Requirement of autocrine signaling by bone morphogenetic protein-4 for chondrogenic differentiation of ATDC5 cells

Chisa Shukunami[a], Haruhiko Akiyama[b], Takashi Nakamura[b], Yuji Hiraki[a,*]

[a] *Department of Molecular Interaction and Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, 53 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan*
[b] *Department of Orthopedic Surgery, Kyoto University School of Medicine, Kyoto 606-8507, Japan*

Received 6 January 2000; received in revised form 3 February 2000

Edited by Masayuki Miyasaka

Abstract Mouse EC cell line ATDC5 undergoes differentiation to form cartilage nodules via the cellular condensation stage in the presence of insulin. ATDC5 cells expressed transcripts for bone morphogenetic protein-4 (BMP-4), and type IA and type II BMP receptors. Moreover, cells retained responsiveness to BMP-4, which induced the formation of chondrocytes in the culture. When transfected with a kinase domain-truncated type IA BMP receptor construct, cells failed to undergo differentiation beyond the condensation stage even in the presence of insulin. The soluble form of type IA BMP receptor also blocked the formation of chondrocytes in a dose dependent manner. These lines of evidence suggested that autocrine BMP-4 signaling is required for the conversion of chondrogenic precursor cells into chondrocytes.

Key words: Chondrogenic differentiation; Bone morphogenetic protein-4; Autocrine bone morphogenetic protein signaling; Mesenchymal condensation

1. Introduction

Mouse embryonal carcinoma-derived cell line ATDC5 provides an excellent in vitro model that exhibits the multistep chondrogenic differentiation observed during endochondral bone formation [1]. In the presence of 5% fetal bovine serum (FBS) and 10 μg/ml insulin, ATDC5 cells differentiate into type II collagen-expressing chondrocytes (early-phase differentiation) via the cellular condensation stage to form cartilage nodules in culture [2]. After the growth and expansion of cartilage nodules, hypertrophic chondrocytes appeared in association with type X collagen gene expression and elevation of alkaline phosphatase activity followed by matrix mineralization (late-phase differentiation) [1]. Thus, ATDC5 cells sequentially differentiate to give rise to condensing prechondrocytes, type II collagen-expressing chondrocytes, type X collagen-expressing hypertrophic chondrocytes and mineralizing chondrocytes in the presence of insulin or insulin-like growth factor-I (IGF-I) without any additional growth/differentiation factors [1,2].

It is intriguing to note that insulin or IGF-I first induces the formation of regions of cellular condensation in which differentiated chondrocytes appear [2]. Thus, numerous spotty nodules of chondrocytes are formed in ATDC5 cell cultures in the presence of insulin. Bone morphogenetic protein-2 (BMP-2), however, induced the conversion of undifferentiated ATDC5 cells into chondrocytes (early-phase differentiation) as well as cellular hypertrophy and matrix mineralization (late-phase differentiation) [3]. Treatment with BMP-2 induced chondrogenic conversion all over the culture of confluent undifferentiated cells to form a nearly continuous sheet of chondrocytes even in the absence of insulin [3]. The stage of cellular condensation was skipped out in the cultures treated with exogenous BMP-2.

Here we report that autocrine BMP-4 signaling is required for the conversion of prechondrocytes into chondrocytes in the regions of cellular condensation during insulin-induced differentiation of ATDC5 cells by inhibiting autocrine BMP signaling with the expression of dominant negative type IA BMP receptor (BMPR-IA) or with treatment of cells with the soluble form of BMPR-IA. However, the blockade of autocrine BMP signaling did not interfere with the induction of cellular condensation.

2. Materials and methods

2.1. Materials

Recombinant human BMP-4 was purchased from R and D Systems (Minneapolis, MN, USA) and diluted with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA).

