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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 osteo-chondroprogenitors. 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, TNW 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

## Introduction

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.

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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 osteochondroprogenitor cells.

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 TNW 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 $1 \times 10^6$ 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 hours 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'-GACTCATCCCTATGGCTCGTG-3' and 5'-GGTAGGGAGCTGGGTTAAGG-3' ; *TN-W* , 5'-AGGTGGGACATCACAGTCT-3' and 5'-TGATGGGACATCACTCTTGG-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 the mean ± 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 $1 \times 10^6$ 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. 1 A 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. 1 C). 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. 1 D, *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 a process of endochondral bone formation *TN-W* was first expressed in the perichondrium of E13.5 embryos, but was not detected in the condensed mesenchymal cells ( Fig. 2 A). In E16.5 embryos, *TN-W* was detected in perichondrium/periosteum, but not in cells associated with bone trabeculae and in chondrocytes.

### Expression of TN-W during a process of bone fracture repair in adult mice

During a process of bone fracture repair, mesenchymal cells around bone fracture lesions initially form cartilaginous callus surrounded by newly formed perichondrium/periosteum. As shown in Fig. 2B-D, *TN-W* transcripts were detected in periosteum of the unfractured rib. During a repair process, the levels of *TN-W* transcripts increased in cells of newly formed perichondrium/periosteum surrounding the cartilaginous callus 7 days after fracture. *TN-W* was not detected in cells associated with trabecular and cortical bones throughout a process of bone fracture repair.

### Expression of TN-W in perichondrium/periosteum of Runx2-null and Osterix-null mouse embryos

Previous studies showed that both *Runx2*-null and *Osterix*-null mice completely lack bone formation due to block of differentiation of preosteoblasts into functional osteoblasts, indicating that these two transcription factors are required for early osteoblast differentiation in mesenchymal cells [4, 6, 7]. To better characterize the cell types that express *TN-W* during endochondral bone formation, we analyzed the expression of *TN-W* in bones of *Runx2*-null and *Osterix*-null mouse embryos by *in situ* hybridization ( Fig. 3 A-F ). In E17.5 both mutant embryos, *TN-W* was detectable in perichondrium/periosteum. We next assessed the effect of Runx2 and Osterix on the expression of *TN-W* in MC3T3-E1 cells by RT-PCR ( Fig.3 G). Runx2 and Osterix had no effect on the level of *TN-W* expression, respectively, while the expression of *Osteocalcin* was increased by Runx2 and Osterix. Thus, these results indicate that *TN-W* is a marker of preosteoblasts and that its expression is independent of *Runx2* and *Osterix*.

### Effects of TN-W on cell proliferation and differentiation in MC3T3-E1 cells , ATDC5 cells, *and bone marrow cells*

To assess the effects of TN-W on cell proliferation, MC3T3-E1 osteoblastic cells and ATDC5 osteo-chondrogenitors were cultured with various amounts of the conditioned media containing TN-W protein. As shown in Fig. 4A and B, TN-W inhibited BrdU incorporation in MC3T3-E1 cells and ATDC5 cells in a dose-dependent manner. We next assessed the effects of TN-W by RT-PCR on the expression of osteoblast specific marker

genes, *Runx2, Col1a1 and Osterix,* in MC3T3-E1 cells. As shown in Fig. 4 C, the level of *Col1a1* expression was decreased in MC3T3-E1 cells by the conditioned media containing TN-W protein, while the steady-state level of *Runx2* and *Osterix* expression had no change. The expression levels of these genes did not change with TN-W in ATDC5 cells (data not shown). We further investigated the effects of TN-W on initiation of osteogenesis by CFUF and -O assays ( Fig. 4 D and E). The positive stained areas of ALP and alizarin red staining were comparable. Thus these results suggest that TN-W inhibits cell proliferation and differentiation of preosteoblasts, although TN-W has no significant effect on initial stage of osteogenesis.

### Effects of TN-W on canonical Wnt signaling

Recent studies show that canonical Wnt signaling pathway regulates osteoblast proliferation and differentiation [17, 18]. We speculate that TN-W may inhibit cell proliferation and differentiation mediated by canonical Wnt signaling pathway. To test this hypothesis, we assessed the effect of TN-W on canonical Wnt signaling by luciferase assays using TCF-responsive reporter, Topflash, under exposure to exogenous Wnt3A, the activator of Wnt signaling (Fig.4F). TN-W suppressed 25% of the luciferase activity of Topflash, which was activated by canonical Wnt signaling induced by Wnt3A, indicating that TN-W has an inhibitory effect on canonical Wnt signaling.

