Biochemical and Biophysical Research Communications 424 (2012) 793–800

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

TGF-β but not BMP signaling induces prechondrogenic condensation through ATP oscillations during chondrogenesis

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ARTICLE INFO

Article history:
Received 9 July 2012
Available online 16 July 2012

Keywords:
Transforming growth factor-beta (TGF-β)
Bone morphogenetic proteins (BMPs)
Prechondrogenic condensation
Chondrogenic differentiation
ATP oscillations

ABSTRACT

Although both TGF-β and BMP signaling enhance expression of adhesion molecules during chondrogenesis, TGF-β but not BMP signaling can initiate condensation of uncondensed mesenchymal cells. However, it remains unclear what causes the differential effects between TGF-β and BMP signaling on prechondrogenic condensation. Our previous report demonstrated that ATP oscillations play a critical role in prechondrogenic condensation. Thus, the current study examined whether ATP oscillations are associated with the differential actions of TGF-β and BMP signaling on prechondrogenic condensation. The result revealed that while both TGF-β1 and BMP2 stimulated chondrogenic differentiation, TGF-β1 but not BMP2 induced prechondrogenic condensation. It was also found that TGF-β1 but not BMP2 induced ATP oscillations and inhibition of TGF-β but not BMP signaling prevented insulin-induced ATP oscillations. Moreover, blockage of ATP oscillations inhibited TGF-β1-induced prechondrogenic condensation. In addition, TGF-β1-driven ATP oscillations and prechondrogenic condensation depended on Ca^{2+} influx via voltage-dependent calcium channels. This study suggests that Ca^{2+}-driven ATP oscillations mediate TGF-β-induced the initiation step of prechondrogenic condensation and determine the differential effects between TGF-β and BMP signaling on chondrogenesis.

1. Introduction

In vertebrate limb development, mesenchymal cells first aggregate, become closely packed, and differentiate into chondrocytes within these aggregates to form cartilage elements that are ultimately replaced by bone tissues [1]. This fact indicates that prechondrogenic condensation is a critical step for skeletal pattern formation. Many studies have described that the skeletal pattern formation depends on a number of signaling molecules including transforming growth factor-beta (TGF-β) superfamily, fibroblast growth factor family, Hedgehog (HH) family and Wingless family [2].

TGF-β superfamily members play diverse roles in all aspects of skeletal development [3,4]. TGF-β signaling has been shown to stimulate cartilage formation in a variety of *in vitro* models [5,6]. Studies in micromass cultures have demonstrated that TGF-β signaling stimulates prechondrogenic condensation by producing both fibronectin and N-cadherin and also enhances expression of chondrogenic markers [7–9]. Furthermore, TGF-β signaling has been shown to drive isolated adult mesenchymal cells to a chondrocytic fate and is thus used to stimulate cartilage formation in dedifferentiated chondrocytes and mesenchymal stem cells [10]. Bone morphogenetic proteins (BMPs), which are members of TGF-β superfamily, also regulate formation of the cartilage elements and promote chondrogenesis in non-chondrogenic cells in the limb [11,12]. Moreover, it was demonstrated that BMP signaling is required for prechondrogenic condensation [13].

In contrast, it has been demonstrated that TGF-β signaling operates prior to BMP signaling and effectively initiates condensation of uncondensed cells, whereas BMP signaling is more effective in condensed cells [8] and has little effect on mesenchymal cells at low density [14]. Furthermore, it was shown that TGF-β signaling increases not only the size and but also the number of prechondrogenic condensations, while BMP signaling increases the condensations size rather than the condensations number [7]. These results reveal the differential effects between TGF-β and BMP signaling on prechondrogenic condensation. However, it has remained unclear what underlie these distinct actions between TGF-β and BMP signaling on prechondrogenic condensation.

Our previous study demonstrated that ATP oscillations play a critical role in prechondrogenic condensation by inducing oscillatory secretion [15,16]. This stimulated us to postulate that the action of TGF-β and BMP signaling on prechondrogenic condensation is related to ATP oscillations. Thus, the present work has focused on whether ATP oscillations mediate the actions of TGF-β and BMP signaling on prechondrogenic condensation. In this study,

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.07.041

prechondrogenic cell line ATDC5 which differentiates into chondrocyte to form condensations in the presence of insulin [17] and primary mouse mesenchymal stem cells (mMSCs) which differentiate into chondrocytes in the micromass culture were used as *in vitro* chondrogenesis models [18]. As described in the previous studies [15,16], bioluminescence reporter gene of *Phrixothrix hirtus* luciferase emitting red light (PxRe) fused to an ACTIN promoter (P_{ACTIN}-PxRe) was used in monitoring intracellular ATP level during chondrogenesis. Herein, this study showed the differential effects between TGF-β and BMP signaling on prechondrogenic condensation that TGF-β but not BMP signaling induced prechondrogenic condensation. In parallel to this result, the result showed the differential actions between TGF-β and BMP signaling on ATP oscillations that TGF-β but not BMP signaling induced ATP oscillations. Furthermore, suppression of ATP oscillations blocked TGF-β-induced prechondrogenic condensation. In addition, both TGF-β-driven ATP oscillations and prechondrogenic condensations depended on Ca^{2+} influx via voltage-dependent calcium channels (VDCCs). This study suggests that ATP oscillations mediate inductive action of TGF-β signaling on prechondrogenic condensation.