2.2. Cell culture

ATDC5 cells were maintained in a 1:1 mixture of DME and Ham's F-12 (DME/F12) medium (Flow Laboratories, Irvine, UK) containing 5% FBS (JRH Biosciences, Lenexa, KS, USA), and ITS, i.e. 10 μg/ml bovine insulin (I; Boehringer Mannheim, Gaithersburg, MD, USA), 10 μg/ml human transferrin (T; Boehringer Mannheim), and 3×10^{-8} M sodium selenite (S; Sigma), as previously described [2]. The inoculum size of cells was 6×10^4 cells/well in six-multiwell plates (Corning Glass, Corning, NY, USA) at 37°C under 5% CO_2 in air. On day 21, the culture medium was switched to αMEM (Flow Laboratories) containing 5% FBS plus ITS, and the CO_2 concentration was shifted to 3% for the facilitation of cellular hypertrophy and mineralization in culture [1]. Balb/c 3T3 cells and C3H10T1/2 cells were plated in six-multiwell plates at a density of 6×10^4 cells/well and cultured in DME containing 10% FBS. MC3T3-E1 cells were plated in 6-multiwell plates at a density of 6×10^4 cells/well and cultured in αMEM containing 10% FBS. The medium was replaced every other day.

In some experiments, ATDC5 cells were plated at a density of 1×10^4 cells/well in 48-multiwell plates and cultured in DME/F12 medium containing 5% FBS and ITS. On day 3, the cultured medium was replaced by DME/F12 medium containing 5% FBS, ITS and soluble BMP type IA receptor (sBMPR) [4]. The medium was replaced every other day. On day 11, cells were fixed with 95% methanol and stained with 0.1% Alcian blue 8GS (Fluka, Buchs, Switzerland) in 0.1 M HCl overnight.

*Corresponding author. Fax: (81)-75-751 4633.

PII: S0014-5793(00)01251-5

2.3. RNA extraction and hybridization analysis

Total RNA was prepared from the cultures by the single-step method of Chomczynski and Sacchi [5]. For Northern hybridization, 20 μg of total RNA was denatured with 6% formaldehyde, separated by 1% agarose gel (SeaKem GTG, FMC Bioproducts, Rockland, ME, USA) electrophoresis, and transferred onto Nytran membranes with Turboblotter (Schleicher and Schuell, Dassel, Germany). Hybridization was performed overnight at 42°C with an appropriate cDNA probe (10^6 cpm/ml) in a solution containing 50% formamide, 6×SSPE (0.9 M NaCl, 20 mM NaH_2PO_4, 6 mM EDTA at pH 7.4), 0.1% BSA, 0.1% Ficoll 400 (Pharmacia, Uppsala, Sweden), 0.1% polyvinylpyrolidone (Wako Pure Chemical, Osaka, Japan), 0.1% SDS, and 200 μg/ml denatured salmon sperm DNA. Hybridization probes were prepared by the random-primer method with a BcaBEST labeling kit (Takara, Shiga, Japan) using the appropriate cDNA fragments: a 2.0 kb *Eco*RI fragment of pKT1809 [6] as a probe for α1(I) collagen mRNA; a 1.4 kb *Eco*RI fragment of pKT1180 [6] as a probe for α1(II) collagen mRNA, and a 2.2 kb *Eco*RI–*Xho*I fragment of pcDNA1R15B [7] as a probe for PTH/PTHrP receptor mRNA. The following cDNA fragments were amplified by reverse transcription-polymerase chain reaction (RT-PCR) with a specific primer set as described below, and used as a probe for Northern hybridization: a 0.6 kb fragment of BMP-4 cDNA, a 0.34 kb fragment of BMP type IA receptor cDNA. After hybridization, the filters were washed for 30 min at 55°C in 2×SSPE and 0.1% SDS, then washed for 30 min at 55°C in 0.1×SSPE, 0.1% SDS, and then exposed to X-OMAT film (Eastman Kodak, Rochester, NY, USA) at −80°C with a Cronex lightening plus intensifying screen (DuPont, Boston, MA, USA).

2.4. DNA transfection

For construction of the expression vector for the dominant-negative mutant of mouse BMP type I A receptor (DN-BMPR-IA), a *Hin*dIII/*Sac*I fragment (670 bp) of pBΔmBMPR [8] was subcloned into pcDNA3 (Invitrogen, San Diego, CA, USA). The resultant expression vector, pCMVΔmBMPR, was transfected to undifferentiated ATDC5 cells by lipofection using Tfx-50 Reagent (Promega, Madison, WI, USA). 2 days later, the cells were diluted 10-fold and incubated with the medium containing 400 μg/ml geneticin (GIBCO). After 10 days, six drug-resistant clones were selected and expanded. Cells from each clone were plated in 12-multiwell plates at a density of 4×10^4 cells/well, and cultured in DME/F12 medium containing 5% FBS and ITS for 15 days. Chondrogenic differentiation of cells was examined by Alcian blue staining [2].