## Discussion

Tenascins are a family of four extracellular matrix glycoproteins [19]. In mesenchymal tissues, Tenascins contribute to matrix structure mediated by proper deposition of collagen fibers, and regulate cell morphology, growth, and migration by activating diverse intracellular signaling pathways [20]. During endochondral bone formation, Tenascin-C (TN-C) is initially expressed in condensed mesenchymal cells, and then is in both chondrocytes and osteobalsts [21]. Taking account of the lack of skeletal phenotypes in *TNC*-null mice [22], one can speculate that other Tenascins might possess functional redundancy in endochondral bone formation. A recent study showed that *TN-W* is expressed in bones and regulates cell adhesion mediated by α8 integrin, and that Bmp2 treatment leads to the accumulation of TN-W in the conditioned medium of C2C12 myogenic cells in vitro [9, 23]. In this study, we identify *TN-W* upregulated by Bmp2 in ATDC5 osteochondroprogenitors, and *TN-W* is expressed in cells of perichondrium/periosteum in endochondral bones and during a process of bone fracture repair in vivo, suggesting that *TN-W* may be involved in endochondral bone formation.

During endochondral bone formation, multipotential mesenchymal cells are initially committed to osteo-chondroprogenitors. These cells aggregate to form mesenchymal condensations, and then osteoblastic cells and chondrogenic cells are separated to form perichondrium/periosteum and growth plate cartilage, respectively. Bmp2 and Bmp4, and their receptors, Bmp type IA and IB receptors are all expressed in condensed mesenchymal cells, and the conditional null mutation of both Bmp type IA and IB receptors results in complete lack of bone formation after mesenchymal condensation [24]. Moreover, Bmps induce the expression of *Runx2* and *Osterix*, both of which are essential for osteobalst differentiation [5, 25, 26]. Thus, these findings indicate that Bmp signaling is required for initiation of osteoblast differentiation in mesenchymal cells. We previously described a model of an osteoblast differentiation pathway [4]: Multipotential mesenchymal progenitors first differentiate into preosteoblasts, a process for which *Runx2* is needed. These cells do not express osteoblast marker genes, except low levels of *Col1a1*, an early marker of osteoblast differentiation. Preosteoblasts then differentiate into functional osteoblasts expressing high levels of osteoblast marker genes, a process which requires *Osterix*. In vivo, *TN-W* is expressed in perichondrium/periosteum but not in cells of mesenchymal

condensations and of trabecular and cortical bones during endochondral bone formation. Furthermore, expression of *TN-W* is detectable in perichondrium/periosteum of *Runx2*-null and *Osterix*-null mice. In addition, overexpression of *Runx2* or *Osterix* does not increase the expression of *TN-W* in MC3T3-E1 cells. Moreover, in this study, we show that *TN-W*, which was upregulated by Bmp2 in ATDC5 osteo-chondroprogenitors, inhibits cell proliferation in both MC3T3-E1 cells and ATDC5 cells and the expression of *Col1a1,* a marker gene of early stage of osteogenesis, in MC3T3-E1 cells. In ATDC5 cells, osteoblast marker genes are not upregulated by TN-W. Since ATDC5 cells are osteochondroprogenitor-like cells, providing an excellent in vitro model that exhibits the multistep chondrogenic differentiation[27], TN-W does not affect the expression of osteoblast marker genes in ATDC5 cells. Indeed, it is likely that TN-W has no effect on initial stage of osteogenesis in bone marrow cells. Thus, these lines of evidence indicate that *TN-W* is expressed in preosteoblasts after Bmp initiates osteoblast differentiation in condensed mesenchymal cells during endochondral bone formation and that *TN-W* may be involved in retaining the phenotypes of preosteoblasts by inhibiting cell proliferation and differentiation of preosteoblasts into functional osteoblasts.

Recently, canonical Wnt signaling is essential for chondrocyte and osteoblast differentiation[17, 18]. Canonical Wnt signaling regulates lineage commitment between chondrocytes and osteoblasts, inhibits chondrocyte differentiation, and promotes osteoblast differentiation. Our study shows that TN-W suppresses canonical Wnt signaling in MC3T3-E1 cells, strongly suggesting that TN-W inhibits cell proliferation and differentiation mediated by canonical Wnt signaling pathway during osteogenesis. Further studies are needed to elucidate the molecular mechanism of the inhibition of TN-W on canonical Wnt signaling.