2. Materials and methods

2.1. Cell culture and light microscopic observation

The ATDC5 cell line was obtained from the RIKEN cell bank (Tsukuba). The cells were cultured in maintenance medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM-F12) (Invitrogen) supplemented with 5% fetal bovine serum, 10 mg/ml human transferrin (Roche Molecular Biochemicals), and 3×10^{-8} M sodium selenite (Sigma–Aldrich) in polystyrene dishes at 37 °C under 5% CO_2. For chondrogenic induction, when ATDC5 cells maintained in the maintenance medium reached confluency, the medium was replaced with the chondrogenic medium supplemented with 10 μg/ml insulin (Sigma–Aldrich). For examining the effects of growth factors and chemical compounds on ATDC5 cells, the medium was replaced with the medium supplemented with 1 μg/ml BMP2 (Peprotech), 100 ng/ml TGF-β1 (Peprotech), and 10 μg/ml insulin plus one of the following inhibitors: 100 ng/ml Noggin (R&D Systems) and 10 μM SB-431542 (Sigma–Aldrich) respectively. After each medium was replaced every other day for 1 week, microscopic observation was performed with a phase contrast microscope (Nikon). mMSCs (Invitrogen), which are produced from bone marrow isolated from C57BL/6 mice, were maintained in DMEM-F12 with GlutaMAX-I supplemented with 10% MSC-qualified FBS (Invitrogen). For chondrogenesis, mMSCs were cultured in a high-density micromass (8×10^6 cells/ml) with the chondrogenic medium (Invitrogen). For examining the effects of growth factors and chemical compounds on mMSC, the medium was replaced with the medium supplemented with 100 ng/ml BMP2 (Peprotech), 5 ng/ml TGF-β1 (Peprotech), and 5 ng/ml TGF-β1 plus one of the following inhibitors: 1 mM 2-deoxy glucose (2-DG) (Sigma–Aldrich), 50 μM nifedipine (Sigma–Aldrich) and 100 μM 2-aminoethoxydiphenyl borate (2-APB) (Calbiochem), respectively. After 1 day culture in each medium, microscopic observation was performed with a phase contrast microscope (Nikon).

2.2. Transfection into the cells with reporter gene and bioluminescence monitoring

Bioluminescence reporter gene of *Phrixothrix hirtus* luciferase emitting red light (PxRe) fused to an ACTIN promoter (P_{ACTIN}-PxRe) was inserted into retrovirus vectors (P_{ACTIN}-PxRe). ATDC5 cells were transfected using retrovirus infection (Clontech) and then were selected by puromycin. mMSCs were transiently transfected using Lipofectamine LTX (Invitrogen). The cells transfected stably with P_{ACTIN}-PxRe were seeded in 35 mm dishes. After replacing the medium by a recording medium (DMEM/F12 with 5% FBS, 0.1 mM luciferin (Wako), and 50 mM HEPES-NaOH, pH = 7.0) (time = 0 h), bioluminescence intensity was continuously measured at 37 °C in air using a dish-type luminescencer, Kronos (ATTO) for 1 min at 1–30 min intervals.

2.3. Real-time PCR analysis

The total RNA was isolated from the ATDC5 cells cultured under various conditions for 7 d using the RNeasy Mini Kit (Qiagen). The reverse transcription reactions were performed from 0.2 μg of total RNA using a cDNA synthesis kit (Takara). The real-time PCR reactions for GAPDH, collagen II, and aggrecan were conducted using the SYBR green system. The primer sequences were as follows: type II collagen gene (*Col2a1*) forward primer 5′-AGGGCAACA GCAGGTTCACATAC-3′, reverse primer 5′-TGTCCACACCAAATTCCTG TTCA-3′; aggrecan gene (*Agc*) forward primer 5′-AGTGGATCGGTCT-GAA TGACAGG-3′, reverse primer 5′-AGAAGTTGTCAGGCTGGTTTG GA-3′; GAPDH (*Gapdh*) gene forward primer 5′-TGTGTCCGTCGTGG ATCTGA-3′, and reverse primer 5′-TTGCTGTTGAAGTCGCAGGAG-3′. The real-time PCR reactions were performed using a thermal cycler dice real time system (Takara). The samples were held at 95 °C for 10 min, followed by 40 amplification cycles consisting of a denaturation step at 95 °C for 15 s, and an extension step at 60 °C for 1 min. The expression level of the gene was normalized to GAPDH.