2.5. RT-PCR

The RT-PCR was performed as described previously [2]. Briefly, first-strand cDNA was synthesized using SuperScript II RNase H^- reverse transcriptase (GIBCO BRL, Gaithersburg, MD, USA) with total RNA extracted from ATDC5 cells transfected with pCMVΔmBMPR. To detect the transgene, T3 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-GATTTAGGTGACACTATAG-3′) primers (Invitrogen) were used. The PCR conditions were 94°C for 30 s, 55°C for 1 min, 72°C for 30 s for 32 cycles, and final extension at 72°C for 5 min.

3. Results

3.1. Expression of BMP-4, BMPR-IA, BMPR-II transcripts in ATDC5 cells

The transcripts for the *BMP-2* and *BMP-4* genes are expressed spatially and temporally at the sites of skeletogenesis such as limb buds and vertebrae during embryonic development [9–11]. We studied the expression of *BMP-4* mRNA in several mouse mesenchymal cell lines by Northern blot analysis (Fig. 1A). Transcripts for the *BMP-4* gene were not detected in the Balb/c 3T3 cells, which are typical fibroblastic cells. However, the expression of *BMP-4* mRNA was clearly detected in a mesodermal progenitor cell line, C3H10T1/2, which is capable of differentiation into myoblasts, adipocytes and chondrocytes [12]. The expression of the mRNA was also readily detected in the confluent monolayer culture (day 3) of

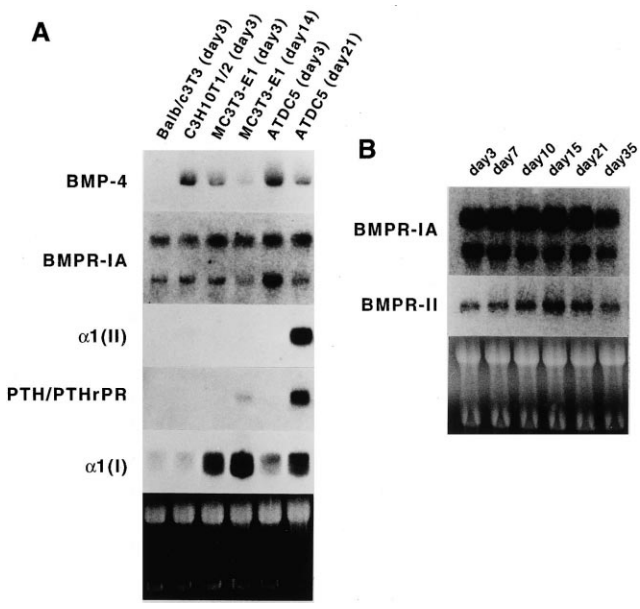

Fig. 1. Expressions of *BMP-4*, *BMPR-IA*, and *BMPR-II* transcripts in mesenchymal cells. Cells were maintained as described in Section 2. Total RNA was prepared from Balb/c 3T3 cells on day 3, C3H10T1/2 cells on day 3, MC3T3-E1 cells on day 3 and on day 14, and ATDC5 cells on the indicated days of culture. A: the same membrane filter was used for hybridization with the probes of mouse *BMP-4*, mouse *BMPR-IA*, rat *type II collagen*, rat *PTH/PTHrP receptor*, and rat *type I collagen* cDNAs. B: the same membrane filter was used for hybridization with the probes of mouse *BMPR-IA* and *BMPR-II* cDNAs. The equivalent loading of RNA (20 μg/lane) was verified by ethidium bromide staining, as shown in the bottom panels.

osteoblastic MC3T3-E1 cells isolated from mouse calvariae (Fig. 1A) [13]. Thereafter, MC3T3-E1 cells gradually matured after a prolonged culture as shown by the expression of *PTH/PTHrP receptor* mRNA (day 14). The *BMP-4* mRNA level declined as the cells became mature on day 14 (Fig. 1A). The mRNA level of *BMP-4* gene is expressed at a relatively high level in undifferentiated ATDC5 cells (Fig. 1A). In association with the formation of cartilage nodules, the mRNA level of *BMP-4* gene slightly declined (Fig. 1A).

