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Characterization of the Role Nuclear Bmp2 (nBmp2) Plays in Regulating Gene Expression

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ABSTRACT

Characterization of the Role Nuclear Bmp2 (nBmp2) Plays
in Regulating Gene Expression

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The nBmp2 protein was first identified in a DNA affinity chromatography/mass spectrometry screen designed to detect proteins that interact with a cartilage-specific enhancer element (called D/E) from the type XI collagen gene *Col11a2*. The transcription factor SOX9, a protein from the Sox (SRY-related HMG box) family, binds to and activates gene expression from this enhancer. nBmp2 has no transcriptional activity of its own on this enhancer, but when co-transfected with SOX9 it increases SOX9's activation of D/E nearly 2-fold. SOX9 also activates cartilage-specific enhancer elements from the *Col2a1*, *Col27a1*, and *Col9a1* genes. The purpose of this project was to determine 1) whether nBmp2 similarly effects SOX9-dependent expression from these enhancers, and 2) whether it does so by binding (either directly or indirectly) to the *Col2a1*, *Col27a1*, and *Col9a1* enhancers. The work described in this thesis has shown that nBmp2 increases luciferase levels produced from three enhancer/reporter plasmids, but it does so without binding directly to the enhancers. This work has opened up a new area of exploration into the function of the novel protein nBmp2 to examine its potential effects on a variety of different gene regulatory processes.

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I am also grateful to Dr. Stark and Dr. Reynolds for all their help. I want to express special thanks to Elaine Rotz for her work. I couldn't have made it through without the support of the department and also the support of the BYU Cancer Research Center from their summer fellowships. Finally, I am grateful to my wonderful family who helped me never give up on my dreams and goals by encouraging me every day.

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Abbreviations

AMH	Anti-Mullerian hormone
Bmp2	Bone Morphogenetic Protein 2
Bmp4	Bone Morphogenetic Protein 4
Col9	The type XI collagen gene
CD	Campomelic dysplasia
CD-RAP	Cartilage-derived retinoic acid sensitive protein
cNLS	Classical Nuclear Localization Signal
D-MEM F-12	Dulbecco's modified Eagle medium nutrient mixture F-12 HAM
DBT	DNA binding buffer
EMSA	Electrophoretic Mobility Shift Assay
HEK 293T	Human embryonic kidney cells
HMG	High mobility group
KCL	Potassium Chloride
nBmp2	Nuclear Bone Morphogenetic Protein 2
nBmp4	Nuclear Bone Morphogenetic Protein 4
NLS	Nuclear Localization Signal
NP-40	Tergitol ® Type NP-40
PMSF	Phenylmethanesulfonylfluoride
PKA	The cyclic AMP-dependent protein kinase (PKA)
SRY	Sex determining factor
Sox	SRY-related HMG box
TGF-β	Transforming growth factor beta

Introduction

nBmp2

Bone morphogenetic proteins (BMPs) are secreted signaling molecules of the transforming growth factor beta (TGF beta) superfamily (Ducy and Karsenty 2000; Mishina 2003). They were first identified as proteins that play a central role in developmental processes, especially in cartilage and bone formation. More than thirty BMP family members are acknowledged to date. Bmp2 is involved in bone formation (Wozney, Rosen et al. 1988; Hogan 1996; Ducy and Karsenty 2000; Lories and Luyten 2005). Bmp2 also plays a critical role in cardiac cushion formation and in the epithelial-mesenchymal transition (Ducy and Karsenty 2000) and is involved in heart and myocardial patterning (Zhang and Bradley 1996; Ma, Lu et al. 2005). BMP2 is also involved in neural development (Goldstein, Brewer et al. 2005), limb patterning (Schlange, Arnold et al. 2002) and induction of osteoblast differentiation (Reddi, Muthukumaran et al. 1989; Luyten, Cunningham et al. 1992) .

Recently in Dr. Bridgewater's laboratory, Jenny Felin and others demonstrated the presence of a nuclear form of Bmp2 (nBmp2) in several different cell lines (Fig. 1) (Felin, Mayo et al. 2010). Previously, BMP2 was recognized only as a secreted growth factor derived from a pro-protein that undergoes cleavage to release the mature protein from the propeptide while it passes through the secretory pathway. Mutational analyses showed that translation of nBmp2 is initiated from a downstream alternative start codon to generate a protein that lacks the N-terminal signal peptide and is thus translated in the cytoplasm rather than the endoplasmic reticulum. This protein contains a nuclear

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localization signal (NLS) that overlaps the cleavage site, which directs translocation of the uncleaved nBmp2 to the nucleus (Fig.2) (Felin, Mayo et al. 2010).