3. Results

3.1. Differential effects of TGF-β and BMP signaling on prechondrogenic condensation

Effects of TGF-β and BMP signaling on chondrogenesis were examined in the micromass culture of mMSCs. It was shown that TGF-β1 induced prechondrogenic condensation and also stimulated chondrogenic differentiation with increasing gene expression of chondrogenic markers such as type II collagen and aggrecan (Fig. 1A and B). However, BMP2 did not induce prechondrogenic condensation despite increasing type II collagen and aggrecan (Fig. 1A and B). Consistent with this result, it was also shown in ATDC5 cells that TGF-β1 but not BMP2 induced cellular condensation although both TGF-β1 and BMP2 stimulated chondrogenic differentiation (Fig. 1C and D). These results clearly confirmed differential effects between TGF-β and BMP signaling on prechondrogenic condensation and revealed that *in vitro* culture system using mMSC and ATDC5 cells are excellent systems to study on the differential effects between TGF-β and BMP signaling.

3.2. TGF-β but not BMP signaling drive ATP oscillations

It was reported that ATP oscillations play an essential role in prechondrogenic condensation (Kwon et al. [15]). Thus, we examined whether the actions of TGF-β and BMP signaling on prechondrogenic condensation depend on ATP oscillations. In the micromass culture of mMSCs, bioluminescence monitoring showed that TGF-β1 induced P_{ACTIN}-PxRe oscillations, while BMP2 did not (Fig. 2A). Consistent with this result, it was also found in ATDC5 cells that TGF-β1 but not BMP2 induced P_{ACTIN}-PxRe oscillations (Fig. 2B). These results indicate that TGF-β but not BMP signaling drives ATP oscillations in chondrogenesis. This data, combining with the result that TGF-β but not BMP signaling induces prechondrogenic condensation, implicates that ATP

