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Tenascin-W inhibits proliferation and differentiation of preosteoblasts during endochondral bone formation

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Abstract

We identified a cDNA encoding mouse Tenascin-W (TN-W) upregulated by bone morphogenetic protein (Bmp)2 in ATDC5 osteochondroprogenitors. In adult mice, *TN-W* was markedly expressed in bone. In mouse embryos, during endochondral bone formation *TN-W* was localized in perichondrium/periosteum, but not in trabecular and cortical bones. During bone fracture repair, cells in the newly formed perichondrium/periosteum surrounding the cartilaginous callus expressed *TN-W*. Furthermore, *TN-W* was detectable in perichondrium/periosteum of *Runx2*-null and *Osterix*-null embryos, indicating that *TN-W* is expressed in preosteoblasts. In CFU-F and -O cells, TN-W had no effect on initiation of osteogenesis of bone marrow cells, and in MC3T3-E1 osteoblastic cells TN-W inhibited cell proliferation and *Col1a1* expression. In addition, TN-W suppressed canonical Wnt signaling which stimulates osteoblastic differentiation. Our results indicate that *TN-W* is a novel marker of preosteoblasts in early stage of osteogenesis, and that TN-W inhibits cell proliferation and differentiation of preosteoblasts mediated by canonical Wnt signaling.

Keywords: Tenascin-W; Osteogenesis; Bmp; Preosteoblast

Osteoblast differentiation is initiated in cells in periphery of mesenchymal condensation. These cells are first committed to the osteoblast lineage, differentiate into preosteoblasts and then into functional osteoblasts that produce a bone-specific matrix. Accumulating evidence indicates that bone morphogenetic protein (Bmp) signaling plays an important role in these sequential cellular events. Bmp2 and Bmp4 are localized in mesenchymal condensation and then in the perichondrium/periosteum, and they function as potent stimulators of osteoblast differentiation *in vitro* and *in vivo* [1–3]. In addition, recent studies indicate that Bmps induce *Runx2* and *Osterix*, both of which are essential for osteoblast differentiation, and that Runx2 activity is enhanced by Bmp-mediated Runx2/Smads interactions [4–7]. However, it is expected that there is another molecule induced by Bmp signaling in the early phase of osteoblast differentiation of mesenchymal cells.

We previously showed that clonal mouse EC cells, ATDC5, display a number of characteristics as committed osteo-chondroprogenitors [1]. When cultured in the presence of insulin, ATDC5 cells form discrete cartilaginous nodules, and enter into sequential chondrocytic maturation processes. In these cells, autocrine Bmp signalings are potentially required for the transition of undifferentiated cells into chondrocytes. Indeed, ATDC5 cells express Bmp4 as well as Bmp type IA and II receptors, and exogenous Bmp2 and Bmp4 dramatically accelerate the differentiation programs of ATDC5 cells. In addition, transfection with a dominant-negative Bmp type IA receptor as well as treatment of cells with the soluble form of Bmp type IA receptor block cell differentiation in these cells [8]. Thus, the advantage of ATDC5 cells as an *in vitro* model for Bmp-mediated mesenchymal differentiation is substantiated by the detection of Bmp downstream genes in osteo-chondroprogenitor cells.

Abbreviations: TN-W, Tenascin-W; dpc, days post coitum; bp, base pair(s); Bmp, bone morphogenetic protein.

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In this study, we designed a simple screening to isolate cDNAs that are upregulated by Bmp2. We compared mRNAs expressed in Bmp2-untreated and Bmp2-treated ATDC5 cells and identified a cDNA encoding Tenascin-W (TN-W). A recent study shows that TN-W is upregulated by Bmp2 in C2C12 myogenic cells *in vitro* and is expressed in periosteum of adult mice *in vivo* [9]. We demonstrated that TN-W, that is expressed in preosteoblasts in perichondrium/periosteum during endochondral bone formation, has inhibitory effects on proliferation and differentiation of preosteoblasts mediated by canonical Wnt signaling.

Materials and methods

Cells and culture conditions. ATDC5 cells, MC3T3-E1 cells and bone marrow cells were cultured as previously described [1,10].

RNA extraction and suppression subtractive hybridization. ATDC5 cells were cultured for a total of 5 days and were exposed to 1000 ng/ml Bmp2 or vehicle for10 h. Poly(A)$^+$ RNA was isolated from Bmp2-untreated and Bmp2-treated ATDC5 cells as previously described [11] and analyzed by suppression subtractive hybridization (PCR-Select cDNA Subtractions Kit, Clontech Laboratories, Inc., Palo Alto, CA) and differential hybridization (differential screening kit, Clontech Laboratories, Inc.) according to the manufacture's instructions. The cDNA fragment of approximately 500-bp expressed at a high level in Bmp2-treated ATDC5 cells was identified. Oligo(dT) primed cDNA library from poly(A)$^+$ RNA of Bmp2-treated ATDC5 cells was constructed in λZAP Express vector (Stratagene, La Jolla, CA), and 1×10^6 plaques were screened with the 500-bp fragment as a probe as previously described [12].