Two kinds of type I BMP receptors (BMPR-IA and BMPR-IB) have been identified in vertebrates [14]. These type I receptors bind BMP-2, BMP-4, and BMP-7 in combination with type II BMP receptor (BMPR-II) or type II activin receptor (ACTR-II). As shown in Fig. 1, ATDC5 cells expressed two types of transcripts for *BMPR-IA*. The sizes of these transcripts were identical to those found in mouse tissues [15]. The level of these transcripts did not change significantly during differentiation of ATDC5 cells (Fig. 1). The expression of the receptor transcripts was also detected in other osteoblastic and fibroblastic cells including Balb/c 3T3 cells (Fig. 1A). Similarly, transcripts for the *BMPR-II* gene were detected at all stages of differentiation of ATDC5 cells. The level of transcripts was upregulated during maturation of differentiated cells from day 10 to day 21 of culture (Fig. 1B). The *BMP-6* gene was expressed in parallel with the induction of type II collagen and aggrecan mRNAs in differentiated ATDC5 cells, as previously reported [16]. However, we did not detect transcripts for *the BMP-2* or *BMPR-IB* genes in ATDC5 cells (data not shown).

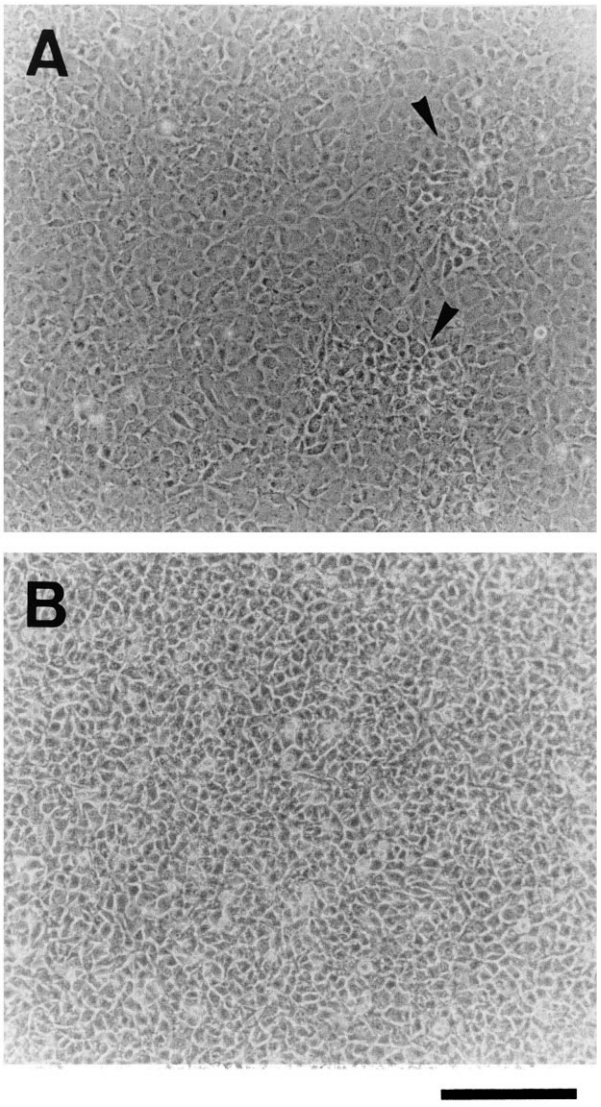

Fig. 2. Stimulation of early-phase chondrogenic differentiation of ATDC5 cells by BMP-4. Cells were plated at a density of 4×10^4 cells per well in 12-multiwell plates, and grown to confluence in DME/F12 medium containing 5% FBS and ITS for 3 days. Cells were then incubated in the absence (A) or presence of 200 ng/ml BMP-4 (B) for additional 2 days. Bar, 200 μm.