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## Abbreviations

| | |
|---|---|
| **TN-W** | Tenascin-W |
| **dpc** | days post coitum |
| **bp** | base pair(s) |
| **Bmp** | bone morphogenetic protein |

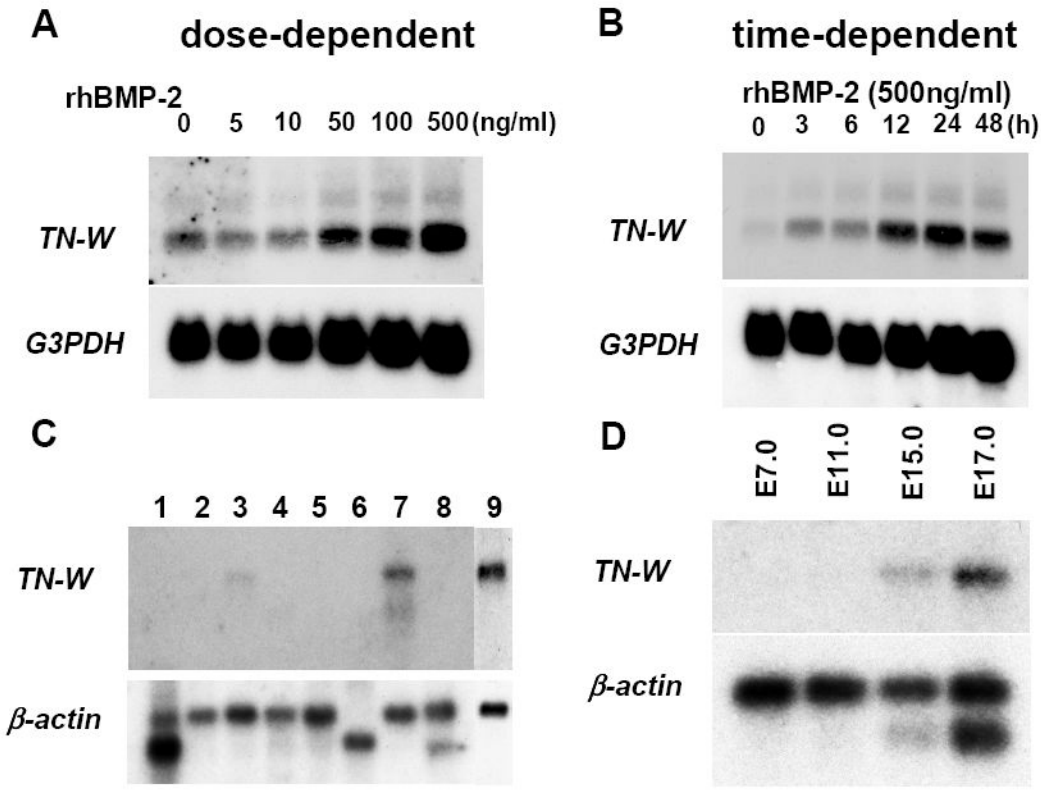


**Fig.1 . Effect of Bmp2 on *TN-W* expression in ATDC5 cells and the expression of *TN-W* in adult mouse tissues and mouse embryos**

(A) Undifferentiated ATDC5 cells were exposed to Bmp2 (0-500ng/ml) for 36 hours, or (B) 500ng/ml of Bmp2 at indicated time periods (0-48 h). Twenty μg per lane of total RNA was analyzed by northern blot hybridization. (C) MTN Blots containing 2 μg of poly $(A)^+$ RNA from various adult mouse tissues (1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis) and from calvaria of new born C57BL/6 mice (9, bone) were hybridized with *TN-W* cDNA. (D) MTN Blots containing 2 μg of poly $(A)^+$ RNA from mouse embryos in various stages of development (E7, 11, 15 and 17) were analyzed by northern blot hybridization.

