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# Regulation of Tenascin Expression in Bone

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## ABSTRACT

Tenascins regulate cell interaction with the surrounding pericellular matrix. Within bone, tenascins C and W influence osteoblast adhesion and differentiation, although little is known about the regulation of tenascin expression. In this study we examined the effect of osteogenic differentiation, bone morphogenetic protein (BMP) and Wnt growth factors, and mechanical loading on tenascin expression in osteogenic cells. Osteogenic differentiation increased tenascin C (*TnC*), and decreased tenascin W (*TnW*), expression. Both growth factors and mechanical loading increased both *TnC* and *TnW* expression, albeit via distinct signaling mechanisms. Both BMP-2 and Wnt5a induction of tenascin expression were mediated by MAP kinases. These data establish a role for BMP, Wnts, and mechanical loading in the regulation of tenascin expression in osteoblasts. J. Cell. Biochem. 112: 3354–3363, 2011. © 2011 Wiley Periodicals, Inc.

**KEY WORDS:** TENASCIN; BONE MORPHOGENETIC PROTEIN; WNT; MAPK; OSTEOBLAST; OSTEOCYTE; MECHANOTRANSDUCTION

The skeletal system is critical for structural support and mineral homeostasis, while also providing a protected environment conducive to hematopoiesis. To fulfill these multiple functions, bones adapt to a variety of signals, including systemic hormones and localized biophysical forces. These forces are generated during the loading and unloading of long bones, and include oscillatory flow of interstitial fluid, substrate strain, and streaming potentials [Robling et al., 2006]. These signals trigger mechanosensitive cells resident within the skeleton, such as mineral depositing-osteoblasts, to activate a variety of signal transduction pathways that alter growth factor release, gene expression, and mineral deposition in order to minimize tissue strain [Robling et al., 2006]. The organic portion of bone extracellular matrix is composed of both large structural proteins and smaller matricellular proteins, which modulate cell behavior and cell surface interactions through direct and indirect mechanisms.

Tenascins are matricellular glycoproteins that are highly expressed during tissue development and remodeling. Tenascin expression is increased under a plethora of pathological conditions including skin wounds, atherosclerosis, asthma, and cancer [Chiquet-Ehrismann and Chiquet, 2003]. Of the four vertebrate tenascins—C, W, R, and Xb—a function in the skeleton has been previously assigned to tenascins C and W. Tenascin C is widely, although transiently, expressed during embryogenesis and organogenesis [Chiquet-Ehrismann, 2004]. Tenascin W is also transiently expressed during development, but is largely restricted to the musculoskeletal system. In adult bone both tenascin C and W localize to the periosteum, while tenascin C is also found in the endosteum [Mackie et al., 1987; Scherberich et al., 2004]. Expression of both tenascin C and W increase during fracture healing [Kimura et al., 2007; Kilian et al., 2008]. Functionally, tenascins C and W are implicated in osteoblast differentiation and proliferation [Mackie and Ramsey, 1996; Meloty-Kapella et al., 2008]. Tenascin X expression is observed embryonically within the mandible [Kurihara and Sato, 2004; Bristow et al., 2005], and, in the adult, tenascin X is described in muscle and loose connective tissue. Its deletion is implicated in the connective tissue disorder Ehlers–Danlos syndrome [Burch et al., 1997]. The expression of tenascin R or Xb have not been widely described in bone. The distinct expression pattern of each member of the tenascin family suggests possible unique transcriptional regulation, yet the reported control of tenascins in bone, as well as the body, remains poorly defined.

The osteoblast-derived proteins and molecules that demonstrate anabolic effects upon the skeleton do so by increasing proliferation and matrix production and by decreasing apoptosis. Such factors include ATP [Orriss et al., 2010], prostaglandins [Pilbeam et al., 2002], bone morphogenetic proteins (BMPs) [Wan and Cao, 2005], and Wnt glycoproteins [Westendorf et al., 2004]. BMPs were recognized in the 1960s for their osteoinductive effect [Urist, 1965], and BMP-2 and BMP-7 are currently in clinical use for spinal fusion

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and non-unions [Axelrad and Einhorn, 2009]. Similarly, Wnt signaling through cognate Lrp5 or Lrp6 receptors, is implicated in bone formation [Gong et al., 2001]. Both BMP and Wnt signaling drive the embryologic development of bone [Wozney, 1992], are increased during fracture repair [Marsell and Einhorn, 2009; Secreto et al., 2009], and are activated by mechanical loading of bone cells both in vitro and in vivo [Hens et al., 2005; Lau et al., 2006; Kido et al., 2010]. Further, these pathways have been implicated in tenascin regulation in other tissues [Scherberich et al., 2005; Cohen et al., 2009].

Because tenascins are differentially expressed in the skeleton during various phases (e.g., embryogenesis, fracture repair, and post-natal homeostasis), and because tenascin expression is influenced in other tissues by factors known to influence bone cell behavior, we examined tenascin expression in response to osteogenic differentiation, growth factors, and biophysical signaling. BMP-2, Wnt5a, and fluid shear stress each induced the expression of tenascins C and W, albeit via* distinct signaling pathways involving de novo protein synthesis, phospholipase C (PLC), and MAP kinases. These data indicate that tenascins C and W are regulated by multiple distinct mechanisms in osteoblasts.

## MATERIALS AND METHODS

### REAGENTS

Chemical inhibitors of MEK1/2 (U0126), p38 (SB 203580), JNK (JNK Inhibitor II), PLC (U-73122), and calcineurin (cyclosporin A, CsA) were purchased from EMD Biosciences. Cycloheximide (CHX) was purchased from Sigma, BMP-2 was from Peprotech, and Wnts were from R&D Systems. All antagonists were added 1 h prior to the beginning of each experiment, and were present in experimental media.

### CELL CULTURE

Murine pre-osteoblastic cells (MC3T3-E1; provided by Norman J. Karin, Pacific Northwest National Laboratory) were seeded on tissue culture-treated plastic at a density of 10,000 cell/cm$^2$ in α-MEM supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S) for all static experiments. Cells were maintained in a standard humidified incubator at 37°C with 5% $CO_2$. Media was replaced with α-MEM supplemented with 2% FBS and 1% P/S overnight prior to performing all experiments. For osteogenic differentiation, cells were cultured in the presence of osteogenic media (standard media supplemented with 50 μg/ml ascorbic acid-2-phosphate and 5 mM β-glycerol phosphate).