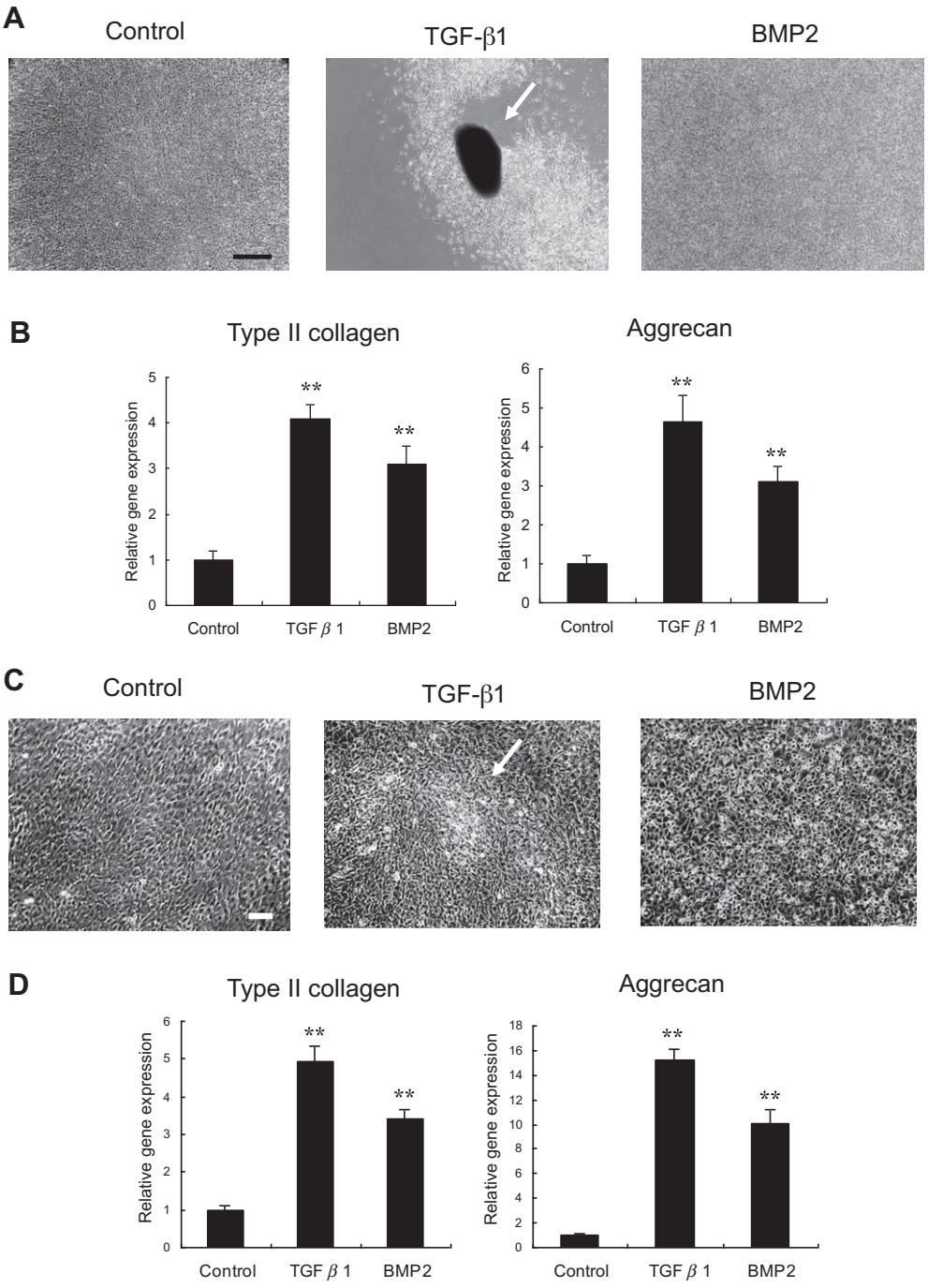

Fig. 1. TGF-β signaling but not BMP signaling induces prechondrogenic condensation. (A) Effect of TGF-β1 and BMP2 treatment on prechondrogenic condensation in the micromass culture of mMSCs. The cells were observed after 1 week culture in the maintenance medium (control) and the medium supplemented with TGF-β1 or BMP2. White arrow represents cellular condensations. Scale bars, 500 μm. (B) Effect of TGF-β and BMP signaling on gene expression of type II collagen and aggrecan in the micromass culture of mMSCs. Gene expression was analyzed after 1 day in the maintenance medium (control) and the medium supplemented with TGF-β1 or BMP2. The values shown are the mean ± SD ($N = 4$). $^{**}p < 0.01$. Dunnett's test. (C) Effect of TGF-β1 and BMP2 on prechondrogenic condensation in prechondrogenic ATDC5 cells. The cells were observed after 1 week culture in the maintenance medium (control) and the medium supplemented with TGF-β1 or BMP2. White arrow represents a cellular condensation. White arrow represents cellular condensations. Scale bars, 100 μm. (D) Effect of TGF-β and BMP signaling on gene expression of type II collagen and aggrecan in ATDC5 cells. Gene expression was analyzed after 1 week in the maintenance medium (control) and the medium supplemented with TGF-β1 or BMP2. The values shown are the mean ± SD ($N = 4$). $^{**}p < 0.01$. Dunnett's test.

oscillations mediate the inductive actions of TGF-β signaling on prechondrogenic condensation.

3.3. Inhibition of TGF-β signaling blocks both ATP oscillations and prechondrogenic condensation

It was explored how the inhibition of TGF-β and BMP signaling influences ATP oscillations and chondrogenesis during insulin-induced chondrogenesis of ATDC5 cells. It was shown that an inhibitor of TGF-β signaling SB431542 prevented induction of P_{ACTIN}-PxRe oscillation in the presence of insulin and also suppressed the insulin-induced P_{ACTIN}-PxRe oscillations which occurred prior to the SB431542 treatment (Fig. 3A). However, an inhibitor of BMP signaling Noggin revealed no inhibition effect on the insulin-induced P_{ACTIN}-PxRe oscillations (Fig. 3A). These results indicate that ATP oscillations depend on TGF-β signaling but