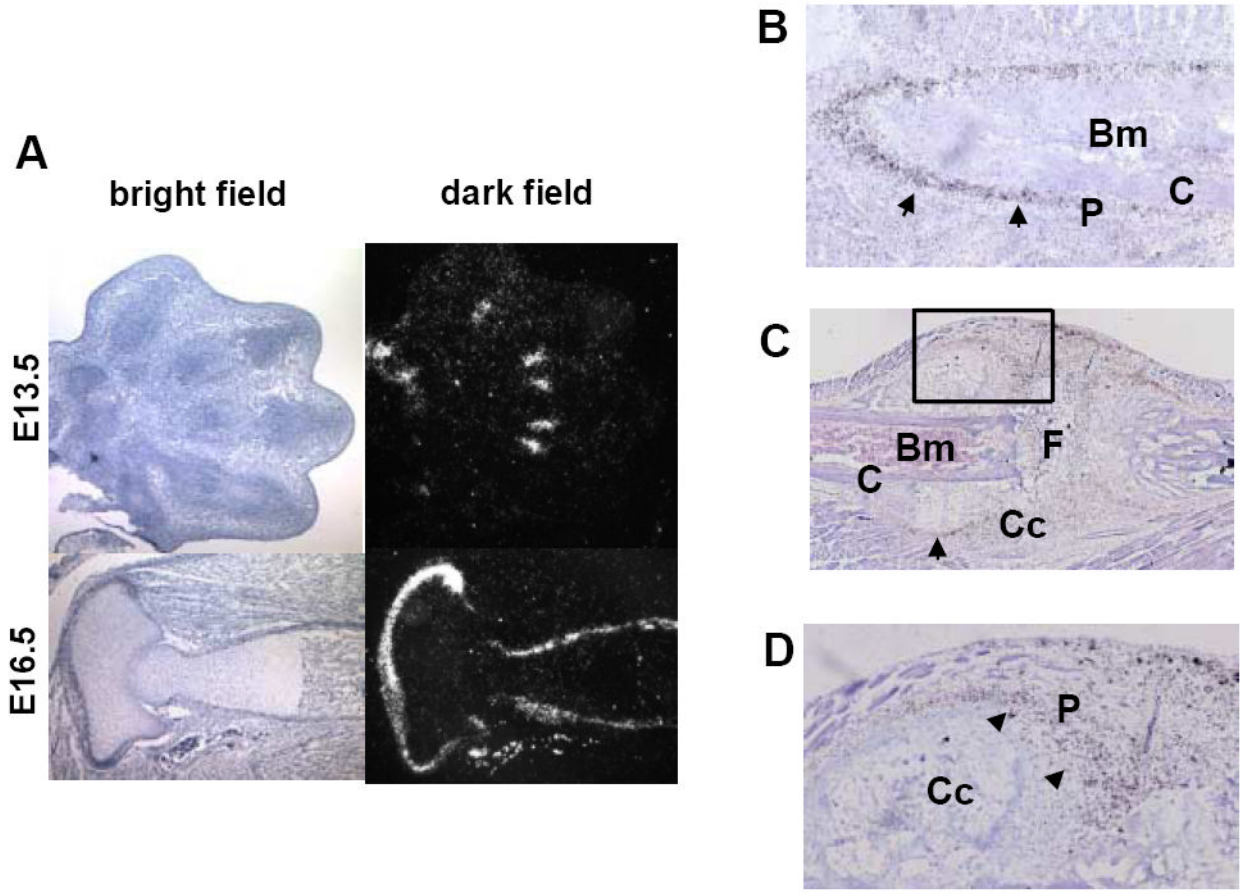


**Fig.2 . *in situ* hybridization analysis of *TN-W* expression in forelimbs of mouse embryos and in adult mouse rib fracture repair**

(A) *in situ* hybridization analysis of *TN-W* expression during endochondral bone formation in E13.5 and E16.5 mouse embryos. (B) Expression of *TN-W* is detected in periosteum of unfractured ribs. (C) At day 7 after fracture, expression of *TN-W* is localized in perichondrium/periosteum surrounding the cartilaginous callus around the fracture of the rib. (D) The boxed region in (C) is shown at a higher magnification.

Arrowheads indicate the expression of *TN-W*. Bm, bone marrow; C, cortex; P, periosteum; Cc, cartilaginous callus; F, fracture.