### OSCILLATORY FLUID FLOW

MC3T3-E1 cells were seeded at a density of 5,000 cell/cm$^2$ onto 7.5 × 3.8 cm$^2$ glass slides in α-MEM supplemented with 10% FBS, and 1% P/S. Cells were cultured in a standard humidified incubator at 37°C with 5% $CO_2$. Media was replaced with α-MEM supplemented with 2% FBS and 1% P/S overnight prior to the application of fluid flow. Cell-seeded slides were placed into a custom-made parallel plate flow chamber, modified from that described by Frangos et al. [1988], immediately prior to flow experiments. Flow chambers were maintained in a humidified incubator at 37°C throughout the flow period. Fluid flow was delivered using 500 μl Hamilton glass syringes mounted into a mechanical loading device (TestBench, Bose) as previously described [Jacobs et al., 1998]; this system generates sinusoidal oscillatory fluid flow at a frequency of 1 Hz which produces a peak shear stress of 15 dyn/cm$^2$. The flow rate was confirmed with an in-line ultrasonic flow meter (Transonic Systems). Flow medium (αMEM + 2% FBS + 1% P/S) was supplemented with 10 mM HEPES and 50 μg/ml gentamicin. Slides were removed from flow chambers after 2-h exposure and placed in tissue culture dishes with α-MEM supplemented with 2% FBS, and 1% P/S for the indicated post-flow incubation time.

### QUANTITATIVE PCR

RNA was isolated from mid-diaphysial sections of murine femurs (aged 14–16 weeks) according to Genetos et al. [2010]. Alternately, RNA was isolated and purified using RNeasy Mini Kit per the manufacturer's instructions (Qiagen). Total RNA was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen), which includes a genomic DNA digestion step. Quantitative PCR was performed using QuantiFast PCR Master Mix (Qiagen) on a Mastercycler Realplex2 (Eppendorf). Proprietary primers and probes for tenascins C (*TnC*), W (*TnN*), R (*TnR*), and Xb (*TnXb*) and *Rpl13* were purchased from Applied Biosystems. Amplification conditions were 95°C for 3 min followed by 40 cycles of 3 s at 95°C and 60°C for 30 s. The ribosomal gene *Rpl13* was used to normalize samples for comparison. Gene expression was calculated relative to *Rpl13* ($2^{-\Delta C_t}$), and was occasionally further normalized to matched control ($2^{-\Delta\Delta C_t}$) [Schmittgen and Livak, 2008].

### WESTERN BLOT

Protein was collected in RIPA (0.1% Triton X-100, 10 mM Tris, pH 8, 1 mM EDTA, 200 nM $Na_3VO_4$) with HALT protease and phosphatase inhibitors (Pierce). Protein concentration was determined using the DC Protein Assay (Biorad) according to the manufacturer's instructions. Equal amounts of protein were loaded in each well and run out on a 10% SDS gel before transfer to a nitrocellulose membrane. Membranes were probed with primary antibody overnight at 4°C and an appropriate secondary antibody (1:1,000) at room temperature for 1 h prior to development with enhanced chemiluminescent substrate (Denville). Anti-mouse and anti-rabbit secondary antibodies were purchased from Jackson Laboratories. Primary antibodies against p-JNK (1:1,000), JNK (1:1,000), p38 (1:500), and P-p38 (1:1,000) were purchased from Cell Signaling while p-ERK1/2 (1:1,000) and ERK1/2 (1:1,000) antibodies were purchased from Santa Cruz Biotechnology.

### STATISTICAL ANALYSIS

Each data set is the result of a minimum of three independent experiments. Unless otherwise indicated, data were normalized to vehicle control samples in the absence of growth factor. Data were analyzed by Student's *t*-test, one-way, or two-way ANOVA. Dunnet or Tukey's post hoc tests were performed when significant differences were detected by ANOVA. Statistical significance was considered for $P < 0.05$.

## RESULTS

### TENASCIN EXPRESSION

Tenascin expression was examined in a variety of tissues by qPCR. Expression of *TnC*, *TnW*, *TnR*, and *TnXb* was observed at varying levels in samples from adult murine heart, kidney, liver, and prostate (Fig. 1A). *TnW* expression was observed in heart, kidney, and prostate, but not in the liver. Similarly, there was differential expression of each tenascin isoform in adult murine femurs from mice strains (CD-1 vs. C57BL/6) and clonal cells of varying osteoblastic phenotype (pre-osteoblastic MC3T3-E1 vs. osteocyte-like MLO-Y4) (Fig. 1B). *TnC* and *TnW* were detected in each tissue or cell sample. *TnR* and *TnXb* transcripts were occasionally detected at low levels but expression was not confirmed at the protein level. For all skeletal-derived samples, *TnC* and *TnW* were consistently expressed at higher levels than were *TnR* and *TnXb*. Similarly, tenascin expression was higher in femur-derived RNA than in clonal cell lines.

Tenascin expression was also examined during the course of in vitro osteogenic differentiation. MC3T3-E1 cells were cultured with osteogenic media for 0, 7, 14, and 21 days. By day 14 *TnC* transcript was significantly increased compared to expression on day 0 (Fig. 1C). *TnW* transcript levels decreased over time and were significantly decreased from day 0 at all time points examined (Fig. 1D). These data illustrate that *TnC* and *TnW* transcripts are present in the MC3T3-E1 pre-osteoblast cell line and are differentially expressed during the course of osteoblastic differentiation.

### BMP REGULATION OF TENASCIN EXPRESSION

Tenascins C and W are localized within the periosteum, where BMP-2 plays a role in differentiation of osteoprogenitors [Zhao, 2003; Wan and Cao, 2005; Kimura et al., 2007], thus we chose to examine whether BMP signaling influenced expression of *TnC* or *TnW*. MC3T3-E1 pre-osteoblastic cells were exposed to BMP-2 at 0, 100, or 250 ng/ml for 6 h. qPCR revealed an inductive effect of BMP-2 upon *TnC* and *TnW* expression (Fig. 2A). One hundred nanograms/ml BMP-2 induced *TnW* expression, and 250 ng/ml BMP-2 significantly increased both *TnC* and *TnW* compared to treatment-matched controls.

We next examined whether de novo protein synthesis was required for induction of *TnC* or *TnW* by BMP-2. Super-induction of *TnC* was observed with 10 μg/ml CHX in the presence or absence of 250 ng/ml BMP-2. Similar super-induction was observed when using another protein synthesis inhibitor, emetine (data not shown). A significant interaction between growth factor and CHX treatment indicated that these effects are not additive. In contrast to *TnC*, we observed no super-induction in response to CHX (Fig. 2B) or emetine (data not shown). CHX pre-treatment prevented BMP-2-induced *TnW* expression (Fig. 2B), indicating that BMP-2-induced tenascin W requires de novo protein synthesis. Thus, BMP-2 induces both *TnC* and *TnW*, via a differential requirement for protein synthesis.

