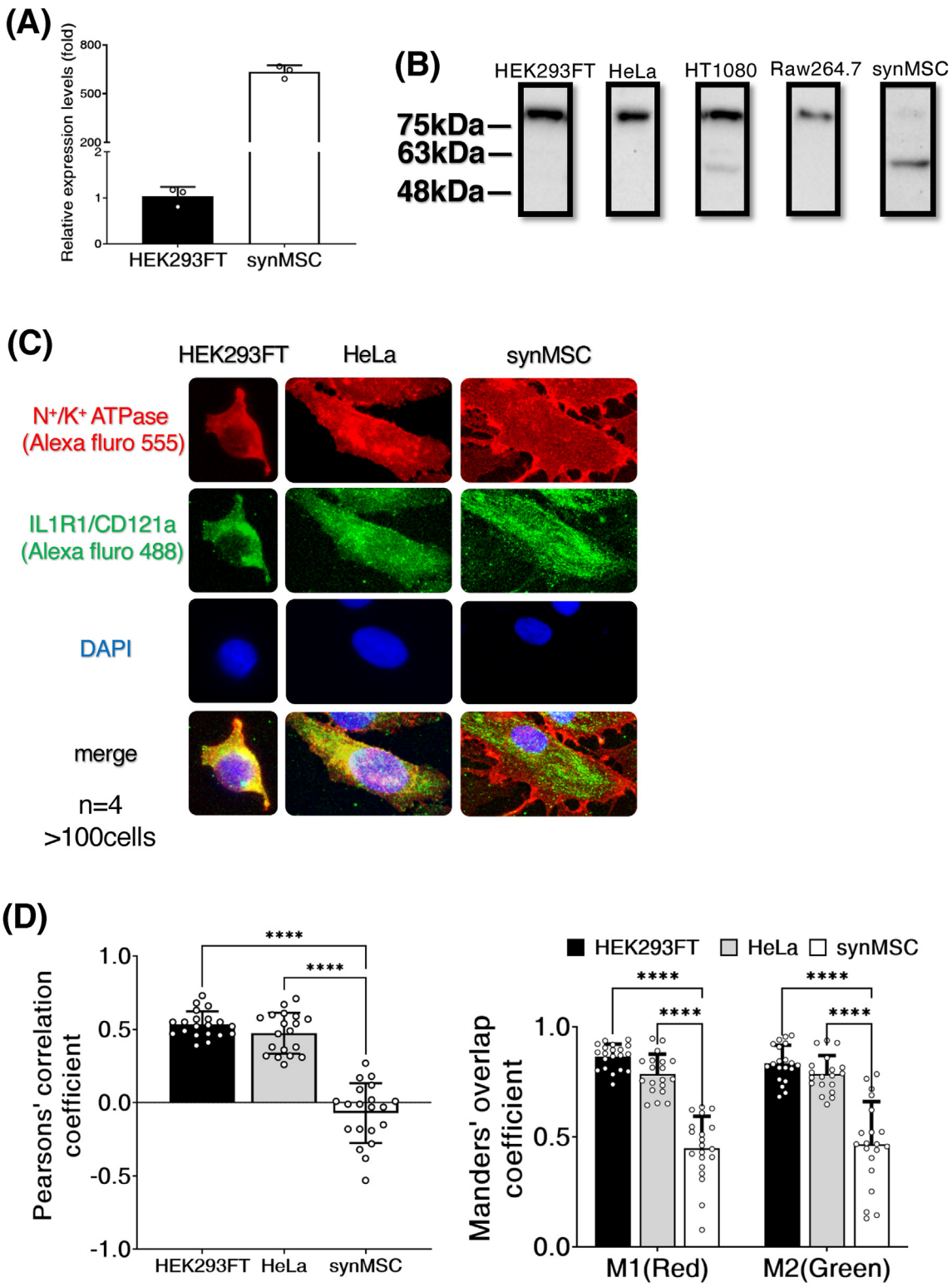

Figure 2. Low molecular weight of IL1R1/CD121a is specifically expressed in synMSCs which is majorly existed in the cytoplasmic region. (A)IL1R1/CD121a mRNA is expressed in synMSCs. Relative expression levels were analyzed by Q-PCR. Data are presented as mean ± SD (n = 3). (B) A short isoform of IL1R1/CD121a protein was detected in synMSCs (Western blot). To reconfirm the short isoform of IL1R1/CD121a in synMSCs, cells from 6 patients were subjected for the analyses and obtained the similar results (data not shown). See Supplemental figure 2 for uncropped images. (C) IL1R1/CD121a (in green) was co-stained with Na+/K + ATPase (in red) which was selected as marker of the plasma membrane. Cell nuclei was stained by DAPI (n = 4). (D) Pearson's correlation coefficient and Mander's overlap coefficient were calculated by the coloc2 plugin in Image J, and each point represents one region of interest. Selected regions of interest included over 100 discernible cells in total for each cell line [18, 19, 20]. Data are presented as mean ± SD (n = 4). **** indicates $p < 0.001$ by One-way ANOVA followed by the Tukey–Kramer method.