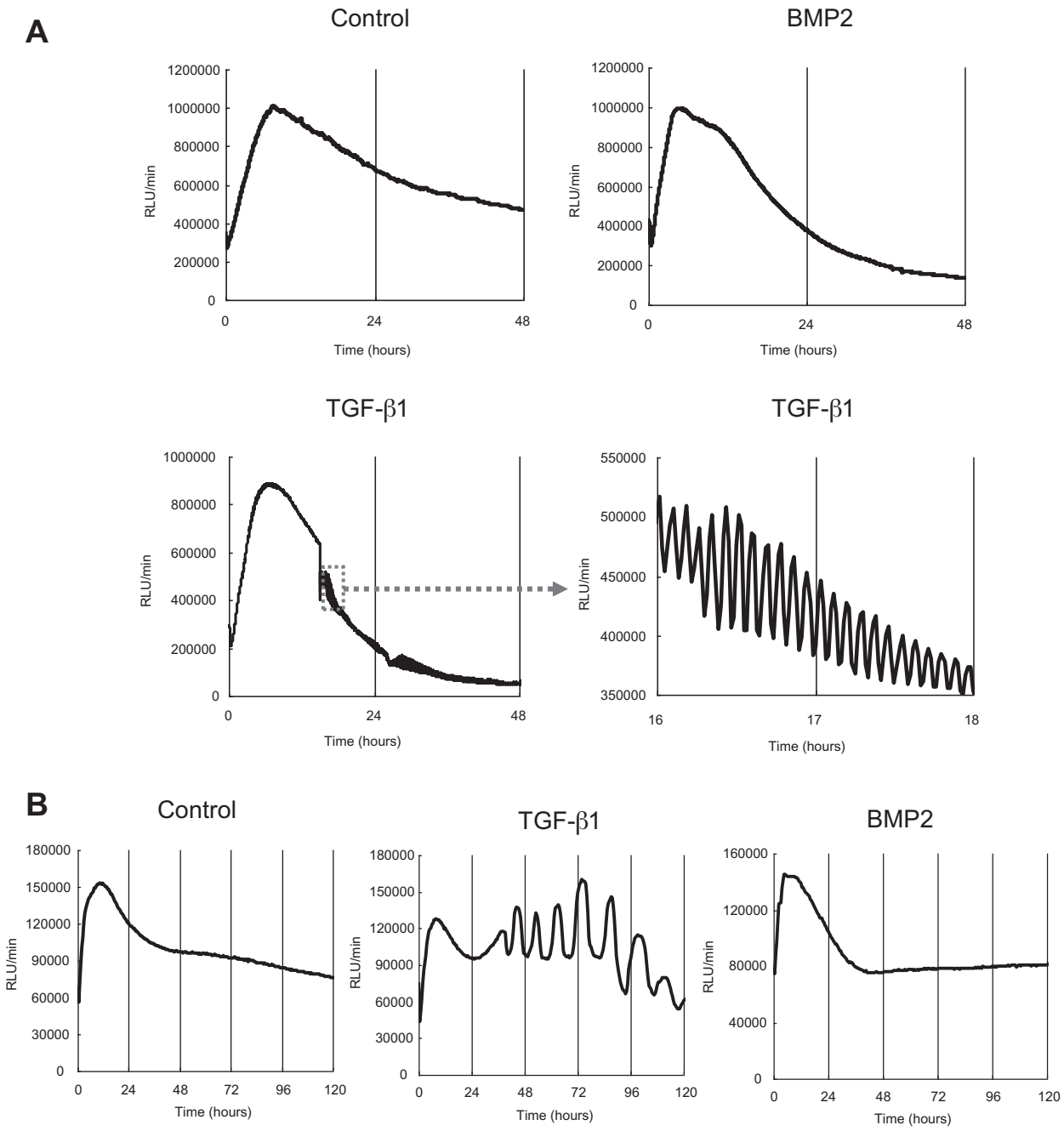

Fig. 2. TGF-β signaling but not BMP signaling induces ATP oscillations. (A) Effect of TGF-β1 and BMP2 on P_{ACTIN}-PxRe intensity in the micromass culture of mMSCs. Bioluminescence monitoring of P_{ACTIN}-PxRe intensity was performed after replacing the culture medium by the maintenance medium (control) and the medium supplemented with TGF-β1 or BMP2. (B) Effect of TGF-β1 and BMP2 on P_{ACTIN}-PxRe intensity in prechondrogenic ATDC5 cells. Bioluminescence monitoring of P_{ACTIN}-PxRe intensity was performed after replacing the culture medium by the maintenance medium (control) and the medium supplemented with TGF-β1 or BMP2.

not on BMP signaling. In addition, SB-431542 abrogated prechondrogenic condensation even in the presence of insulin, while Noggin had little inhibitory effect on the condensation (Fig. 3B). However, both SB431542 and Noggin suppressed expression of type II collagen and aggrecan in the presence of insulin (Fig. 3C). These data are consistent with the results that TGF-β and BMP signaling promote chondrogenic differentiation but have differential effects on prechondrogenic condensation (Fig. 1A–D). This strong positive correlation between ATP oscillations and prechondrogenic condensation suggests that TGF-β signaling mediates prechondrogenic condensation through ATP oscillations. In addition, the present result that ATP oscillations are independent of BMP signaling which stimulated chondrogenic differentiation but not prechondrogenic condensation corroborates the previous proposal that ATP oscillations are critical in prechondrogenic condensation but not chondrogenic differentiation [15].

3.4. ATP oscillations mediate TGF-β-induced prechondrogenic condensation

To clearly demonstrate that the inductive action of TGF-β signaling on prechondrogenic condensation is mediated by ATP oscillations, it was examined how inhibition of ATP oscillations influences TGF-β-induced prechondrogenic condensation. It was