SOX 9 transcription factor

Gene transcription is a complex process involving many elements that work together to appropriately activate and transcribe a gene. Transcription factors bind to DNA and interact with the promoter to regulate gene expression. Activators and repressors are specific transcription factors. They can activate or repress the transcription of individual genes (Nelson 2000).

Activators bind to enhancer or *cis*-acting elements and increase gene expression by inducing conformational changes. Enhancers have one or more consensus sequences to which activators can bind. These enhancer elements in DNA can be located upstream, downstream, or in a transcriptional entity (Nelson 2000). When the DNA is ready for transcription, the enhancer sequences become available for activator proteins to bind. These interactions between transcription factors, co-activators and the RNA Pol II complex initiate transcription (Carey 1999).

Most SOX proteins are transcription factors that are involved in pre-B and T cell development, sex determination, neural induction, and skeletal formation (Mertin, McDowall et al. 1999). SOX9 is one of the SOX proteins that regulates cartilage collagen (Col) genes by binding to the enhancer element regions of these genes (Lefebvre, Huang et al. 1997; Bridgewater, Lefebvre et al. 1998; Zhou, Lefebvre et al. 1998; Liu, Li et al. 2000; Lefebvre, Behringer et al. 2001; Bridgewater, Walker et al. 2003; Zhang, Jimenez

et al. 2003).

SOX9 also is expressed in brain and testis cells and activates the anti-Mullerian hormone (AMH) gene (Mertin, McDowall et al. 1999; Harley, Clarkson et al. 2003; Stolt, Lommes et al. 2003). Campomelic dysplasia (CD) is caused by haploinsufficiency of SOX9 protein in human. It is a fatal disease characterized by XY sex reversal and endochondral bone malformation (Huang, Zhou et al. 2000; Harley, Clarkson et al. 2003; Mori-Akiyama, Akiyama et al. 2003). Patients with CD have impaired gonadal and skeletal development because either the anti-Mullerian hormone gene or other genes that exist downstream of SOX9 are not sufficiently activated (Huang, Zhou et al. 2000).

As mentioned before, SOX9 is a member of Sox (SRY-related HMG box) superfamily, with high mobility group (HMG) domain that helps them to bind DNA. Sox proteins share >50% of similarity with the HMG domain of SRY (sex determining factor) (Harley, Clarkson et al. 2003). In SOX9 protein, the HMG domain has three α-helices that form a twisted L-shaped supported by a hydrophobic core. Sox9 protein binds to the minor groove of DNA by its consensus sequence which is heptameric (A/T)(A/T)CAA(A/T)G. After binding to DNA, it induces bending and conformational changes in the RNA Pol II complex, which causes unwinding of DNA and initiating transcription (Wegner 1999; Dailey and Basilico 2001). When the cyclic AMP-dependent protein kinase (PKA) phosphorylates *SOX9*, it increases SOX9's transcriptional activity (Huang, Zhou et al. 2000).

SOX9 protein, like SOX8 and SOX10 proteins, has an HMG domain, at least one transactivation domain, and a dimerization domain. Removing the PQS transactivation

domain of these proteins causes transcription reduction. The transcription activation

domain of the SOX9 is located at the C terminus of the protein. It cooperates directly or

indirectly with the Pol II complex. In addition to PQS domain, only SOX9 has another

domain, PQA. However, all these proteins, SOX9, SOX8 and SOX10, have dimerization

domains that help binding between these proteins forming DNA-dependent heterodimers

and homodimers (Sudbeck, Schmitz et al. 1996; Bernard, Tang et al. 2003; Sock, Pagon

et al. 2003).

Dimerization of SOX9 is required for its function as a transcriptional activator

protein in cartilage genes. However, SOX9 binds as a monomer in sex determination

gene regulation (Bernard, Tang et al. 2003). As mentioned before, dimerized SOX9

controls the regulation of some cartilage collagen genes (*Col2a1, Col11a2, Col9a2,*

Col27a1, and *CD-RAP*) by binding to the enhancer element regions of these genes. The

enhancer elements in these genes contain SOX9 sites paired in opposite orientation to

each other with three base pairs in between (Lefebvre, Huang et al. 1997; Bridgewater,

Lefebvre et al. 1998; Zhou, Lefebvre et al. 1998; Liu, Li et al. 2000; Lefebvre, Behringer

et al. 2001; Bridgewater, Walker et al. 2003; Zhang, Jimenez et al. 2003).