### MAP KINASES MEDIATE BMP-2 INDUCED TENASCIN TRANSCRIPT

To further elucidate the mechanism behind BMP-2-induced tenascin expression, we examined the role of MAPK signaling in BMP-2-

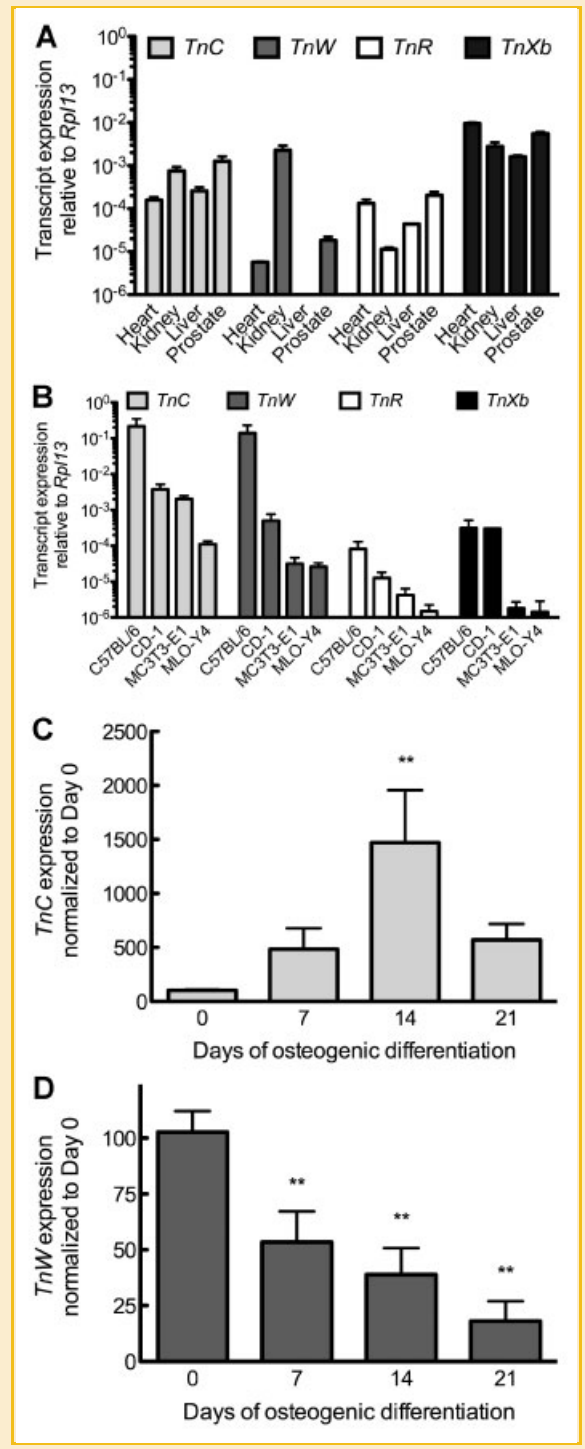


Fig. 1. Tenascin expression in murine bone. A: qPCR of tenascin expression in a range of murine tissues. Data are expressed relative to control gene *Rpl13*. Bars represent mean ± SEM, n ≥ 3. B: qPCR analysis of tenascin expression in the femur from two strains of mice, pre-osteoblastic MC3T3-E1 cells, and osteocyte-like MLO-Y4 cells. Data are expressed relative to control gene *Rpl13*. Bars represent mean ± SEM, n ≥ 3. C: qPCR analysis of *TnC* in MC3T3-E1 cells after 0, 7, 14, or 21 days of osteogenic differentiation. Data are normalized to control gene *Rpl13*, then to day 0. Bars represent mean ± SEM, n ≥ 8. D: qPCR analysis of *TnW* expression in MC3T3-E1 cells after 0, 7, 14, or 21 days of osteogenic differentiation. Data are normalized to the control gene *Rpl13* and then to day 0. $^{**}P < 0.01$ compared to day 0. Bars represent mean ± SEM, n ≥ 8.

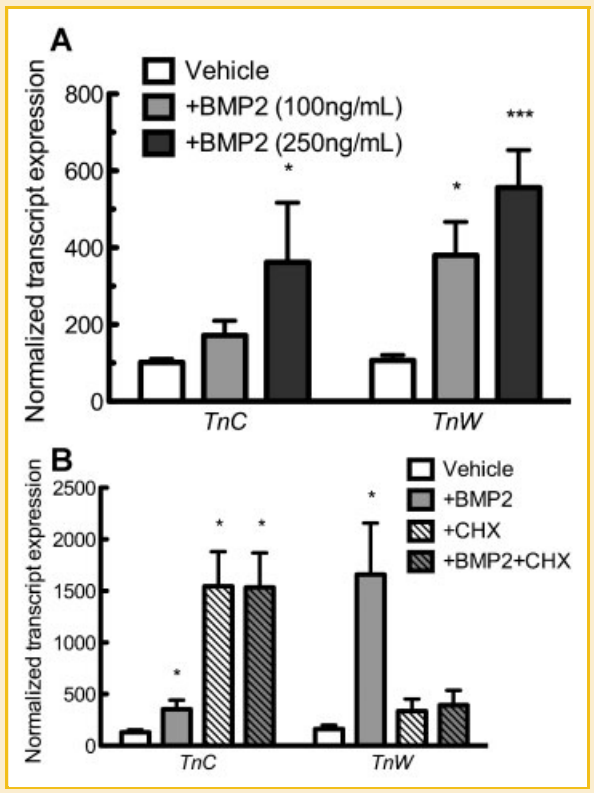


Fig. 2. BMP-2 induces tenascin expression. A: *TnC* and *TnW* expression in MC3T3-E1 cells in response to a 6-h exposure to BMP-2 as measured by qPCR. $^{*}P < 0.05$ or $^{***}P \leq 0.001$ compared to control. Bars represent mean ± SEM, $n \geq 8$. B: *TnC* and *TnW* expression induced by BMP-2 (250 ng/ml) in the presence or absence of CHX (10 μg/ml). CHX was applied to cells 1-h prior to the 6-h application of BMP-2. $^{*}P < 0.05$ compared to vehicle control. The effects of CHX and BMP-2 were not additive when analyzed by two-way ANOVA. All data are normalized to control gene *Rpl13*, and then to the vehicle control. Bars represent mean ± SEM, $n \geq 9$.