3.2. *Induction of early-phase differentiation of ATDC5 cells by BMP-4*

Undifferentiated ATDC5 cells rapidly proliferate with a fibroblastic morphology and ceased to grow at confluence [2]. In the presence of 5% FCS and 10 μg/ml insulin, cells give rise to chondrocytes to form cartilage nodules via the cellular condensation stage (early-phase differentiation). Then hypertrophic chondrocytes appear in the cartilage nodule, and matrix mineralization follows (late-phase differentiation) [1]. When added in the culture, BMP-2 facilitated the progression of early-phase and late-phase differentiation of ATDC5 cells [3]. Similarly, BMP-4 induced differentiation to turn confluent undifferentiated cells into rounded chondrocytic cells all over the culture (Fig. 2). The expression of type II collagen gene was induced by the BMP-4 treatment (data not shown). Once induction of early phase chondrogenic differentiation of ATDC5 cells occurred after transient exposure to BMP-4

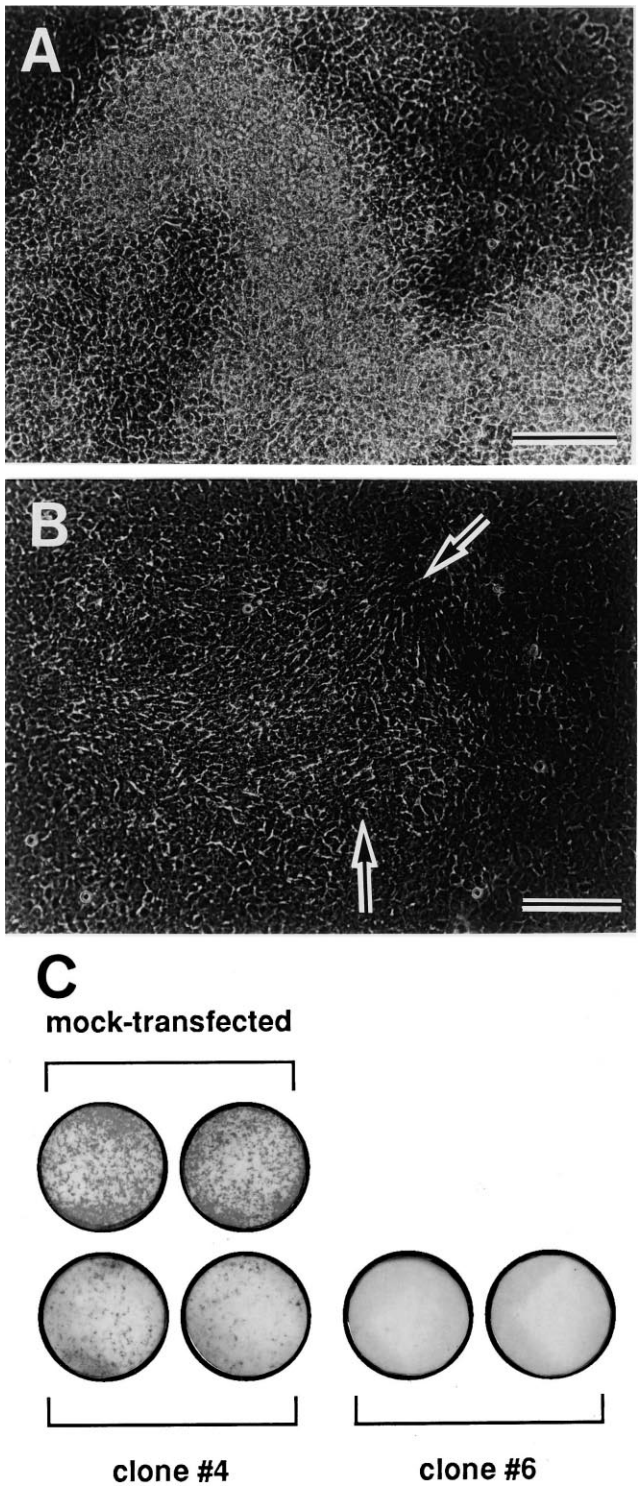

Fig. 3. Inhibition of early-phase chondrogenic differentiation of ATDC5 cells transfected with DN-BMPR-IA. Six ATDC5 clones transfected with the dominant negative construct for BMPR-IA were plated at a density of 4×10^4 cells per well in 12-multiwell plates, and grown in DME/F12 medium containing 5% FBS and ITS for 15 days. Phase-contrast micrographs of the mock-transfected cells (A) and the DN-BMPR-IA transfected cells (B; clone #3) are shown. B: arrows indicate the region of cellular condensation formed in the culture of the DN-BMPR-IA transfected cells. Bars represent 200 μm for (A) and (B). C: chondrogenic differentiation of cells was examined by Alcian blue staining on day 15.