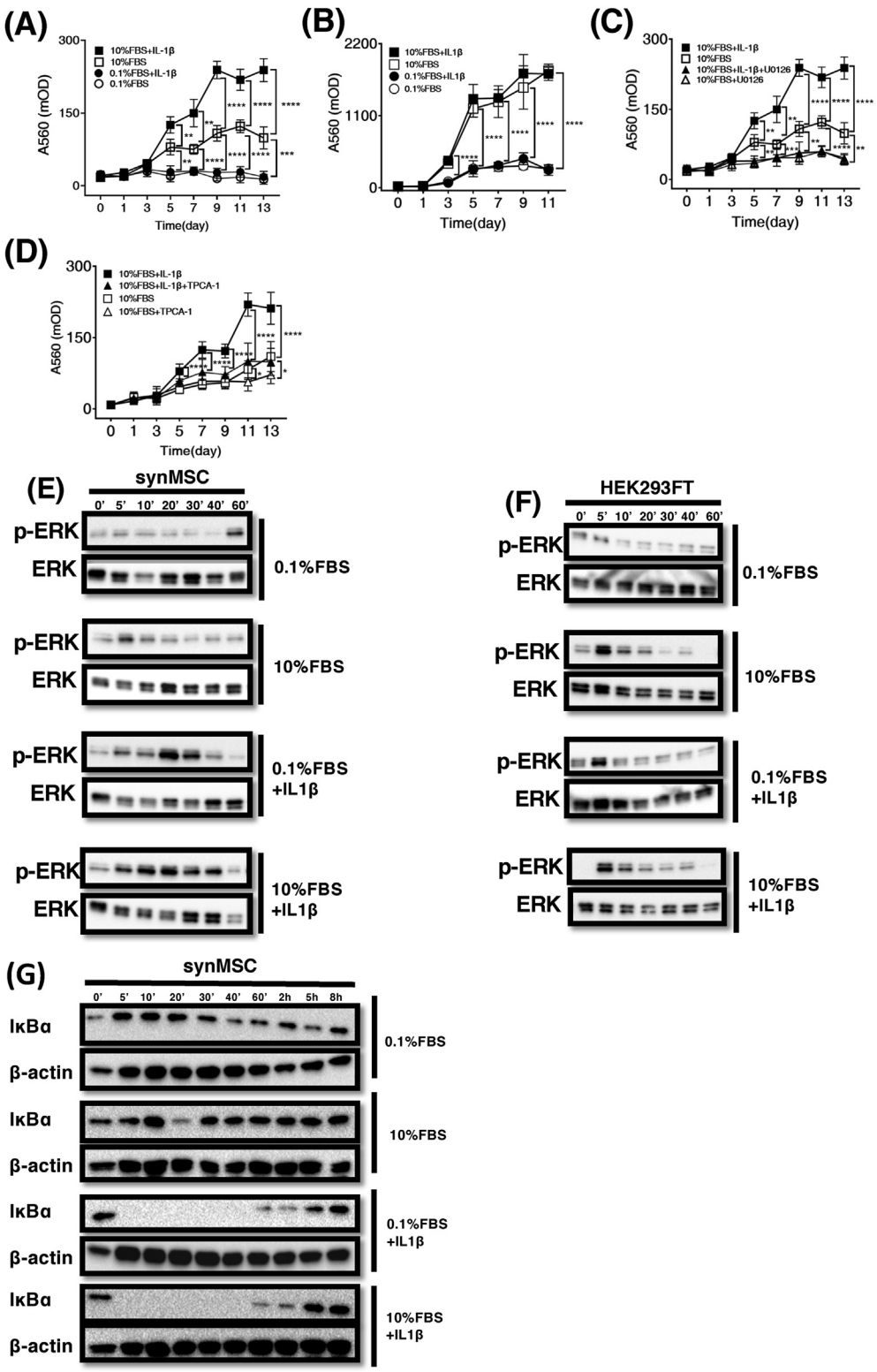

Figure 3. Analysis of intracellular signals in synMSCs stimulated by IL1β. (A) IL1β (10 ng/mL) can promote synMSCs proliferation only in the presence of 10% FBS (MTT assay). Data are presented as mean ± SD (n = 6). (B) The growth-promoting effect of IL1β (10 ng/mL) was not observed in HEK293FT cells (MTT assay). Data are presented as mean ± SD (Octuplicate). (C) An ERK1/2 inhibitor U0126 (20μM) completely inhibited the growth-promoting effect of IL1β (10 ng/mL) in synMSCs (MTT assay). Data are presented as mean ± SD (n = 6). (D) A NFκB inhibitor TPCA-1 (1μM) partially inhibited of the growth-promoting effect of IL1β (10 ng/mL) in synMSCs (MTT assay). Data are presented as mean ± SD (n = 6). **; $p < 0.01$, ***; $p < 0.005$, ****; $p < 0.001$ by Two-way ANOVA followed by the Tukey–Kramer method. (E) Delayed phosphorylation of ERK1/2 was observed in the presence of IL1β (10 ng/mL) in synMSCs (Western blot). See Supplemental figure 3 for uncropped images. Results were reconfirmed by 6 independent experiments. (F) 10%FBS and IL1β (10 ng/mL) enhanced ERK1/2 phosphorylation at 5 to 10 min in HEK293FT cells (Western blot, n = 1). See Supplemental figure 4 for uncropped images. (G)Western blot of IκBα showed IL1β (10 ng/mL) induced the degradation of IκBα from 5′ to 40′ in synMSCs (n = 1). See Supplemental figure 5 for uncropped images.

3.2. Low molecular weight of CD121a is specifically expressed in synMSCs which is majorly existed in the cytoplasmic region

Based on these results, we investigated CD121a expression in synMSCs. Quantitative PCR (qPCR) experiments indicated that CD121a expression was detectable in MSCs and the relative mRNA expression level was higher than that in HEK293FT cells, which were positive for CD121a on the cell surface (Figures 2A and 2C). Western blot analyses indicated that the short isoform of approximately 50–60 kDa was detected in MSCs. This short isoform of CD121a seems specifically expressed in synMSCs since we observed a long isoform in other types of cells with surface expression of CD121a (Fig 2B and C). Immunocytochemical analyses indicated that CD121a was co-localized with Na^+/K^+ ATPases, which are cell membrane-associated proteins, in HEK293FT