induced tenascins. Cells were pre-treated with individual MAPK inhibitors for 1 h prior to BMP exposure; the efficacy of each of these inhibitors has been previously demonstrated in osteoblasts [Kozawa et al., 2001; Guicheux et al., 2003; Patil et al., 2004]. BMP-2-induced *TnC* response persisted in the presence of the MEK1/2 inhibitor U0126, but was abrogated by pre-treatment with inhibitors of p38 (SB203580) or JNK (JNKi II). BMP-2-induced *TnW* transcript was induced in the presence of all three MAPK inhibitors (Fig. 3B), although it was significantly reduced in cells in which p38 was inhibited. These data suggest that BMP-2-induced *TnC* is dependent upon p38 and JNK, whereas *TnW* induction is partially p38-dependent.

## Wnt REGULATION OF TENASCIN EXPRESSION

Similar to BMPs, Wnts demonstrate powerful effects upon the osteogenic differentiation of MSCs [Westendorf et al., 2004]. We next examined the influence of both non-canonical and canonical Wnts upon expression of *TnC* and *TnW*. A non-canonical Wnt, Wnt5a, significantly increased both *TnC* and *TnW* transcript after 6 h (Fig. 4A) and this was maintained through 24 h of Wnt5a treatment (data not show). A canonical Wnt, Wnt3a, demonstrated trends toward induction of both *TnC* and *TnW* after a 6-h exposure (Fig. 4B). After 24 h there was a significant increase in both *TnC* and *TnW*, in response to Wnt3a (Fig. 4C).

We next examined the role of de novo protein synthesis in Wnt5a-induced *TnC* and *TnW* expression. *TnC* transcript was induced by the protein synthesis inhibitor CHX in the absence or presence of Wnt5a (Fig. 4D); there was a significant interaction between growth factor treatment and CHX treatment suggesting the effects were not additive. Conversely, CHX blocked Wnt5a-induced *TnW* expression (Fig. 4D). These data indicate that Wnt5a, similar to BMP-2, utilizes distinct signaling pathways in order to increase *TnC* and *TnW* expression.

## MAP KINASE SIGNALING IS REQUIRED FOR Wnt5a-INDUCED TENASCIN TRANSCRIPT

Non-canonical Wnts, such as Wnt5a, activate the MAPK family in other cell types [Yamanaka et al., 2002; Ma and Wang, 2007]. In order to confirm this activation in osteoblasts we measured the phosphorylation of ERK1/2, p38, and JNK after exposure to 250 ng/ml Wnt5a for 5–120 min. Wnt5a transiently increased the phosphorylated forms of both ERK1/2 (Tyr 204) and p38 (Thr180/

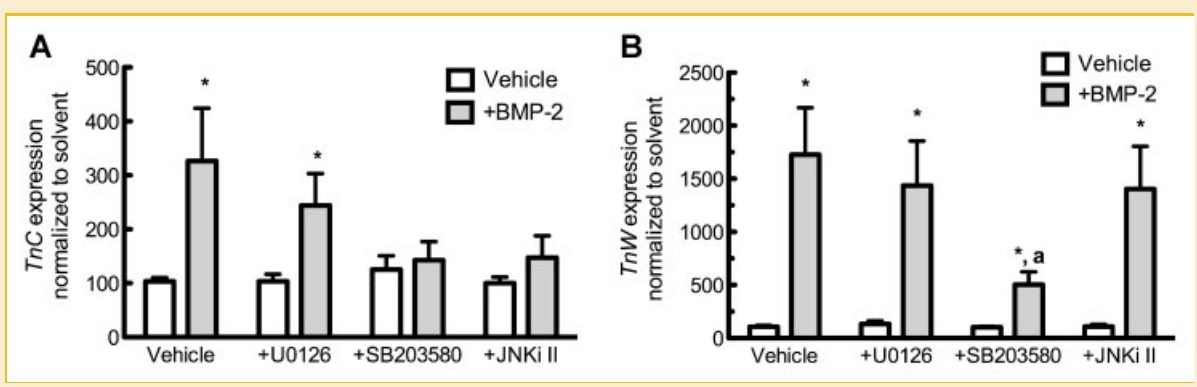


Fig. 3. The differential role of MAP kinases in BMP-2-induced tenascin expression. *TnC* (A) and *TnW* (B) expression induced by BMP-2 (250 ng/ml) in the presence of inhibitors for the MAP kinases MEK1/2 (U0126, 10 μM), p38 (SB203580, 10 μM), and JNK (JNKi II, 20 μM). All inhibitors were applied for 1 h prior to the addition of BMP-2. qPCR was performed after 6 h. $^{*}P < 0.05$ compared to solvent-matched control. $^{a}P < 0.05$ compared to BMP-2-treated vehicle control. All data are normalized to control gene *Rpl13*, then to the solvent-matched control. Bars represent mean ± SEM, $n \geq 9$.