Cartilage-specific collagen genes and their enhancers

Type II collagen (*Col2a1*) is a homotrimeric molecule that contains three α1(II)

chains. It is the core component of cartilage collagen fibrils. Collagen fibrils are a

fundamental part of the interactions of proteins in the extracellular matrix of the cartilage.

Type II collagen, after post-translational modification, contacts other triple-helical

collagen genes (*Col9a1* and *Col11a1*) to form fibrils (Zhang et al., 2003).

In the published article where the 48-bp *Col2a1* cartilage-specific enhancer was first identified, the author identified SOX9 binding sites in a 48-base pair enhancer from the *Col2a1* gene (Lefebvre, Zhou et al. 1996). These sites are required for chondrocyte-specific enhancer activity in transgenic mice and transient transfections. Dimeric SOX9 binds at these paired sites that are directed in opposite orientation to each other with three base pairs in between. Mutations in any of the SOX9 sites inhibited chondrocyte-specific expression of reporter genes in transgenic mice and in transiently transfected cells. That shows that dimerized SOX9 plays an essential role in *Col2a1* gene activation (Zhou, Lefebvre et al. 1998; Dailey and Basilico 2001).

Type XXVII collagen (*Col27a1*) is a minor fibrillar collagen. It is highly expressed in cartilage and weakly expressed in the ear, eye, lung, and colon tissues (Cheah, Lau et al. 1991; Tsumaki, Kimura et al. 1996; Tsumaki, Sugimoto et al. 1996; Pace, Corrado et al. 2003). Jenkins et al. (2005) demonstrated that *Col27a1* has at least two similar enhancer elements that bind SOX9 at paired sites. Mutations in any of the sites or changing the spacing between the sites disabled enhancer function (Jenkins, Moss et al. 2005).

Type IX collagen is a heterotrimeric molecule like type II and type XI collagens. It is composed of the *Col9a1*, *Col9a2*, and *Col9a3* gene products. Its function in the inner part of the fibril is to limit the fibril diameter as well as stabilize cartilage collagen fibrils. An enhancer from the *Col9a1 gene* was identified, which contains four separate SOX9-binding sites. The sites are arranged as two pairs with each pair resembling those in the *Col27a1* and *Col2a1* enhancers, but in this case, all four sites are required for enhancer

activity. All four sites bind SOX9, and mutation of any one of the four sites abolished enhancer activity (Zhang, Jimenez et al. 2003; Genzer and Bridgewater 2007).

Rationale for this project

The nBmp2 protein was first identified in a DNA affinity chromatography/mass spectrometry screen designed to detect proteins that interact with a cartilage-specific enhancer element (called D/E) from the type XI collagen gene *Col11a2*. The transcription factor SOX9 binds to and activates gene expression from this enhancer. Another student in our lab, Brandt Nichols, recently performed reporter assays which demonstrated that although nBmp2 has no transcriptional activity of its own, when co-transfected with SOX9, it increases SOX9's activation of the D/E enhancer, and of two other *Col11a2* enhancers, as much as 2-fold. Since SOX9 also activates cartilage-specific enhancer elements from the *Col2a1*, *Col27a1,* and *Col9a1* genes, this project focused on those enhancers. The purpose of this project was to determine 1) whether nBmp2 similarly effects SOX9-dependent expression from these enhancers, and 2) whether it does so by binding directly to the *Col2a1*, *Col27a1*, and *Col9a1* enhancers.

The results show that nBmp2 increases SOX9-regulated reporter gene expression from the *Col27a1* 27C/D/E and 27F/G enhancers and the *Col9a1* M1/M2/D1/D2 enhancer. It does not, however, act as a transcription activator by itself.

Electrophoretic Mobility Shift Assay (EMSA) experiments revealed that even though nBMP2 increases SOX9-dependent gene expression, it does not bind directly to the enhancer DNA sequences. It is possible, then, that nBmp2 increases reporter gene

expression by binding indirectly to SOX9 or perhaps by post-transcriptional mechanisms such as stabilizing mRNA or inhibiting protein degradation.