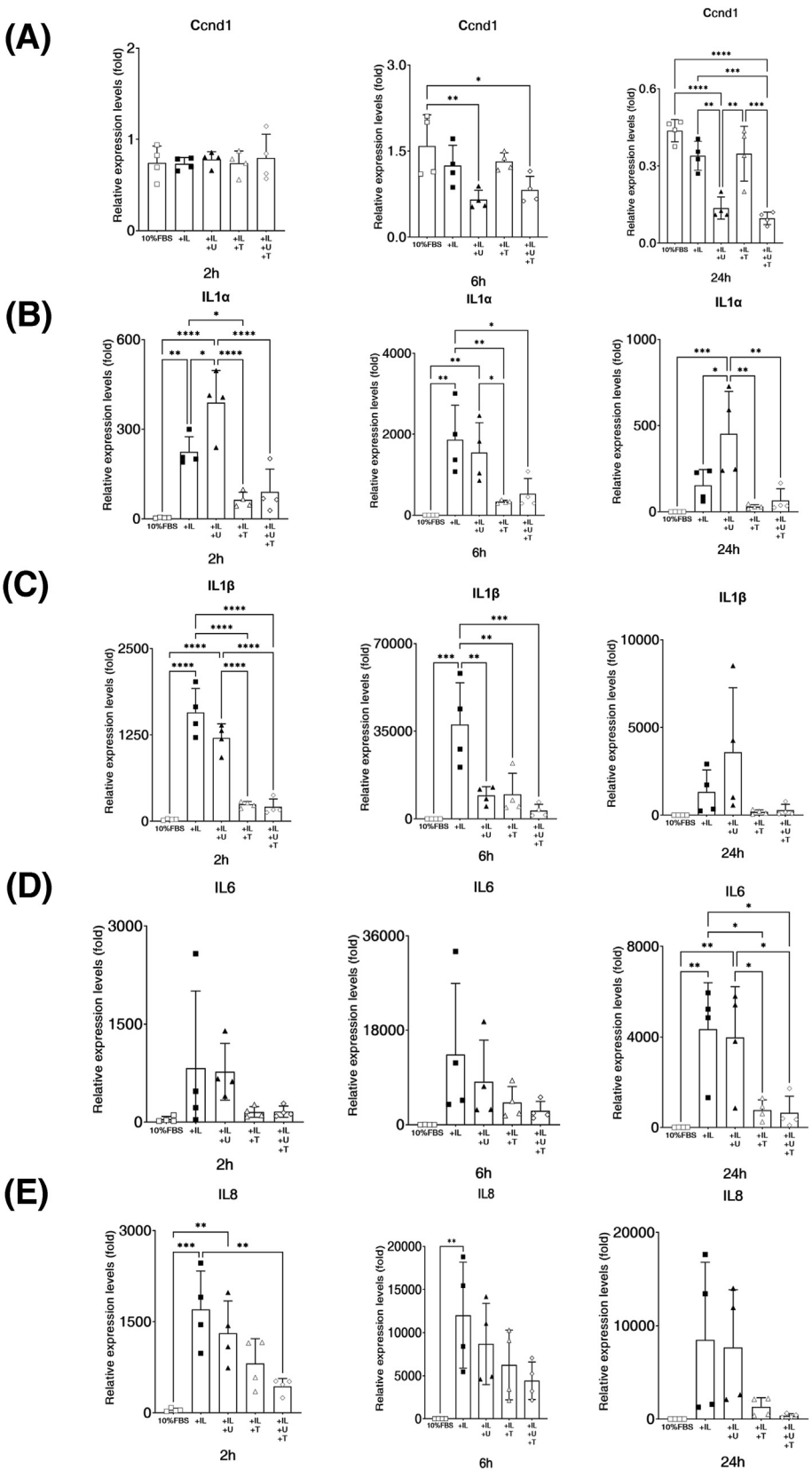

Figure 4. Effects of ERK1/2 inhibitor U0126 (20μM) and/or NFκB pathway inhibitors TPCA-1 (1μM) on the expression of IL1β target genes in synMSCs. (A) Ccnd1 (cyclin D1) expression at 2, 6, and 24 h after FBS (10%) and IL1β (10 ng/mL) stimulation in synMSCs. (B-D) Cytokine (IL1α, IL1β and IL6) expressions at 2, 6, and 24 h after FBS (10%) and IL1β (10 ng/mL) stimulation in synMSCs. (E) Chemokine (IL8) expressions at 2, 6, and 24 h after FBS (10%) and IL1β (10 ng/mL) stimulation in synMSCs. All data are presented as mean ± SD (n = 4). *; $p<0.05$, **; $p<0.01$, ***; $p<0.005$, ****; $p<0.001$ by One-way ANOVA followed by the Tukey–Kramer method. IL: IL1β, U: U0126, T: TPCA-1.

and HeLa cells (Figure 2C). CD121a protein was also detected in MSCs; however, it was majorly localized in the cytoplasm (Figure 2C). These results were quantitatively confirmed by Pearson's correlation, Mander's overlap coefficient analyses, and profile plots [18, 19, 20] (Figure 2D and Supplemental figure 1).