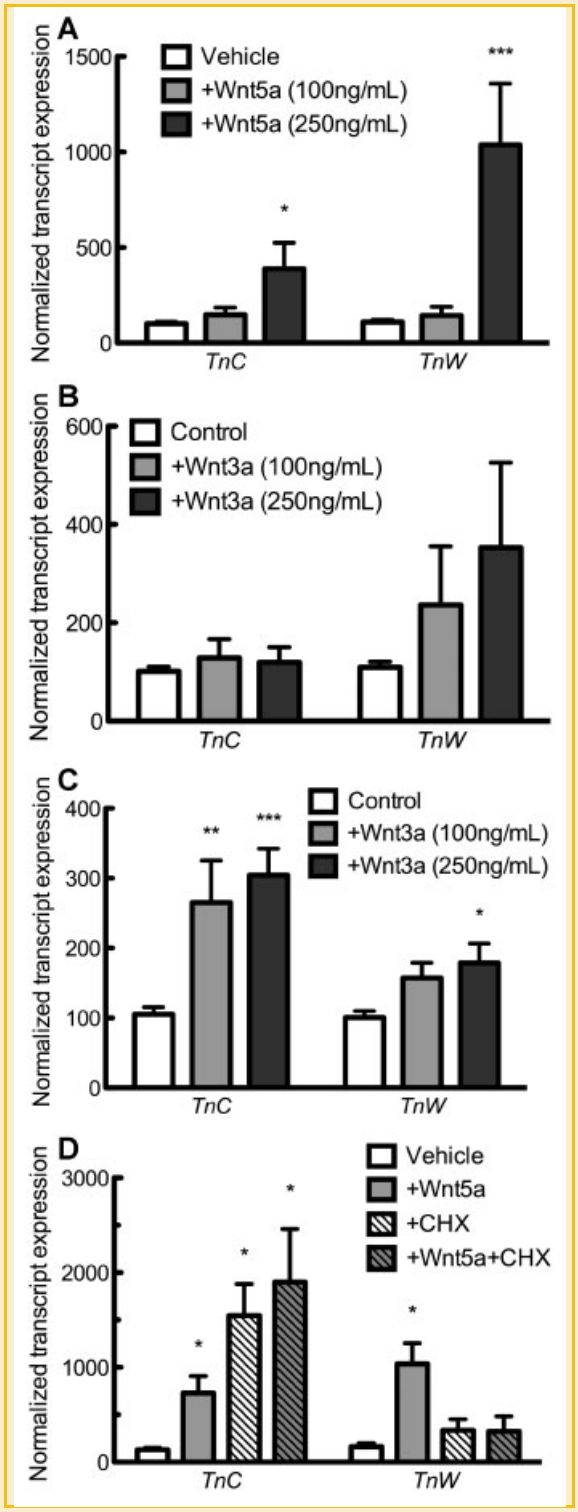


Fig. 4. Wnt induces tenascin expression. *TnC* and *TnW* induction by Wnt3a at 6 h (A) and Wnt5a at 6 h (B) and 24 h (C) as measured by qPCR. $^{*}P < 0.05$ compared to control. *TnC* and *TnW* expression induced by Wnt5a (250 ng/ml) in the presence or absence of CHX (10 μg/ml) (D). CHX was applied to cells 1-h prior to a 6-h exposure to Wnt5a. $^{*}P < 0.05$ compared to vehicle control; The effects of CHX and BMP-2 were not additive when analyzed by two-way ANOVA. All data are normalized to control gene *Rpl13*, then to the vehicle control. Bars represent mean ± SEM, $n \geq 8$.

Tyr182) (Fig. 5A), while the phosphorylated form of JNK (Thr183/Tyr185) was not detected (data not shown). Next, we examine whether Wnt5a induction of *TnC* or *TnW* was dependent upon MAPK signaling. Wnt5a induction of *TnC* remained intact in the presence of each MAPK inhibitor (Fig. 5B); there was a trend for attenuated *TnC* expression in cells treated with Wnt5a in the presence of U0126, although this did not achieve statistical significance. The Wnt5a-induced increase in *TnW* was prevented in cells treated with SB203580 (Fig. 5C), while U0126 or JNKi II did not affect Wnt5a induction of *TnW*.

### PLC AND NFAT IN Wnt5a REGULATION OF TENASCINS

The requirement for PLC in Wnt5a-induced tenascin expression was examined using the PLC inhibitor U73122. U73122 inhibited Wnt5a-induced *TnC* but had no effect on Wnt5a-induced *TnW* (Fig. 6A). These findings suggest that Wnt5a-induced *TnC*, but not *TnW*, is mediated by a PLC-dependent mechanism. We examined whether Wnt5a-induced tenascin expression was mediated by activation of the transcription factor nuclear factor of activated T cells (NFAT), as has been shown in other cell types [Ma and Wang, 2007]. Inhibition of the NFAT-activating phosphatase calcineurin with the immunosuppressant cyclosporin A (5 μg/ml) demonstrated no attenuation of Wnt5a-induced *TnC* and *TnW* expression (Fig. 6B).

### MECHANICAL INDUCTION OF TENASCIN EXPRESSION

Both BMP signaling and non-canonical Wnt signaling are induced by fluid flow across osteoblastic or mesenchymal cells [Lau et al., 2006; Arnsdorf et al., 2009]. Thus, we next sought the effect of oscillatory fluid flow on tenascin expression. MC3T3-E1 cells were exposed to oscillatory fluid flow at 15 dyn/cm$^2$ or maintained under static conditions for 2 h. RNA was subsequently isolated 0, 6, 12, or 24 h thereafter. *TnC* and *TnW* expression transiently increased following oscillatory fluid flow. *TnC* demonstrated a significant increase in expression, compared to time-matched static samples, at 6 or 12 h post-flow, but returned toward baseline levels after 24 h (Fig. 7A). *TnW* expression was significantly increased 12 h after oscillatory fluid flow (Fig. 7A).

We, and others, have previously demonstrated that MAP kinase activation is required for oscillatory fluid flow induced changes in expression matrix proteins such as osteopontin [You et al., 2001] and collagen 1 [Wu et al., 2006]. To test whether the same is true for oscillatory fluid flow-induced *TnC* or *TnW*, MC3T3-E1 osteoblasts were exposed to oscillatory fluid flow for 2 h in the presence of individual MAPK antagonists, and RNA was collected 12 h later. Activation of p38, but not MEK1/2 or JNK, was required for oscillatory fluid flow induction of *TnC*, whereas *TnW* induction required JNK, but not p38 or MEK1/2 (Fig. 7B).

## DISCUSSION

Tenascins modulate osteoblast differentiation and proliferation, and are implicated in skeletal-associated pathologies including breast cancer, giant cell tumors of bone, and osteoarthritis [Yoshida et al., 1995; Hasegawa et al., 2004; Pazzaglia et al., 2010]. Tenascins also play an important role in osteoclast adhesion and fracture repair as