Methods

Cell types and culture

Human embryonic kidney (HEK293) cells were cultured at 37°C with 5% CO_2 in supplemented Dulbecco's modified Eagle medium nutrient mixture F-12 HAM (D-MEM F-12) (Gibco). Supplemented medium contained 10% Fetal Bovine Serum (FBS)(HyClone). Cells were passaged at approximately 90% confluence by using (0.05%) trypsin (HyClone) every 3-4 days but not used after passage 25.

Plasmids

The reporter plasmids were all constructed and described previously (Jenkins, E., J. B. Moss, et al. 200; Genzer, M. A. and L. C. Bridgewater 2007; Zhou, G., V. Lefebvre, et al. 1998). In every case, the enhancer reporter constructs included four tandem copies of the enhancer of interest (see sequences in Table 1) upstream of a minimal promoter that was derived from the *Col2a1* gene but that has no transcriptional activity in the absence of an enhancer element. These elements controlled a firefly luciferase reporter plasmid. The SOX9-pCDNA-5'-UT plasmid was constructed in the lab of Dr. Benoit de Crombrugghe and has been described previously (Zhou, G., V. Lefebvre, et al. 1998). The nBmp2 expression plasmid was constructed for this study by cloning a 5' truncation of the Bmp2 cDNA into the expression vector pcDNA3.1(+). The 5' truncation removed all sequence upstream of the alternative start codon that produces nBmp2, so the construct is not capable of producing secreted Bmp2. The internal control for

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transfection efficiency, pRL, expresses renilla luciferase constitutively and was purchased from Promega.

Enhancer/reporter assays

Transfections were performed in duplicate or triplicate. Briefly, $\sim$ 300,000 cells were seeded to each well of 6-well plates and incubated at 37°C with 5% CO_2 in supplemented Dulbecco's modified Eagle medium nutrient mixture F-12 HAM and grown to a confluence of at least 70%. Then 2µg of plasmid DNA (enhancer/firefly luciferase reporter plasmid (1µg), pRL4 constitutive renilla luciferase expression vector as an internal control for transfection efficiency (0.33 µg), the SOX9 expression vector SOX9-pcDNA-5'-UT (0 or 0.33 µg), the nBmp2 expression plasmid nBMP2-pcDNA-5'-UT (0 or 0.33 µg), and/or the empty vector pcDNA3.1 (0, 0.33 or 0.66 µg) was added to 100 µL OPTI-MEM® I plus 6 µL PLUS reagent and allowed to form complexes for 15 minutes at room temperature.

Meanwhile, 100 µL OPTI-MEM® I plus 4 µL Lipofectamine[TM] were mixed for each sample. After the incubation, diluted Lipofectamine was added to the DNA/PLUS complexes and incubated for 15 minutes at room temperature. During this incubation, the old medium was removed from each well and 2 ml Opti-MEM was added into each well. Finally, the DNA/PLUS/Lipofectamine complexes were added dropwise to the cells and mixed gently. Then, after 6 hours, 200µL (100%) FBS was added to a final concentration of 10% FBS.   Transfected cells were incubated at 37°C with 5% CO_2 for 20-24 hours and then again the old medium was removed from each well and fresh 2ml 10% FBS

media was added to the wells. Transfected cells were allowed to grow for a total of 40-48 hours before being analyzed.

Cell extracts were prepared 40-48 h after the transfection, and enzyme activity assays were performed. First, cells were rinsed (1x) with 2mL 1xPBS (room temperature) and then the Dual Luciferase Assay System (Promega) was used to measure the firefly and Renilla luciferase activity, according to the manufacture's instruction.

Briefly, 100 µL Luciferase Assay Reagent as a substrate was combined with 20 µL cell extract. Results were measured in a TD 20/20 luminometer (Turner Designs). Then in a short period (5 seconds) 100 µL renilla luciferase substrate was added into the mixture. Again, results were measured in a TD 20/20 luminometer (Turner Designs). Finally, all results were calculated as firefly luciferase (x100.000)/ renilla luciferas and data were normalized to 100% activity for reporter plasmid with SOX9 alone. Student's *t*-test was used to analyze for statistical significance All graphs presented include data from 4 or 5 independent experiments, each performed in duplicate or triplicate.