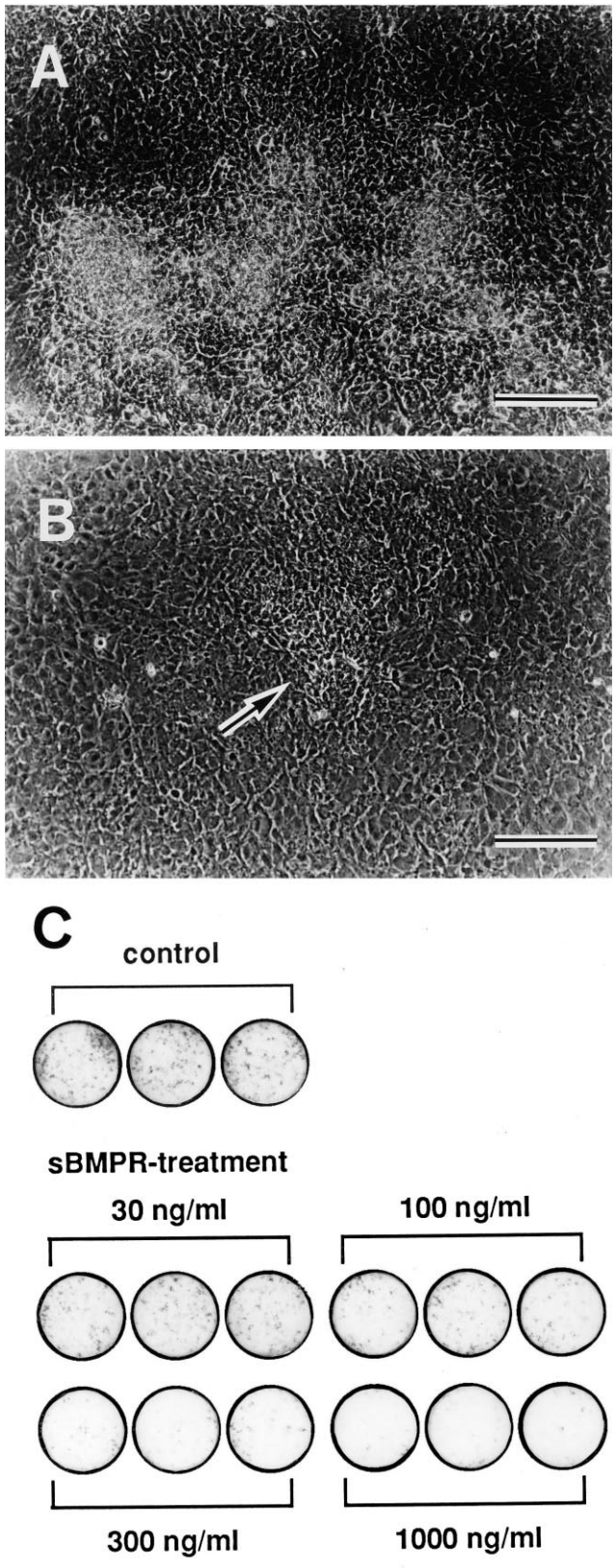

Fig. 4. Inhibitory effect of sBMPR on early-phase chondrogenic differentiation of ATDC5 cells. Cells were plated at a density of 1×10^4 cells per well in 48-multiwell plates, and maintained in DME/F12 medium containing 5% FBS and ITS. On day 3, the culture medium was replaced by DME/F12 medium containing 5% FBS, ITS and various concentrations of sBMPR. On day 11, we examined the formation of cartilage nodules in the control culture (A) and the culture treated with 1000 ng/ml sBMPR (B) under a phase-contrast microscope. B: the arrow indicates the region of cellular condensation formed in the culture. Bars represent 200 μm in A and B. C: chondrogenic differentiation of cells was examined by Alcian blue staining on day 11.
←