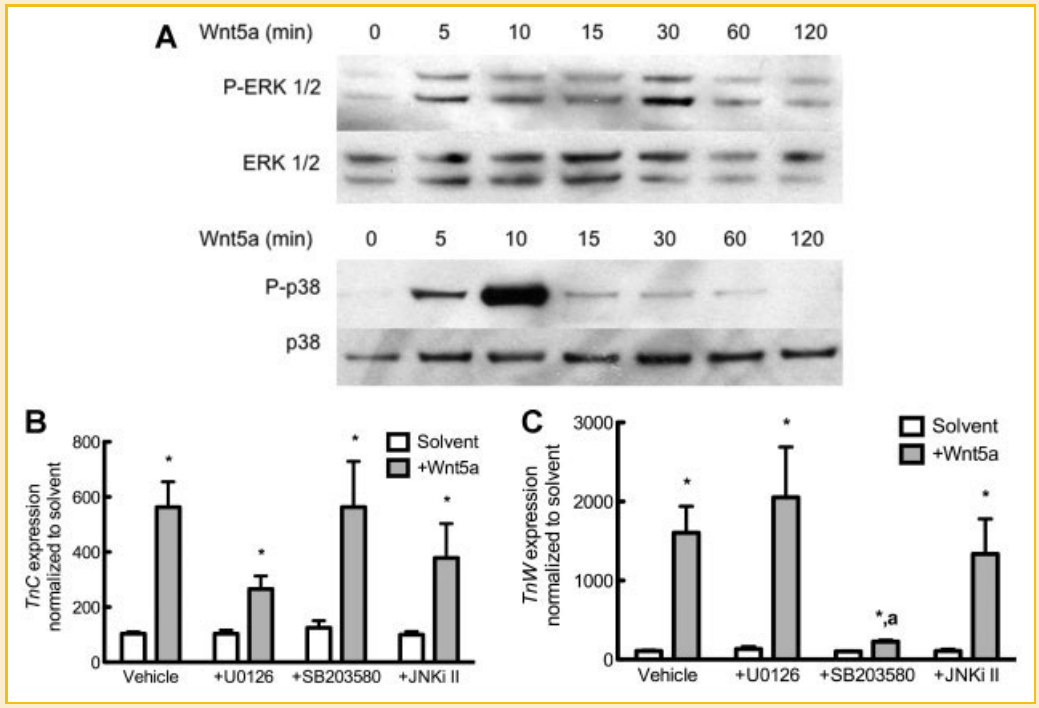


Fig. 5. Wnt5a induces tenascin C and W transcripts through distinct mechanisms. A: Wnt5a (250 ng/ml) transiently induces ERK1/2 and p38 phosphorylation. *TnC* (B) and *TnW* (C) expression induced by Wnt5a (250 ng/ml) in the presence of inhibitors for the MAP kinases MEK1/2 (U0126, 10 μM), p38 (SB203580, 10 μM), and JNK (JNKi II, 20 μM). All inhibitors were applied for 1-h prior to the 6-h exposure to Wnt5a. $^{*}P < 0.05$ compared to solvent-matched static. $^{a}P < 0.05$ compared to Wnt5a treated vehicle control. All data are normalized to control gene *Rpl13* and then to the solvent-matched control. Bars represent mean ± SEM, $n \geq 7$.

illustrated by inappropriate resorption and excessive bone production during fracture repair in the *TnC* knockout mouse [Alford and Hankenson, 2006]. In vivo adult tenascin expression is limited to the bone surface and does not extend into the cortex [Mackie et al., 1987; Scherberich et al., 2004]. Consistent with these observations we observed increased transcript levels for both *TnC* and *TnW* in the osteoblastic cell line, MC3T3-E1, compared to the osteocytic cell line, MLO-Y4 (Fig. 1A). Tenasins C [Mackie and Ramsey, 1996] and W [Meloty-Kapella et al., 2008] are known to induce osteoblastic differentiation, yet the levels of endogenous expression during osteoblastic differentiation are unclear. In the current study we observed decreasing *TnW* levels during the course of osteoblastic differentiation (Fig. 1C). This contrasts with earlier reports of increasing *TnW* expression in clonal myoblast and osteoprogenitor lineages [Scherberich et al., 2004; Mikura et al., 2009]. This discrepancy is possibly due to the less mature nature of the C2C12 and Kusa-A1 cell lines, or the use of BMP-2 to induce osteogenic differentiation, as both this study and others have shown that BMP-2 itself induces *TnW* [Scherberich et al., 2005]. In our study, *TnC* levels increased during osteogenic differentiation until day 14, and decreased thereafter (Fig. 1C). The decreasing trend in tenascin expression by day 21 is consistent with decreased tenascin expression as cells further differentiate towards osteocytes. Thus, we believe our in vitro data accurately reflect in vivo observations, and also establish the MC3T3-E1 pre-osteoblastic cell line as a viable model for the study of osteoblastic tenascin expression.

While several studies have established the importance of tenascins in regulating osteoblast adhesion and ECM interactions, relatively little is known about the regulation of skeletal tenascin expression. This study found a variety of physiologic stimuli including BMPs, Wnts, and mechanical stimulation induced increases in osteoblast expression of *TnC* and *TnW* through distinct signaling mechanisms. BMPs are some of the earliest and most well described osteoinductive proteins. BMP-2 has implications developmentally [Wan and Cao, 2005] and clinically [Axelrad and Einhorn, 2009]. We found that BMP-2 induces *TnC* and *TnW* (Fig. 2A). A previous report [Guicheux et al., 2003] found that BMP-2 treatment of MC3T3-E1 cells induced activation of p38, JNK, and to a much lesser extent, ERK1/2. Consistent with these findings the MEK1/2 inhibitor, immediately upstream of ERK1/2, did not affect BMP-2-induced *TnC* or *TnW* expression in our study. In our hands inhibition of p38 prevented BMP-2-induced *TnC* and *TnW*, while Jnk inhibition only prevented the induction of *TnC* (Fig. 2B). These findings greatly expand on a previous study by Scherberich et al. [2005], which identified p38 as an important mediator of BMP-2-induced *TnW* in fibroblasts. The independent regulation of tenascin C by JNK provides a potential explanation for the distinct but overlapping expression patterns of tenascin C and W. Based on these findings we conclude that BMP-2-induced *TnC* expression is driven by both p38 and JNK signaling while BMP-2-induced *TnW* is driven by only the p38 branch of MAPK signaling (Fig. 8). We have not ruled out additional contribution by the traditional BMP activation of Smad signaling. Previous work suggests that while TGF-β-induced Smad signaling drives *TnC* expression [Jinnin et al., 2004], Smad1/5/8 activation in pulmonary artery smooth muscle cells is inhibitory to *TnC* [Ihida-Stansbury et al., 2006]. The role