In Vitro Transcription/Translation

SOX9 and nBmp2 for EMSA experiments were synthesized by *in vitro* transcription/translation of the SOX9-pcDNA-5'-UT SOX9 expression plasmid and the nBmp2-pcDNA3.1 expression plasmids using the TNT Coupled Reticulocyte Lysate System (Promega). Reticulocyte Lysate (40 µl), expression plasmid (0.5 µg), methionine, and water to a total volume of 50 µl were mixed in a single tube and incubated at $30^{o}C$ for 90 min. The negative control did not have DNA template in the mixture. Control

reactions were prepared as described with the substitution of ^{35}S-methionine for methionine with either DNA template for SOX9 or nBmp2 or no DNA template. Products from reaction containing ^{35}S-methionine were separated by SDS-PAGE (10%) and visualized using autoradiography in order to verify protein production and correct product size (Fig. 8).

Electrophoretic mobility shift assays (EMSA)

EMSA experiments were performed using the *Col9a1 (M1/M2/D1/D2)* and *Col27a1* (27C/D/E and 27F/G) enhancer elements as DNA probes (for sequences, see Table 1). 5' GATC overhangs of each double-stranded oligonucleotide were ^{32}P radiolabeled by end filling with Klenow (Promega) fragment using a ^{32} P-dGTP and dNTP mix (no dGTP). For binding reaction, 1 μL *in vitro* transcribed and translated SOX9 and/or nBmp2 protein and 1 μL radiolabeled probe were combined in 25 μL DNA-binding buffer (20mM Hepes (pH 7.9), 10% glycerol, 50mM KCL, 0.05% NP-40, 0.5 mM DTT and 1 mM PMSF). 0.1 μg of poly (dG-dC). poly(dG-dC) was added as a nonspecific competitor. The mix was incubated with or without SOX9/nBmp2 antibody at room temperature for 30 min. If antibody was included, it was pre-incubated with the proteins and other components for 30 min before the addition of radiolabeled probes. Binding reactions were separated by electrophoresis for 2-3h at 150V on native 8% polyacrylamide gels. Then the gels were dried for 45min at -80 ^{o}C using a Hoefer Slab Gel Dryer. Results were visualized by autoradiography.

Statistical Analysis

Triplicate transient transfection results were averaged and then the averaged results were normalized to 100% for the activity produced by SOX9 alone. The normalized independent repeats were then averaged together and statistical significance was determined using Student's *t*- test.

Results

nBmp2 increases SOX9-dependent reporter gene expression.

To determine whether nBmp2 effects *SOX9*-dependent expression from the *Col27a1*

(27C/D/E and 27F/G)*, Col9a1* (M1/M2/D1/D2)*,* and *Col2a1* (48-bp) enhancers, the

enhancer/reporter assay was performed. The results show that nBmp2 alone cannot activate the

transcription from the *Col27a1, Col9a1* or *Col2a1* enhancers. However, it cooperates with *SOX9*

to further increases transcription from the *Col27a1 (27C/D/E and 27F/G) and Col9a1*

(M1/M2/D1/D2) enhancer elements (Figure 4-7).

It was interesting to see that *nBmp2* activates the unusual *Col9a1* enhancer with

its four SOX9-binding sites. In figure 4 it shows that nBmp2 with cooperation of SOX9

in *Col9a1* (M1/M2/D1/D2) enhancer element significantly increases the transcription ~16

$\pm$ 6% SE (p=0.018) (Fig.4). The nBmp2 protein significantly increased SOX9-dependent

expression from the *Col27a1* (27C/D/E) enhancer element by ~14 $\pm$ 5% SE (p= 0.021)

(Fig.5). The nBmp2 protein also significantly increased SOX9-dependent expression

from the *Col27a1* (27F/G) enhancer element by ~27 $\pm$ 7% SE (p= 0.007) (Fig.6). In

contrast, nBmp2 did not significantly increased SOX9-dependent expression from the

Col2a1 (48-bp) enhancer element (p=0.189) (Fig.7).

nBmp2 does not bind directly to enhancer elements in EMSA experiments.

Having demonstrated that nBmp2 increases SOX9-dependent gene expression

from the *Col27a1* C/D/E and F/G and the *Col9a1* M1/M2/D1/D2 enhancers, we

performed EMSA experiments to see if nBmp2 binds along with SOX9 to these enhancers. The sequences of the enhancer element probes are shown in Table 1.