(48 h), cartilaginous phenotypes of ATDC5 cells were maintained without further administration of BMP-4 (data not shown). Thus the culture plates were covered with a nearly continuous sheet of chondrocytes within 2 days (Fig. 2B). In contrast, cellular condensation took place at this time period under the standard culture condition in the presence of insulin without BMP-4 (Fig. 2A). The formation of condensing areas topically regionalize chondrogenesis to occur, leading to the subsequent appearance of spotty cartilage nodules within the culture plates [2].

3.3. Inhibition of early-phase differentiation of ATDC5 cells by the expression of a kinase-truncated BMPR-IA or the treatment with a soluble form of BMPR-IA

The truncated BMPR-IA without kinase domain have shown to block BMP signaling efficiently in vivo and in vitro [17]. In order to block the BMPR-IA mediated signaling, we transfected the dominant-negative construct of mouse BMPR-IA (DN-BMPR-IA) into undifferentiated ATDC5 cells. Several stable transfectants were cloned. We confirmed the expression of DN-BMPR-IA mRNA in each selected clone by RT-PCR (data not shown). In the mock-transfected cells, chondrocytes were differentiated within the region of condensation to result in the formation of cartilage nodules (Fig. 3A). Cartilage nodules were positively stained with Alcian blue (Fig. 3C). In contrast, the formation of cartilage nodules were evidently impaired in the DN-BMPR-IA-transfected cell clones (Fig. 3B,C). However, it was clearly noted that the formation of condensing areas was successfully induced even in the DN-BMPR-IA-transfected clones (Fig. 3B).

Next, we attempted to block autocrine/paracrine BMP-4 signals due to the endogenous expression of BMP-4 in ATDC5 cells by the treatment of cells with soluble BMPR-IA (sBMPR) [4]. This soluble receptor was in monomer form in solution and bound to BMP-4, but not to activin or transforming growth factor-β1 [4]. When added in culture, sBMPR is considered to bind to BMPs secreted into the culture medium and to act as a antagonist by competing with wild membrane-bound receptors. As shown in Fig. 4, sBMPR inhibited chondrogenic differentiation of ATDC5 cells in a dose dependent manner (30–1000 ng/ml). Cellular condensation of ATDC5 cells was not inhibited by the treatment with sBMPR.

4. Discussion

Endochondral bone formation begins with mesenchymal condensation that defines the regions of subsequent overt chondrogenesis to occur. Thus mesenchymal condensation prefigures the future configuration of skeletal elements in embryo, leading to the formation of cartilaginous bone rudiments. A number of BMP family members are expressed during the early stages of chondrogenesis, including BMP-2, BMP-4, BMP-5, BMP-7, and GDF-5 [9,11,18,19]. Transcripts for the *BMP-4* gene were clearly detected in chondrogenic

ATDC5 cells as well as mesodermal progenitor C3H10T1/2 cells and osteoblastic MC3T3-E1 cells (Fig. 1A). But the expression of BMP-2 transcripts was not detected in ATDC5 cells. The cells expressed transcripts for *BMPR-IA* and *BMPR-II* irrespective of differentiation stages (Fig. 1), suggesting that these BMPRs transmit chondrogenic actions of endogenous and exogenous BMP-4 in ATDC5 cells (Fig. 2).

Upon transfection with the DN-BMPR construct, ATDC5 cells failed to differentiate into chondrocytes (Fig. 3). Similarly, sBMPR inhibited conversion of undifferentiated ATDC5 cells into chondrocytes in a dose dependent manner (Fig. 4). Formation of cartilage nodules was blocked by the impairment of BMP-4 signaling, but condensing regions were clearly established in the cultures (Figs. 3 and 4). These lines of evidence suggested that autocrine BMP-4 signaling was critically required for the conversion of condensing undifferentiated cells into chondrocytes. However, the impairment of BMP signaling did not affect cellular condensation that regionalized the subsequent overt chondrogenesis. Only insulin and/or IGF-I could induce cellular condensation of prechondrogenic cells [3]. In agreement with this observation, Roark and Greer previously suggested that BMPs act only after the condensation stage in chick limb bud micromass cultures [20].