Northern blot analysis. Total RNA and poly(A)$^+$ RNA from ATDC5 cells and calvaria of new born C57BL/6 mice were extracted, respectively, and Northern blot hybridization was performed as previously described [12]. The following cDNA fragments were used as hybridization probes: a 500-bp fragment of *TN-W* cDNA; and a 980-bp fragment of mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA. In analysis of *TN-W* expression in various tissues of adult mice and in various stages of mouse embryogenesis, a labeled cDNA was hybridized to Mouse Multiple Tissue Northern (MTN) Blots (Clontech Laboratories, Inc.).

DNA transfection and preparation of the conditioned media. HEK293 cells were transiently transfected with pME18SFL3/mTN-W (FLJ clone ID: FLJ45101, TOYOBO, Osaka, Japan) or an empty vector using FuGene6 (Roche Diagnostics, Basel Switzerland). The conditioned media were prepared 3 days after transfection.

Analysis of cell proliferation. 5-Bromo-2′deoxy-uridine (BrdU) incorporation was assessed by the cell proliferation ELISA Biotrack kit (Amersham Biosciences, Piscataway, NJ).

Measurement of colony forming unit-fibroblast (CFU-F) and -osteoblast (CFU-O). Bone marrow cells were cultured as described previously [10]. For CFU-F assays, cultures were stained at day 10 with a Sigma alkaline phosphatase kit (Sigma, Missouri, St. Louis). For CFU-O assays, cultures were stained at day 20 with 1% alizarin red S (Wako, Osaka, Japan).

Luciferase reporter assay. Luciferase reporter assays were performed as previously described [13]. Briefly, MC3T3-E1 cells were co-transfected with Topflash and phRL-TK (Promega, Madison, WI) using Fugene 6. Twenty four hours after transfection, Wnt 3A (R&D Systems, Minneapolis, MN) and the conditioned media containing TN-W were added, and luciferase activity was measured 24 h later using the Dual-Luciferase Reporter Assay System (Promega).

RT-PCR. RT-PCR was performed as previously described [1,13]. The sequences of the primers are as follows: *Osterix*, 5′-GACTCATCCCTA TGGCTCGTG-3′ and 5′-GGTAGGGAGCTGGGTTAAGG-3′; *TN-W*, 5′-AGGTGGGACATCACAGTCT-3′ and 5′-TGATGGGACATCACT CTTGG-3′.

Mouse rib fracture model. The right eighth rib of a 5-week-old ICR mouse was fractured as described previously [14]. The fractured rib was collected 7 days after operation.

In situ hybridization. The unfractured and the fractured ribs were fixed with 4% paraformaldehyde and decalcified with 20% EDTA. C57BL/6, *Runx2*-null [7] and *Osterix*-null [4] E17.5 mouse embryos were fixed with 4% paraformaldehyde. The ribs and the embryos were dehydrated in a graded series of ethanol and embedded in paraffin. Sections (7 μm thick) were then processed for *in situ* hybridization as previously described [15]. pBSII-KS(+) (Stratagene, La Jolla, CA) containing the 500-bp mouse *TN-W* cDNA was linealized and labeled. The sections were counterstained with hematoxylin.

Statistical analysis. All results are expressed as means ± SD. Statistical significance was assessed by one-way analysis of variance and unpaired Student's *t* test.

Results

Identification of TN-W upregulated by Bmp2 in ATDC5 cells

To identify genes upregulated by Bmp2 in ATDC5 osteo-chondroprogenitors, we performed a two-step screening consisting of a PCR-based suppression subtractive hybridization followed by differential hybridization using the poly(A)$^+$ RNA extracted from Bmp2-untreated and Bmp2-treated ATDC5 cells. We obtained a 500-bp cDNA fragment corresponding to the 3′-untranslated sequence of *TN-W*, and this cDNA fragment was then used as a probe to screen a mouse cDNA library generated from Bmp-treated ATDC5 cells. Fifteen cDNA clones containing a coding region which was identical with *TN-W* as recently reported [9,16] were obtained from 1×10^6 independent plaques. We closely examined the effects of exogenously administered Bmp2 on the expression of *TN-W* in undifferentiated ATDC5. Undifferentiated ATDC5 cells were treated with either various doses of Bmp2 for 36 h or 500 ng/ml of Bmp2 for the indicated time periods. As shown in Fig. 1A and B, Bmp2 increased the steady-state levels of *TN-W* transcripts in time- and dose-dependent manners, indicating that *TN-W* is a downstream target of Bmp2 in ATDC5 cells.

Expression of TN-W in the adult mouse tissues and in endochondral bone formation during mouse embryogenesis

First, to identify the tissues that expressed *TN-W in vivo*, we performed Northern blot analysis with RNA extracted from bone and with mouse MTN Blots. Among the various adult mouse tissues, *TN-W* was expressed highly in bone, and moderately in kidney and spleen (Fig. 1C). Next, we examined the expression and the distribution of *TN-W* in mouse embryogenesis and in endochondral bone formation by Northern blot analysis and *in situ* hybridization, respectively. As shown in Fig. 1D, *TN-W* was detected from 15.0 dpc embryos, and the levels of its expression increased at 17.0 dpc. *In situ* hybridization analysis showed that during