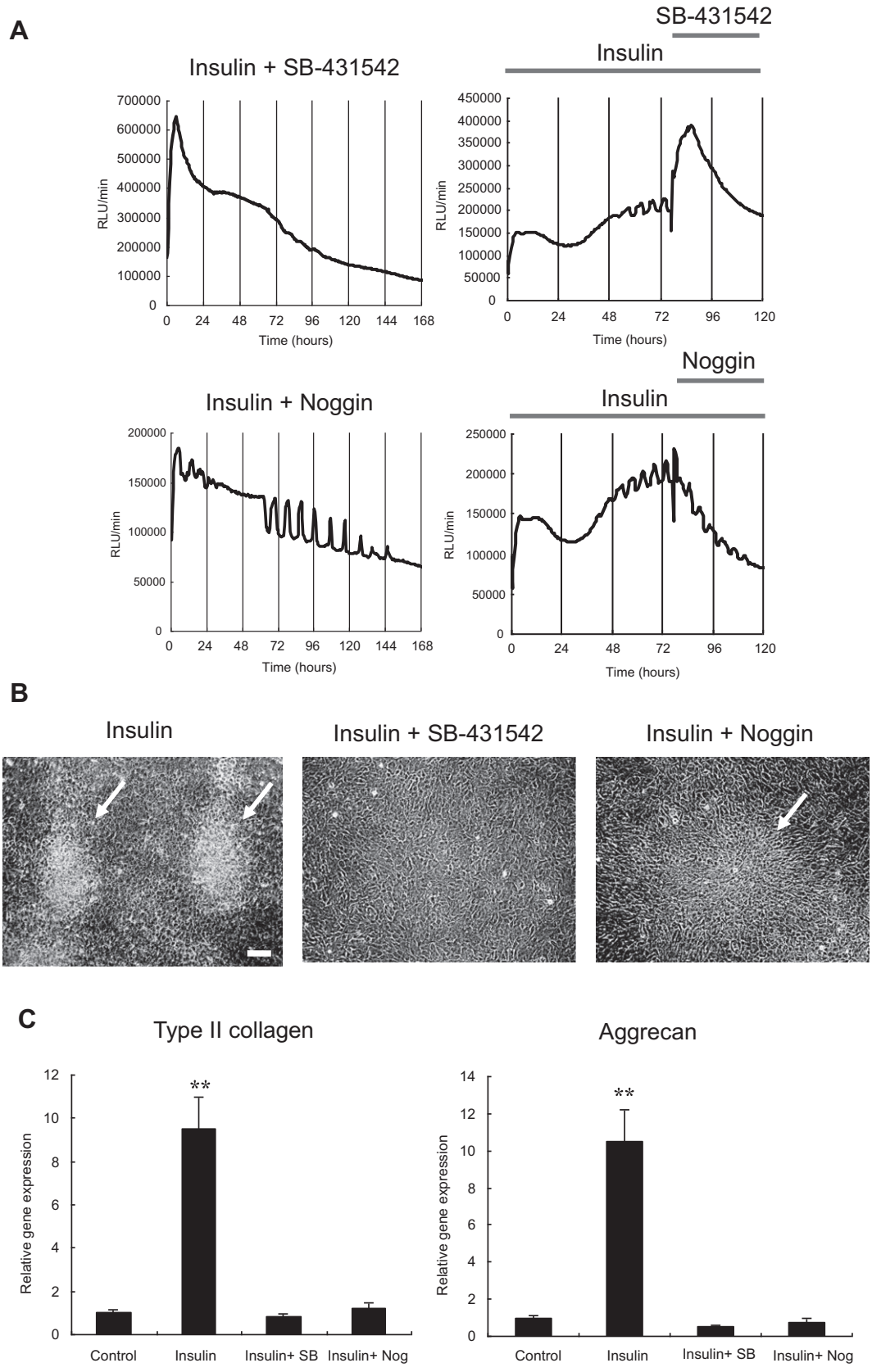

Fig. 3. Inhibition of TGF-β signaling but not inhibition of BMP signaling prevents both ATP oscillations and prechondrogenic condensation. (A) Effect of TGF-β signaling inhibitor SB-431542 and BMP signaling inhibitor noggin on insulin-induced P_{ACTIN}-PxRe oscillations in ATDC5 cells. (B) Effect of SB-431542 and noggin on insulin-induced prechondrogenic condensation in ATDC5 cells. The cells were observed after 1 week culture in the medium supplemented with insulin and insulin plus SB-431542 or noggin. White arrows represent cellular condensations. Scale bars, 100 μm. (C) Effect of SB-431542 (SB) and noggin (Nog) on gene expression of type II collagen and aggrecan in ATDC5 cells cultured in the medium supplemented with insulin. Gene expression was analyzed after 1 week in the maintenance medium (control) and the medium supplemented with insulin and insulin plus SB-431542 or noggin. The values shown are the mean ± SD ($N = 4$). $^{**}p < 0.01$. Dunnett's test.