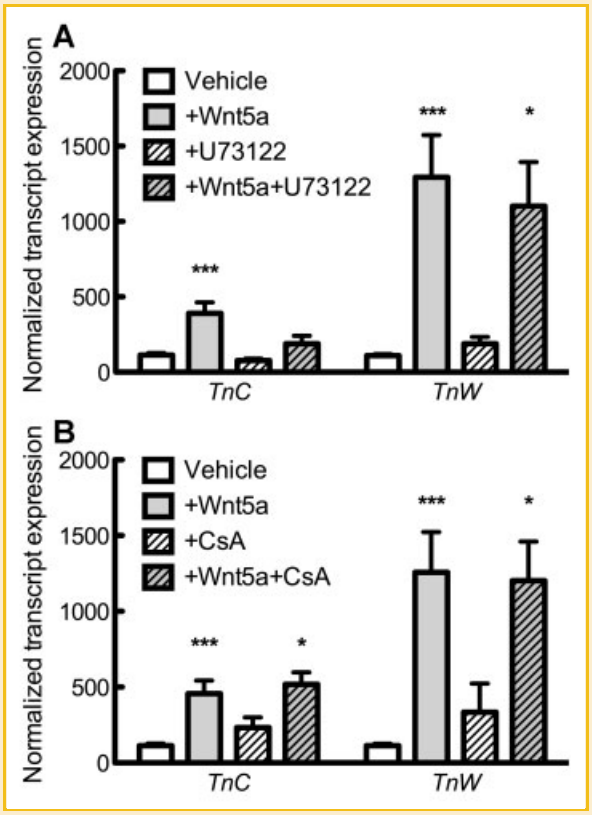


Fig. 6. Role of NFAT and PLC in Wnt5a-induced tenascin expression. A: *TnC* and *TnW* induction by Wnt5a in the presence of a phospholipase C inhibitor (U73122, 10 μM). $^{*}P < 0.05$ compared to treatment-matched control; $^{**}P < 0.01$ compared to solvent-matched static. B: *TnC* and *TnW* expression induction by Wnt5a in the presence of a cyclosporine A (5 μg/ml). $^{*}P < 0.05$ compared to vehicle control; $^{***}P < 0.001$ compared to solvent-matched control. All data are normalized to control gene *Rpl13*, and to the solvent-matched control. Bars represent mean ± SEM, n ≥ 14.

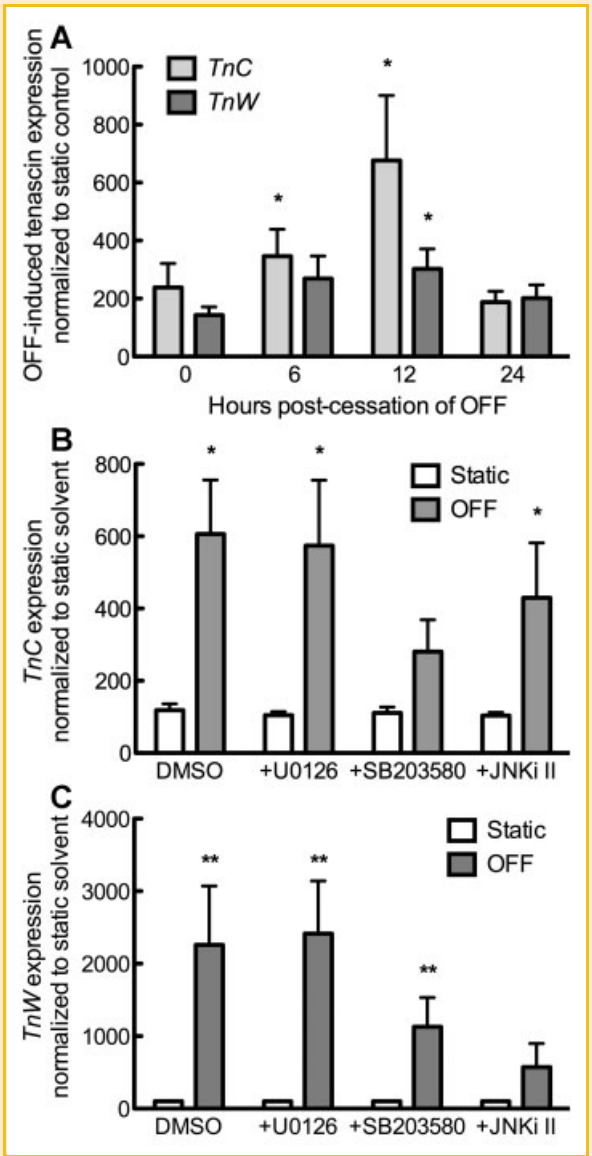


Fig. 7. Mechanical load induces tenascin expression. A: *TnC* and *TnW* induced after a 2-h exposure to 15 dyn/cm$^2$ oscillatory fluid flow. $^{*}P < 0.05$ compared to time-matched static control. All data are first normalized to the control gene *Rpl13* and then to the time-matched static control. *TnC* (B) and *TnW* (C) induction 12 h post-exposure to 2 h of oscillatory fluid flow in the presence of inhibitors for the MAP kinases MEK1/2 (U0126, 10 μM), p38 (SB203580, 10 μM), and JNK (JNKi II, 10 μM). $^{*}P < 0.05$ compared to solvent-matched static. All data are normalized to control gene *Rpl13*, then to the solvent-matched control. Bars represent mean ± SEM, SEM, n ≥ 5.

of Smads in tenascin W expression has yet to be examined. However, that BMP-2-induced *TnW* expression required de novo protein synthesis (detailed below) suggests that Smads do not directly induce *TnW* transcription. While the role of Smad signaling in tenascin expression is an important area of future research, this study highlights the contribution of MAPK signaling in non-canonical BMP regulation of tenascin expression in osteoblastic cells.

Wnts are also critical regulators of bone density. Both canonical Wnt co-receptors Lrp5/6 and the non-canonical Wnt co-receptor Ror2 regulate bone mass [Gong et al., 2001; Liu et al., 2007]. Thus, we were interested in their role in the regulation of *TnC* and *TnW*. Both canonical (Wnt3a) and non-canonical (Wnt5a) Wnts increased *TnC* and *TnW* (Fig. 4A–C), although induction of expression was more rapid (6 h vs. 24 h) and greater in Wnt5a-treated cells versus Wnt3a-treated cells. Similar to BMP-2, Wnt5a induces p38 activation (Fig. 5A), which is a critical step in Wnt5a-induced *TnW* expression, as inhibition of p38 abolished the Wnt5a induction of *TnW*. Conversely, Wnt5a-induction of *TnC* was independent of MAPK signaling (Fig. 5). Wnt5a stimulates PLC through a G-protein-dependent mechanism [Kühl et al., 2000]. We found that inhibition of PLC abolished the induction of *TnC* by Wnt5a (Fig. 6A). This suggests that while *TnW* is induced by Wnt5a through a p38-dependent mechanism, *TnC* induction requires activation of PLC. One down-stream transcription factor from this Wnt5a/PLC pathway, NFAT, was investigated, but inhibition of NFAT signaling did not block *TnC* induction (Fig. 6B). Thus, we believe *TnC* is induced by a PLC-dependent pathway that is independent of NFAT. One candidate is the transcription factor ATF2 which is activated by