When exposed to BMP-4 as well as BMP-2 prior to cellular condensation at confluence, undifferentiated ATDC5 cells in culture evenly responded to them and were rapidly converted to chondrocytes (Fig. 2) [3]. However, once condensed regions were established in the cultures, cells in the inter-nodular space (or outside the condensed regions) failed to respond to the BMP stimuli. BMPs only facilitated the progressive differentiation of cells within condensed areas or cartilage nodules. It is intriguing to address the question why cells in the inter-nodular space failed to become chondrocytes in response to BMP. Our collaborative study suggests that lateral inhibition through Notch signaling play a role in this process (K. Tezuka, submitted elsewhere). However, it is clear that the process of cellular condensation and the conversion of progenitor cells into chondrocytes is differentially regulated by the distinct signals. Autocrine BMP-4 signaling is critically required for the latter process.

Acknowledgements: The soluble form of BMP type I receptor and the dominant-negative construct for BMP type I receptor were kindly provided by Dr. N. Ueno (National Institute for Basic Biology, Okazaki, Japan). Human PTHrP(1–141) was a generous gift from Dr. T.J. Martin (St. Vincent's Institute of Medical Research, Vic., Australia). Bovine PTH(1–34) was from Dr. K. Sato (Chugai Pharmaceutical, Tokyo, Japan). This work was partly supported by The Naito Foundation and Terumo Life Science Foundation.

References

[1] Shukunami, C., Ishizeki, K., Atsumi, T., Ohta, Y., Suzuki, F. and Hiraki, Y. (1997) J. Bone Miner. Res. 12, 1174–1188.
[2] Shukunami, C., Shigeno, C., Atsumi, T., Ishizeki, K., Suzuki, F. and Hiraki, Y. (1996) J. Cell Biol. 133, 457–468.
[3] Shukunami, C., Ohta, Y., Sakuda, M. and Hiraki, Y. (1998) Exp. Cell Res. 241, 1–11.
[4] Natsume, T., Tomita, S., Iemura, S., Kinto, N., Yamaguchi, A. and Ueno, N. (1997) J. Biol. Chem. 272, 11535–11540.
[5] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[6] Kimura, T., Mattei, M.-G., Stevens, J.W., Goldring, M.B., Ninomiya, Y. and Olsen, B.R. (1989) Eur. J. Biochem. 179, 71–78.
[7] Abou-Samra, A.-B. et al. (1992) Proc. Natl. Acad. Sci. USA 89, 2732–2736.
[8] Suzuki, A., Thies, R.S., Yamaji, N., Song, J.J., Wozney, J.M., Murakami, K. and Ueno, N. (1994) Proc. Natl. Acad. Sci. USA 91, 10255–10259.
[9] Lyons, K.M., Pelton, R.W. and Hogan, B.L. (1990) Development 109, 833–844.
[10] Francis, P.H., Richardson, M.K., Brickell, P.M. and Tickle, C. (1994) Development 120, 209–218.
[11] Lyons, K.M., Hogan, B.L. and Robertson, E.J. (1995) Mech. Dev. 50, 71–83.
[12] Taylor, S.M. and Jones, P.A. (1979) Cell 17, 771–779.
[13] Sudo, H., Kodama, H.A., Amagai, Y., Yamamoto, S. and Kasai, S. (1983) J. Cell Biol. 96, 191–198.
[14] Massague, J. (1996) Cell 85, 947–950.
[15] Dewulf, N. et al. (1995) Endocrinology 136, 2652–2663.
[16] Ito, H., Akiyama, H., Shigeno, C. and Nakamura, T. (1999) Biochim. Biophys. Acta 1451, 263–270.
[17] Kawakami, Y. et al. (1996) Development 122, 3557–3566.
[18] King, J.A., Marker, P.C., Seung, K.J. and Kingsley, D.M. (1994) Dev. Biol. 166, 112–122.
[19] Storm, E.E. and Kingsley, D.M. (1996) Development 122, 3969–3979.
[20] Roark, E.F. and Greer, K. (1994) Dev. Dyn. 200, 103–116.