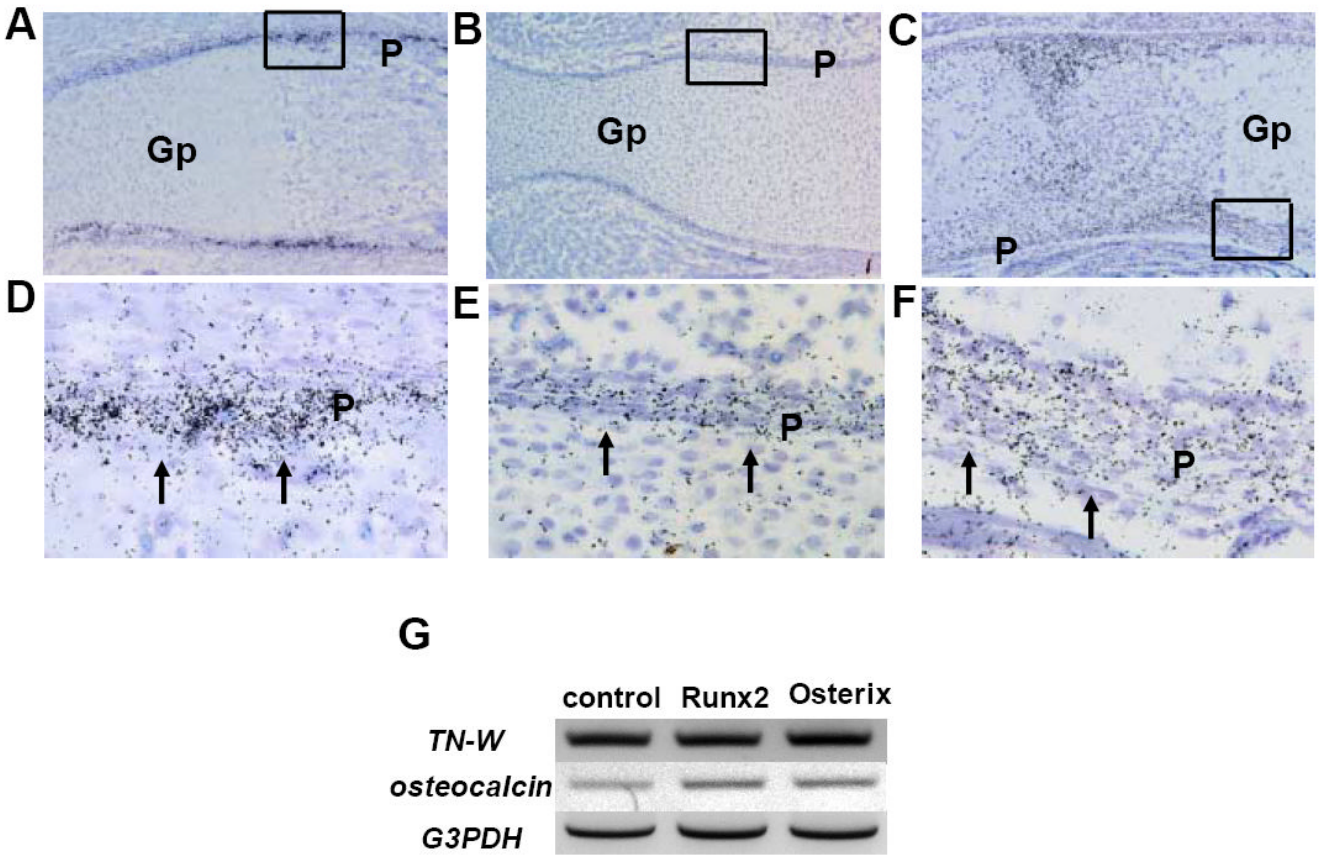


**Fig.3 . *in situ* hybridization analysis of *TN-W* expression in *Runx2*-null and *Osterix*-null mice and effects of *Runx2* and *Osterix* on *TN-W* expression**

(A-C) Expression of *TN-W* is detected in perichondrium/periosteum of the humerus in E17.5 wild type (A), *Runx2*- null (B) and *Osterix*- null (C) mouse embryos. (D-F) The boxed regions in (A), (B) and (C) are shown at a higher magnification in (D), (E) and (F), respectively. Arrows indicate the expression of *TN-W* in perichondrium/periosteum. P, perichondrium/periosteum; Gp, growth plate. (G) MC3T3-E1 cells were transfected with plasmids encoding Runx2 or Osterix. RT-PCR analysis for *TN-W* and *Osteocalcin* were performed.