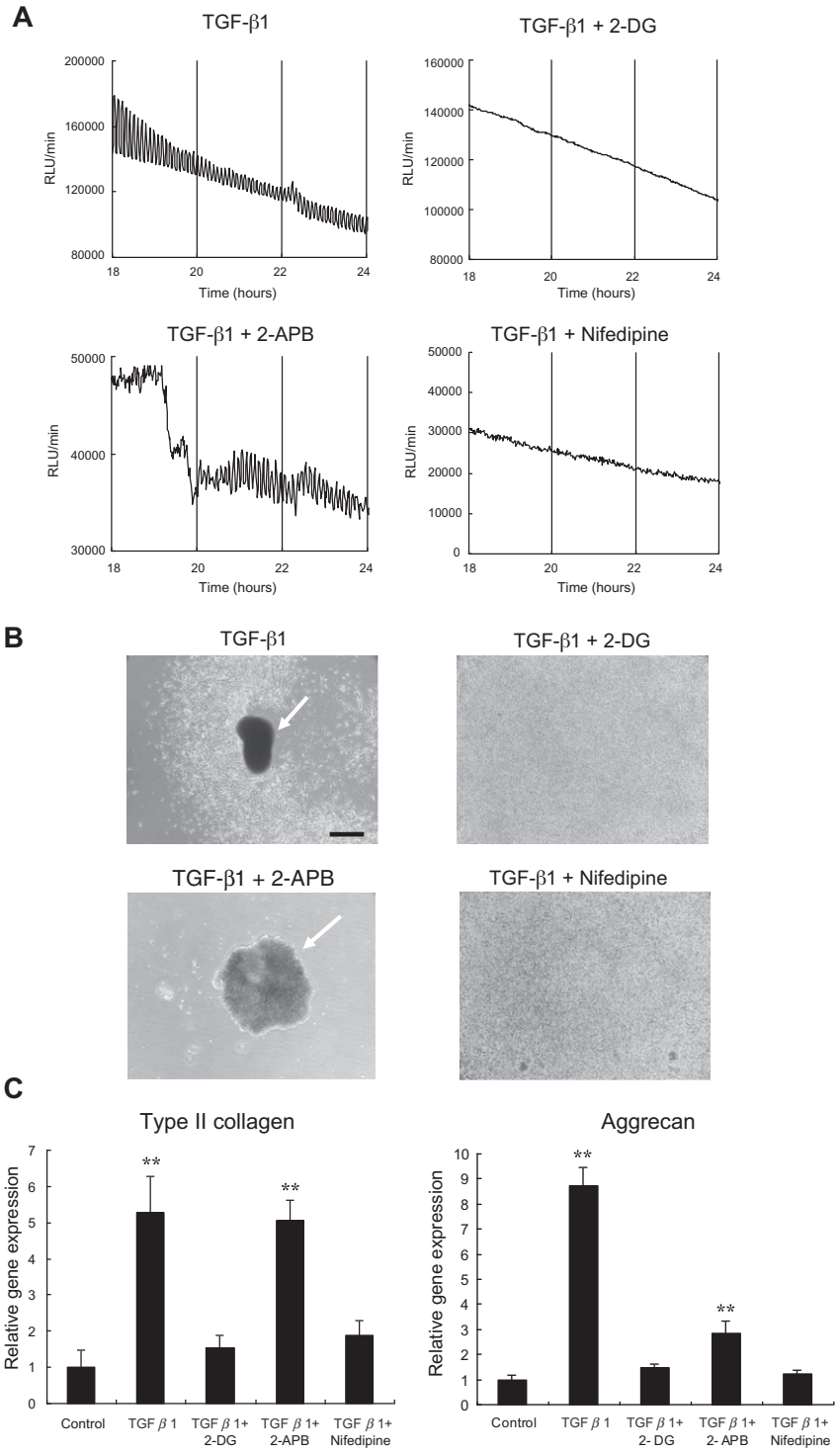

Fig. 4. Blockage of ATP oscillations suppresses TGF-β-induced prechondrogenic condensation. (A) Effect of 2-deoxy glucose (2-DG), 2-APB and nifedipine on TGF-β1-induced P_{ACTIN}-PxRe oscillations in the micromass culture of mMSCs. (B) Effect of 2-DG, 2-APB and nifedipine on TGF-β1-induced prechondrogenic condensation in the micromass culture of mMSCs. White arrows represent cellular condensations. Scale bars, 500 μm. (C) Effect of 2-DG, 2-APB and nifedipine on gene expression of type II collagen and aggrecan in TGF-β1-treated mMSCs. Gene expression was analyzed after 1 day in the maintenance medium (control) and the medium supplemented with TGF-β1 and TGF-β1 plus 2-DG, 2-APB or nifedipine. The values shown are the mean ± SD ($N = 4$). $^{**}p < 0.01$. Dunnett's test.

previously shown in prechondrogenic cells that ATP oscillations depend on glycolysis and Ca^{2+} dynamics [15]. Similarly, the present result showed that glycolysis inhibitor 2-DG prevented TGF-β1-induced PxRe oscillations in the micromass culture of mMSCs (Fig. 4A), indicating that TGF-β-induced ATP oscillations depend on glycolysis. In addition, it was found that a blocker of VDCC nifedipine suppressed TGF-β1-induced PxRe oscillations (Fig. 4A). However, 2-APB, a blocker of the IP_3 receptor and store-operated Ca^{2+} channels (SOC), revealed a little inhibitory effect on the PxRe oscillations. These results indicate that TGF-β-induced ATP oscillations depend on Ca^{2+} influx via VDCC, but not on Ca^{2+} influx via SOC or Ca^{2+} release from intracellular Ca^{2+} stores.

It was then investigated how the inhibition of ATP oscillations influences TGF-β1-induced chondrogenesis. It was found that 2-deoxy glucose (2-DG) and nifedipine, which abrogated ATP oscillations, inhibited TGF-β1-induced prechondrogenic condensation (Fig. 4B). In contrast, 2-APB, which had no inhibitory effect on ATP oscillations, did not suppress TGF-β1-induced prechondrogenic condensation (Fig. 4B). These results suggest that ATP oscillations mediate TGF-β-induced prechondrogenic condensation. However, it was shown that condensations formed by TGF-β1 plus 2-APB were less tightly packed than those by TGF-β1 alone (Fig. 4B). In parallel to these results, both 2-DG and nifedipine significantly suppressed TGF-β1-induced chondrogenic differentiation, whereas 2-APB revealed a little inhibitory effect on the chondrogenic differentiation (Fig. 4C).