3.3. Growth-promoting effect of IL1β is serum-dependent and through extending the phosphorylation duration of ERK 1/2

To further elucidate the underlying molecular mechanisms, we analyzed the intracellular signaling molecules activated by IL1β. IL1β

enhanced the proliferation of MSCs only in the presence of growth factors in serum, and was not observed in HEK293FT cells (Figs 3A and B). The ERK1/2 inhibitor U0126 completely abolished the growth-promoting effect of both serum and IL1β, and decreased to the same levels with 0.1% FBS (Figure 3C). The NFκB pathway inhibitor, TPCA-1, also inhibited the growth-promoting effect of IL1β, however the effect was partial (Figure 3D). Western blotting analyses indicated that serum stimulation enhanced ERK1/2 phosphorylation at 5–10 min in MSCs. In contrast, IL1β increased phospho-ERK1/2 levels after 20 min. As a result, phosphorylation duration was extended after stimulation with serum and IL1β (Figure 3E). Extension of phosphorylation duration seemed unique in synMSCs since both serum and IL1β stimulation enhanced ERK1/2 phosphorylation at 5–10 min in HEK293FT cells (Figure 3F), in which IL1β did not enhance cellular proliferation (Figure 3B). Degradation of IκBα induced by serum stimulation was transiently observed at 20 min in synMSCs. IL1β greatly enhanced the degradation of IκBα regardless of the presence of serum (Figure 3G).

3.4. Differential physiological roles of ERK1/2 phosphorylation and IκBα degradation induced by IL1β on target gene expression in MSCs

To explore the physiological roles of IL1β-induced ERK1/2 phosphorylation and IκBα degradation in MSCs, target gene expression was quantitatively analyzed by qPCR. Expression of Ccnd1, a major cell cycle regulator that promotes G1 to S phase transition, was not increased by IL1β; however, it was significantly downregulated by U0126 at 6 and 24 h after stimulation (Figure 4A). In contrast, TPCA-1 had subtle effects on Ccnd1 expression (Figure 4A). IL1β treatment significantly increased the expression of inflammatory cytokines and chemokines in synMSCs (Fig 4B to 4E). TPCA-1 significantly downregulated the expression levels of IL1α and IL1β at 2 and 6 h, and IL6 at 24h (Fig 4B to 4D) whereas U0126 did not decrease IL1α, IL6, and IL8 mRNA levels up to 24 h (Fig4B to 4E). IL1β expression was significantly inhibited by both U0126 and TPCA-1 cells at 6 h (Figure 4B).

4. Discussion

We have reported that IL1β functions as a potent growth-promoting factor for synMSCs, although most of them do not express the IL1β receptor CD121a on the cell surface. To clarify the underlying molecular mechanisms of this phenomenon, we examined IL1β-mediated signal pathways in synMSCs. Here, we showed that synMSCs express a short isoform of the CD121a protein which is majorly localized in the cytosolic fraction. We further demonstrated that IL1β enhances proliferation in collaboration with growth factors in serum, and IL1β treatment extends the duration of serum induced ERK1/2 phosphorylation in synMSCs.