SOX9 bound each enhancer as has been previously shown and produced a mobility shift (Figures 9, 10 and 11, arrows) (Zhou et al., 1998; Dailey, Basilico, 2001; Jenkins et al., 2005; Zhang et al., 2003; Genzer, Bridgewater, 2007). The nBmp2 protein alone did not shift any of the enhancers, which was not surprising since it also did not independently increase their activity in the reporter assays. It was surprising, however, that when nBmp2 and SOX9 were co-incubated with the enhancers, the shift produced was identical to that caused by SOX9 alone, indicating that nBmp2 does not bind any of the enhancer elements, even in cooperation with SOX9 (Figures 9-11).

Discussion

The SOX9-binding enhancer elements from the *Col27a1*, *Col2a1*, and *Col9a1* genes were used in this project to determine whether nBmp2 plays a role in regulating the expression of other SOX9-responsive enhancer elements besides the ones from *Col11a2*. I determined that nBmp2 increases reporter gene expression when co-transfected with SOX9 in enhancer/reporter assays with the 27C/D/E and 27F/G enhancers from the *Col27a1* gene and M1/M2/D1/D2 enhancer from the *Col9a1* gene. It does not, however, act as a transcription activator by itself. EMSA experiments were then used to determine whether nBmp2 exerts its effect on gene expression by binding to the enhancer elements. I expected that if nBmp2 does function as a traditional transcription factor or co-activator, it would bind to these enhancer elements either independently or in cooperation with SOX9. Surprisingly, the EMSA experiments reveal that nBMP2 does not bind to the enhancer DNA sequences.

The nBmp2 protein clearly increased luciferase expression in the reporter assays, so its failure to bind to the enhancers like a transcriptional activator or co-activator suggests that it has post-transcriptional functions. Some possibilities that could account for nBmp2's ability to increase luciferase levels in our reporter assays include stabilization of SOX9 mRNA or luciferase mRNA. A global stabilization of all mRNAs is a less likely explanation because all results were normalized to the pRL4 renilla luciferase control plasmid.

nBmp2 might also function by slowing degradation of SOX9 or luciferase proteins. A yeast two-hybrid screen that was performed to obtain clues about nBmp2's function by

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identifying protein binding partners found that nBmp2 binds to ROC1, an E3 ubiquitin ligase protein (unpublished data). ROC1 has several known targets including the cell cycle inhibitory proteins p21 and p27, which it marks for degradation by the proteasome pathway. Inactivation of ROC1 in a mouse model caused accumulation of p27, which inhibited cell proliferation and caused embryonic death, demonstrating the critical role that ROC1 plays in protein degradation (Jia L. et al 2009; Tsvetkov D. et al). Perhaps nBmp2's effect on reporter gene expression in our assays is caused by nBmp2's interactions with ROC1 or with other proteins in the ubiquitin/proteasome pathway.

Finally, it is possible that nBmp2 forms a complex with SOX9 that is not strong enough to remain bound during the EMSA assay. It could participate in a complex something like SAGA (Spt-Ada-Gcn5-acetyltrasferase), which activates transcription and includes both a histone acetyltransferase and a ubiquitin protease. (Weake, Dyer et al. 2011).

In summary, the work described in this thesis has shown that nBmp2 increases luciferase levels produced from three enhancer/reporter plasmids, but it does so without binding to the enhancers. This work has opened up a whole new area of exploration into the function of the novel protein nBmp2 to examine its potential effects on a variety of different post-transcriptional regulatory processes.

Table 1

Enhancer element sequence regions with SOX9 binding sites

Element	Sequence
M1/M2/D1/D2	agtgggcacatttttactggaacctca[gccctCTGAAAGcttcCACTGTAttcctata gcagttCTGAAAGctgcCATTGTActcct]atagcagat
27C/D/E	ctccgtTTGTGTAattaAACAAAGctgCCTTTTATttctctccagggctc
27F/G	aaagacttgaagaaaaccTTCTCTGtccCTTTGAAatgctttcccagggt
48-bp	ctgtgaatcgggCTCTGTAtgcgcttgAGAAAAGcccCATTCATgaga