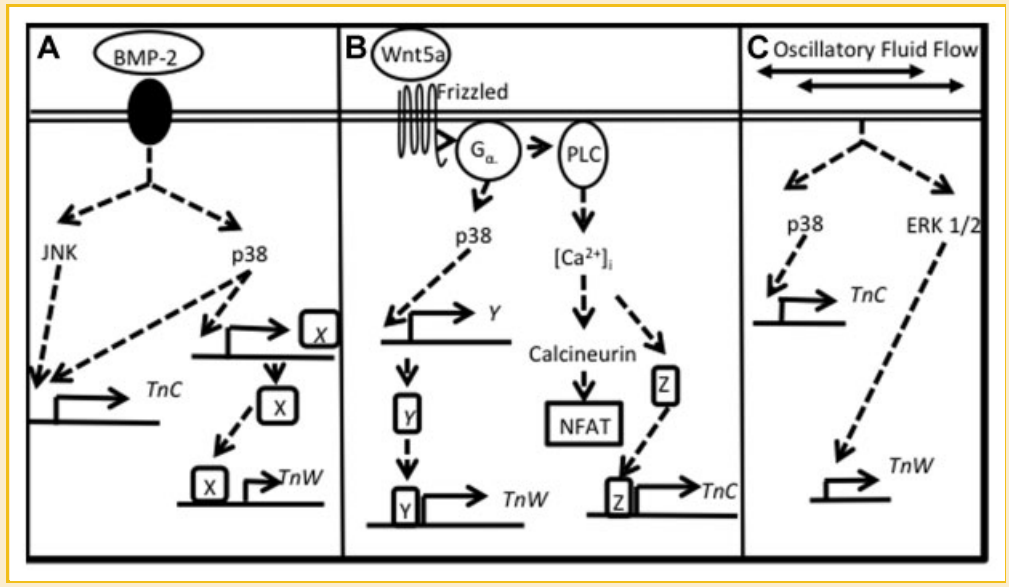


Fig. 8. Working model of osteoblast tenascin induction. A: BMP-2 induces both Smad and MAPK signaling which can drive tenascin expression. We found that p38 and JNK drive *TnC* expression. We propose p38 and Smads drive *TnW* expression through a protein intermediate, represented by X. B: Wnt5a induces *TnW* through p38 and requires a protein intermediate, represented here by Y. We believe that Wnt5a-induced *TnC* occurs through a PLC-dependent pathway independent from NFAT. C: Oscillatory fluid flow induces MAPK signaling, and JNK mediated the induction of *TnW*, while p38 mediated the *TnC* response.

Wnt5a [Ma and Wang, 2007] signaling and important in skeletal growth [Luvalle et al., 2003].

In order to target growth factor pathways to regulate tenascins, it would be important to understand if growth factor-induced tenascin expression is a direct effect of the growth hormone or mediated by a protein intermediate. The increases in *TnW* by either BMP-2 or Wnt5a was blocked by protein synthesis inhibitors, indicating that induction of *TnW* requires a protein intermediate rather than being a direct target of the growth factors. *TnC*, but not *TnW*, was induced by protein synthesis inhibitors alone (Figs. 2B and 4D). *TnC* super-induction in response to CHX was also observed in chick dermal fibroblasts [Chiquet et al., 2004; Jinnin et al., 2006]. *TnC* super-induction likely results from the absence of an inhibitory protein which normally functions to decrease *TnC* transcript; inhibition of protein synthesis would, in turn, prevent expression of this repressor, thereby inducing tenascin expression. Ghatnekar and Trojanowska [2008] recently demonstrated that constitutively expressed GATA-6 represses basal and TGF-$\beta_1$-induced *TnC* expression in fibroblasts. Our data suggests that *TnC* is regulated by a labile repressor, although its induction could still require protein synthesis. We did not observe enhanced transcript expression in the presence of growth factors and CHX, possibly because the induction in response to CHX was greater than to growth factor alone. Understanding endogenous inhibitors of tenascin expression could provide another promising target for intervention and induction of tenascin expression.

As the skeleton experiences constant mechanical loading in vivo it is important to understand the role of mechanical load on tenascin expression. Mechanical load could potentially modulate tenascin expression directly or through the local release of a variety of growth factors. *TnC* and *TnW* were both induced by the application of oscillatory fluid flow to simulate the shear stress experienced by bone cells during locomotion (Fig. 7A). These data support a role for fluid flow as a mechanism to explain previously reported increases in tenascin expression in loaded murine long bone [Webb et al., 1997]. Previous reports also support a role for mechanical regulation of *TnC* expression in fibroblasts and myocytes [Yamamoto et al., 1999; Fluck et al., 2000; Mikic et al., 2000; Chiquet et al., 2004]. To our knowledge this is the first report of mechanically induced *TnW* expression. Previous reports illustrate the capacity of shear stress to induce MAPK signaling in osteoblasts [You et al., 2001; Wu et al., 2006]. We found that p38 inhibition decreased the magnitude of mechanically induced *TnC* (Fig. 7B). A similar trend was observed in *TnW*, although it did not reach statistical significance (Fig. 7C). JNK also plays a role in mechanically induced *TnW* (Fig. 7C). These data support a differential role for the MAP kinases in creating distinct expression mechanisms for the two tenascins.

Consistent with our hypothesis that growth factors and local biophysical signals would regulate tenascin expression in osteoblasts, this study found that *TnC* and *TnW* were induced by BMP-2, Wnt5a, and oscillatory fluid flow. Figure 8 presents a working model for regulation of tenascin expression by these various stimuli. *TnC* and *TnW* were distinctly regulated by each stimulus, which is consistent with the overlapping but unique expression patterns observed in vivo. Further, Each isoform of tenascin had multiple signaling pathways capable of inducing its expression. The variety of signaling pathways that converge upon *TnC* and *TnW* expression supports the notion of a vital role for tenascins in the vertebrate skeleton. Future studies are needed to understand the role of tenascin in mediating some of the long-term effects of these stimuli on osteoblast behavior. Tenascins are known to induce osteoblast differentiation, but whether BMP-2-induced *TnC* is required for

BMP-2-driven osteogenesis has yet to be investigated. Based on the findings of this study we can conclude that these physiologic stimuli regularly adjust tenascin expression. Future studies are needed to examine the role of these stimuli in tenascin regulation in vivo as well as the clinical implications of this increase in tenascin expression.

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