A previous study reported that there are seven transcriptional starting sites in the CD121a gene, one of which is located after the conventional translational start site. Thus, it has been predicted that there are mainly two CD121a protein products: a long isoform and a short isoform [21, 22, 23]. Promoter usage of CD121a mRNA expression varies in each cell type; however, it is believed that transcripts from exon 1C encode the most common (long) isoform of CD121a [22]. The short isoform of the CD121a protein is reported to be a type 3 IL1 receptor (IL1R3), which is reported to be expressed in neural cells [21, 23]. Although the functional differences between the long and short isoforms have not been studied extensively, the short isoform in neural cells is reported to mediate potassium current increase through fast activation of Akt kinase [23]. In this study, we compared CD121a protein expression in HEK293FT, HeLa, HT1080, Raw264.7, and synMSCs by western blotting analyses and showed for the first time that synMSCs dominantly expressed the short isoform of CD121a (around 60kDa). The short isoform of CD121a is expected to start from exon 4; thus, N-terminal signal peptide sequences are truncated. This may be the reason why CD121a majorly localizes to the cytoplasmic region of synMSCs. In other cells tested, the major CD121a product was the long isoform. HEK293FT cells express CD121a on the cell surface. Interestingly, neither extended ERK1/2 phosphorylation nor growth-promoting effects were observed following IL1β treatment in HEK293FT cells. These results suggest that cytoplasmic CD121a may play a pivotal role in IL1β-mediated growth promotion in synMSCs. Previous study reported that the short form of CD121a (IL1R3) in neural cells can bind to IL1β and interact with IL1RAcP (Interleukin 1 receptor accessary protein), a co-receptor for CD121a, to mediate IL1β signal [23]. Thus, we expect that short form of CD121a in synMSCs may bind to IL1β. Our result that growth-promoting effects of IL1β was inhibited by IL1Ra (Figure 1C) also supports our discussion. It is still unclear how IL1β interacts with CD121a in the cytoplasm and why extended ERK1/2 phosphorylation occurs in synMSCs, which are the top of our next experimental plans to be solved.

In this study, we showed that inhibition of ERK1/2 phosphorylation by U0126, a specific inhibitor of MEK1/2 (MAP ERK kinase), significantly downregulated serum and/or IL1β induced Ccnd1 mRNA expression in synMSCs, whereas the IκBα kinase (IKK) inhibitor, TPCA-1, had subtle effects. In contrast, TPCA-1 significantly downregulated IL1α, IL1β, and IL6, whereas U0126 had less effect on the expression of these genes. These data suggest that the growth-promoting effect of IL1β on synMSCs is mainly mediated through the phosphorylated ERK1/2 pathway, whereas catabolic cytokine expression is mainly mediated by the NFκB pathway. Our clinical tests indicated that transplantation of in vitro expanded synMSCs enhanced joint tissue regeneration. These data indicate that an increased number of synMSCs in the joint cavity may have anabolic effects on the cartilaginous tissues in the joint. However, MSCs also produce various catabolic factors, such as inflammatory cytokines and matrix-degrading enzymes, in the inflammatory environment. Since MSCs in synovial fluid are increased after joint damage or inflammation, these cells might be a double-edged sword for the process of joint tissue repair. Our data indicate the possibility that we will be able to enhance the anabolic effects of MSCs using specific NFκB pathway inhibitors. Testing this hypothesis will be considered a priority in our future experimental plan.

In summary, we analyzed the molecular mechanism how IL1β promotes the proliferation of synMSCs and showed that IL1β prolongs the phosphorylation state of ERK1/2 induced by 10%FBS in these cells. These effects seemed specific to synMSCs since we did not observe neither extended ERK1/2 phosphorylation nor growth promotion in HEK293FT cells. We consider that these may be due to the specific regulation of IL1R1/CD121a expression in synMSCs since a short cytoplasmic isoform of IL1R/CD121a is selectively expressed in these cells. In addition, we showed that pro-inflammatory cytokines expression by IL1β is mainly through NFκB but not through ERK1/2 pathway in synMSCs. Thus, we expect that we can use IL1β as a growth factor for synMSCs without inducing pro-inflammatory gene expression in the presence of NFκB inhibitor in vitro. We hope that this study will provide useful information for the optimization of in vitro MSC culture protocols in cell transplantation therapy.

Declarations

Author contribution statement

Kunikazu Tsuji: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yoshinori Asou, Yoichi Ezura, Hiroki Katagiri, Hideyuki Koga, Keiichiro Komori, Kazumasa Miyatake, Yusuke Nakagawa, Tomomasa Nakamura, Ichiro Sekiya: Analyzed and interpreted the data.

Etsuko Matsumura: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Guo Tang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e09476.

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