*brackets indicate the boundaries of the M1/M2/D1/D2 EMSA probe
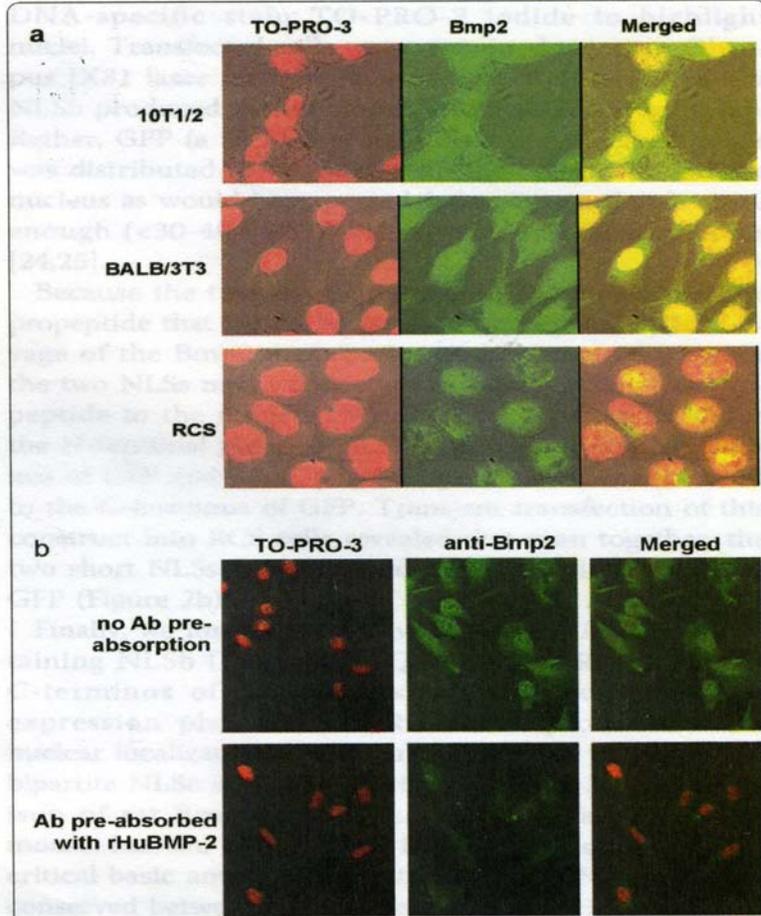
Figure 1. Endogenous Bmp2 is evident in the nuclei of three cell lines. A) 10T1/2 mesenchymal cells, BALB/3T3 fibroblast cells, and RCS cells were cultured on slides and then immunostained using an anti-Bmp2 (green) antibody. TO-PRO-3 (red) was used to stain the nuclei of these cells. Then the cells were examined by laser confocal microscopy. B) Preabsorption of anti-Bmp2 antibody with recombinant human Bmp2 verified the specificity of the Bmp2 antibody.
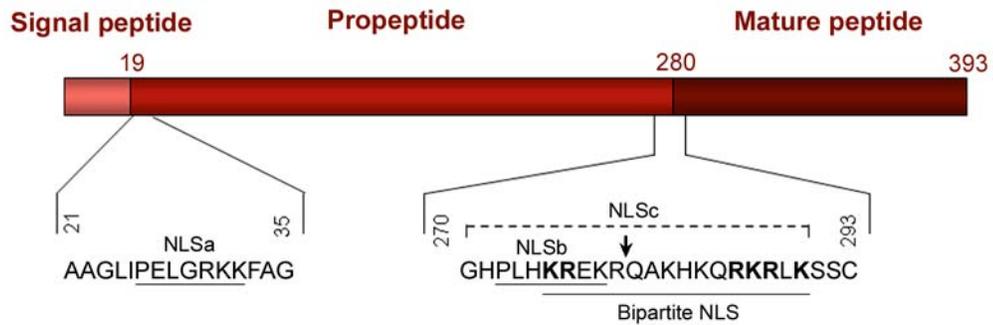
Figure 2. Schematic of the Bmp2 preproprotein. The rat Bmp2 preproprotein map shows the signal peptide, propeptide, and mature chain. The arrow shows the location of proteolytic cleavage to release the mature secreted growth factor. The bold bases indicate the important amino acids in the bipartite NLS (Felin, Mayo et al. 2010).
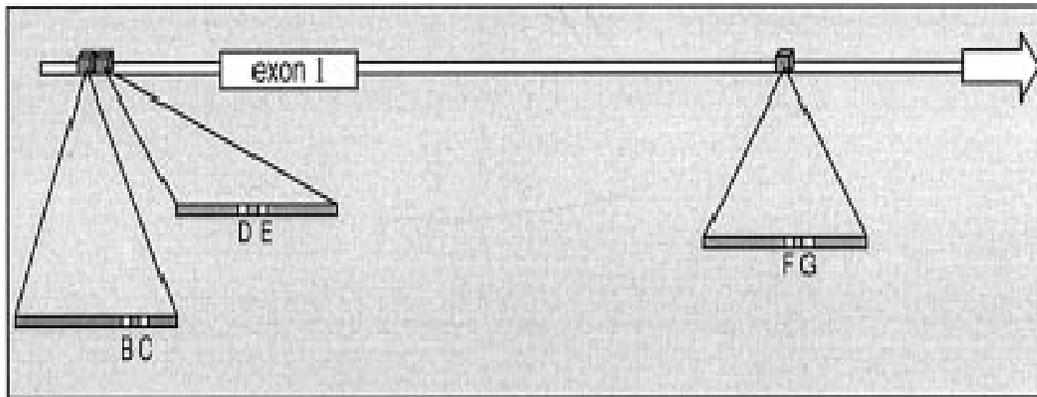