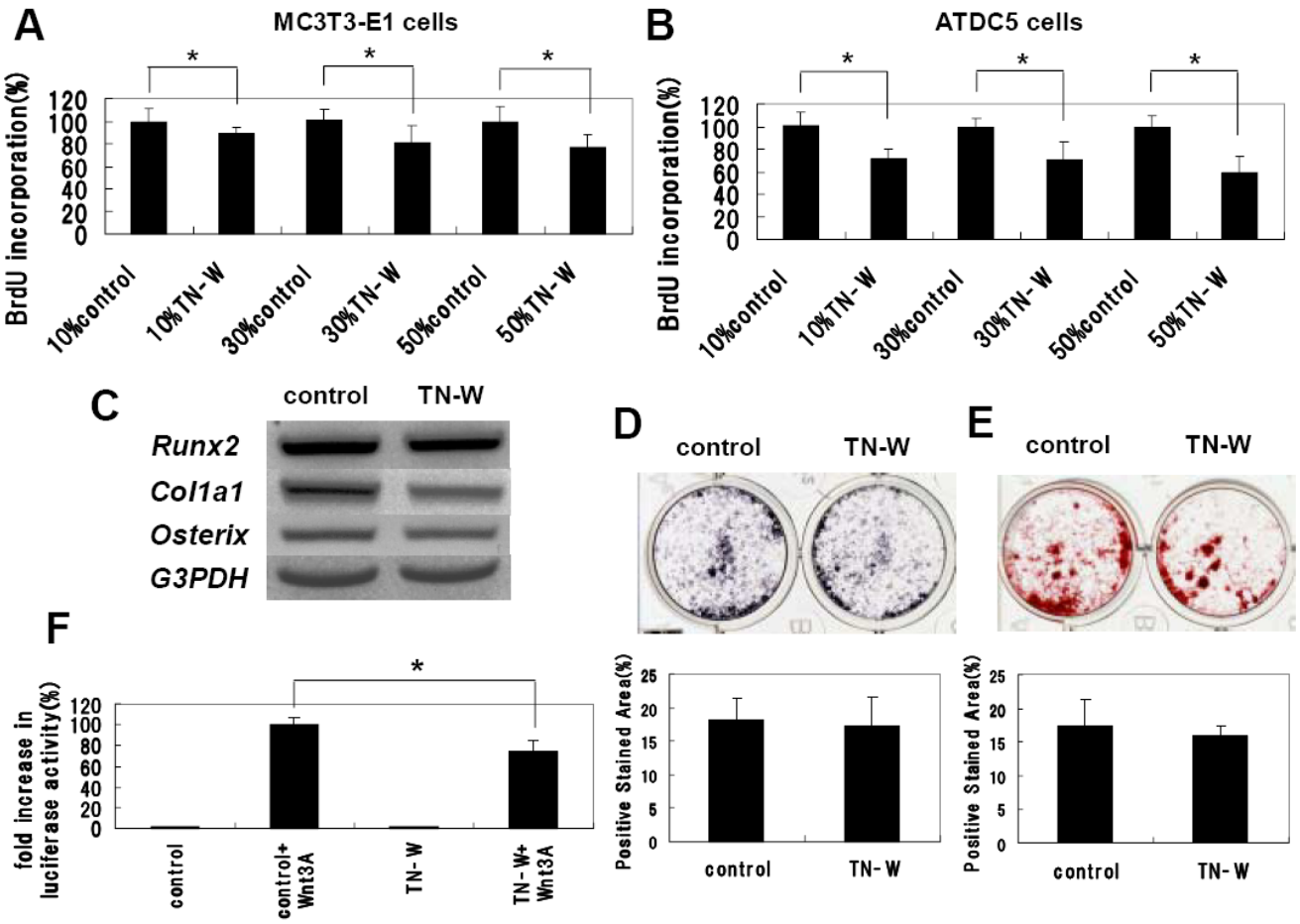


**Fig.4 . Effects of TN-W on cell proliferation, differentiation and canonical Wnt signaling in bone marrow cells and osteoblastic cells**

(A and B) MC3T3-E1 cells (A) and ATDC5 cells (B) were treated with various amounts of the conditioned media containing TN-W for 16 hr, and BrdU incorporation was measured. Data are shown as mean ± SD (* $p < 0.05$). (C) MC3T3-E1 cells were cultured with the conditioned media containing TN-W for 2 days. RT-PCR analysis for *Runx2*,*Col1a1* and *Osterix* were performed. (D and E) Bone marrow cells obtained from Balb/c mice were treated with the conditioned media containing TN-W, and CFU-F (C) and -O (D) assays were performed. Positive stained areas(%) are shown as mean ± SD (* $p < 0.05$). (F) MC3T3-E1 cells were co-transfected with Topflash and phRL-TK, and treated with the conditioned media containing TN-W and 1nM Wnt3A. Data are shown as mean ± SD (* $p < 0.05$).