4. Discussion

It has been known that TGF-βs are localized to sites of embryonic bone and cartilage formation *in vivo* [19] and stimulate prechondrogenic condensation and subsequent chondrogenesis [5,6]. Previous studies have reported that TGF-β signaling regulates chondrogenesis by affecting replication, gene expression, and structural protein synthesis [20,21]. For example, it was shown that TGF-β signaling promotes prechondrogenic condensation by upregulating fibronectin and cell-cell adhesion molecules during chondrogenesis [22–24]. However, in this study, it was demonstrated for the first time that TGF-β signaling regulates prechondrogenic condensation through ATP oscillations. Our previous report showed that ATP oscillations induce oscillatory secretion and thus drive synchronized secretion during chondrogenesis [15]. Therefore, TGF-β signaling can effectively stimulate prechondrogenic condensation through the synergic effect of increased level and synchronized secretion of adhesion molecules.

In the present result, although both TGF-β and BMP signaling stimulated chondrogenic differentiation, it was found that TGF-β1 but not BMP2 induced prechondrogenic condensation and that the inhibition of TGF-β signaling completely prevented prechondrogenic condensation, while the inhibition of BMP signaling showed a weak suppressive effect on the condensation. However, there is some evidence that BMP signaling is required for prechondrogenic condensation [13]: deletion of Bmp2 and Bmp4 within the limb mesenchyme drives the loss of precartilaginous condensations [25] and loss of BMP receptors leads to the absence of most limb condensations [26]. On the other hand, it was also reported that while TGF-β signaling induce Sox genes even in uncondensed mesenchyme cells, BMP signaling is not sufficient to initiate expression of a cartilage condensation marker Sox9 in uncondensed limb mesenchyme but can only induce Sox genes in condensed cells [27]. Similarly, TGF-β signaling induces uncondensed cells to effectively stimulate chondrogenic differentiation, whereas BMP signaling induces condensed cells to effectively stimulate chondrogenic differentiation [8]. Moreover, it was recently reported that BMP-induced chondrogenesis in the limb is dependent on prior activation of the TGF-β signaling [28]. Therefore, it is likely that TGF-β signaling initiates prechondrogenic condensation in the early stages of chondrogenesis, while BMP signaling is not sufficient to initiate the condensation but promotes the growth of the condensations in the later stages of chondrogenesis. Since BMP signaling was shown to enhance the expression of adhesion molecules such N-cadherin and N-CAM [29], the differential effects between TGF-β and BMP signaling cannot be explained by actions of TGF-β and BMP signaling on expression of adhesion molecules. The present data that TGF-β signaling but not BMP signaling drive ATP oscillations suggests that ATP oscillations determine the differential effects of TGF-β and BMP signaling on prechondrogenic condensation. Moreover, the result that inhibition of ATP oscillations blocked TGF-β-induced prechondrogenic condensation indicates that ATP oscillations are essential for TGF-β-induced initiation of prechondrogenic condensation. Therefore, it is speculated that TGF-β signaling play a critical role in initiating prechondrogenic condensation through ATP oscillations, while BMP signaling contributes to growth of prechondrogenic condensation independently of ATP oscillations.

In the present work, it was shown that TGF-β-induced ATP oscillations are dependent upon Ca^{2+} influx via VDCC, but not on Ca^{2+} influx via SOC. Our previous report demonstrated that ATP oscillations in chondrogenesis are driven by Ca^{2+} oscillations which are generated prior to ATP oscillations [15] and are mediated by extracellular ATP signaling [16]. Indeed, TGF-β has been reported to modulate Ca^{2+} influx in a variety of cell types [30]. Moreover, the previous studies showed that TGF-β stimulates extracellular ATP-induced Ca^{2+} mobilization in lung cells [31] and induces Ca^{2+} oscillations in pancreatic β-cells, depending on Ca^{2+} influx via VDCC [32]. These facts suggest that TGF-β signaling drives Ca^{2+} oscillations by stimulating extracellular ATP signaling and modulating Ca^{2+} influx via VDCC and then TGF-β-driven Ca^{2+} oscillations subsequently generate ATP oscillations. In addition, it was previously reported that TGF-β signaling stimulates its own production in various cell lines including mesenchyme cells [33,34]. This TGF-β autoregulation by positive feedback may contribute to generating Ca^{2+} oscillations and subsequent ATP oscillations during chondrogenesis. In other hand, it was shown that 2-APB weakly suppressed both prechondrogenic condensation and chondrogenic differentiation. This result implicates that Ca^{2+} modulations via the IP_3 receptor and SOC also are involved in TGF-β-induced chondrogenesis via ATP oscillation-independent mechanisms.

In conclusion, the present study has reported that ATP oscillations mediate TGF-β-induced prechondrogenic condensation and suggested that the differential effects of TGF-β and BMP signaling on prechondrogenic condensation depend on ATP oscillations. This study will provide a new insight for understanding action of TGF-β signaling on chondrogenesis during skeletal pattern formation.

Acknowledgments

This work was supported by grants from a Grant-in-Aid for Scientific Research (No. 21790195) from the Ministry of Education, Science and Culture, Japan.

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