Figure 3. Schematic diagram of the 5' region of mouse *Col11a2*. The B/C and D/E enhancers in the upstream promoter region and the F/G enhancer in the first intron are represented as boxes, with the expanded views of each enhancer showing approximate locations of two heptameric Sox-binding sites. (This figure was originally published by Bridgewater et al. (Bridgewater, Walker et al. 2003)).
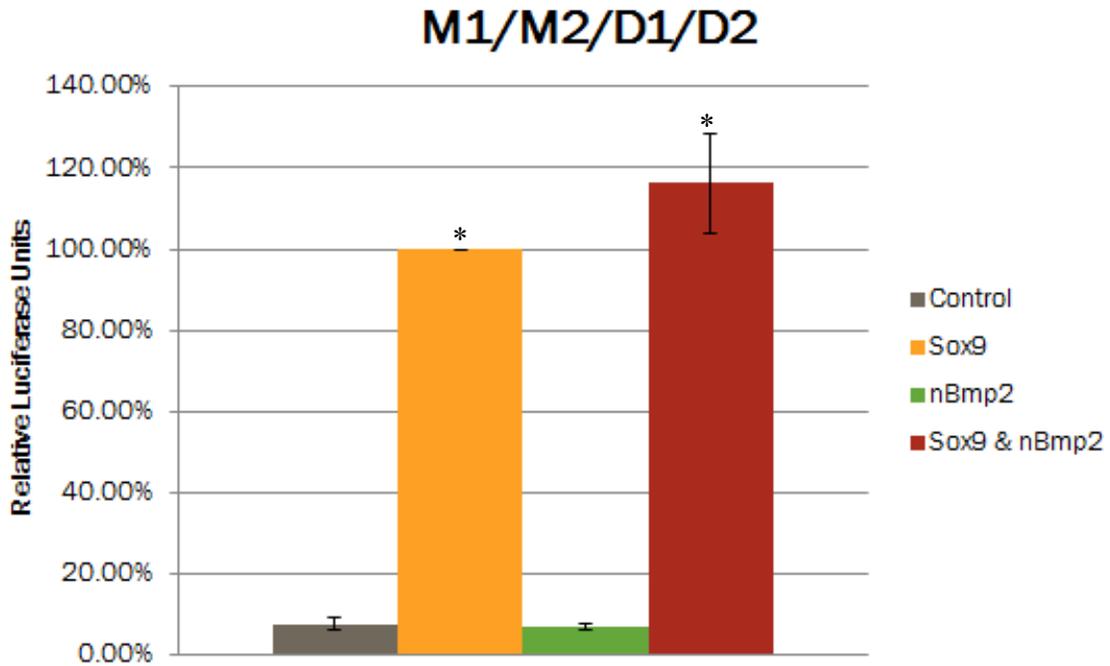
Figure 4. nBmp2 increases SOX9 activation of the *Col9a1* M1/M2/D1/D2 enhancer element. The M1/M2/D1/D2 enhancer/reporter plasmid was transfected into HEK293 cells in combination with Sox9 and/or nBmp2 expression plasmids as shown. The pRL4 renilla luciferase expression plasmid was included in each reaction as an internal control for transfection efficiency. This figure includes data from five independent experiments, each performed in duplicate or triplicate. The results of each independent experiment were normalized to 100% activity for Sox9 (yellow). The control included reporter plasmid with empty pcDNA3.1 vector, without Sox9 or nBmp2 (gray). *(p=0.018)
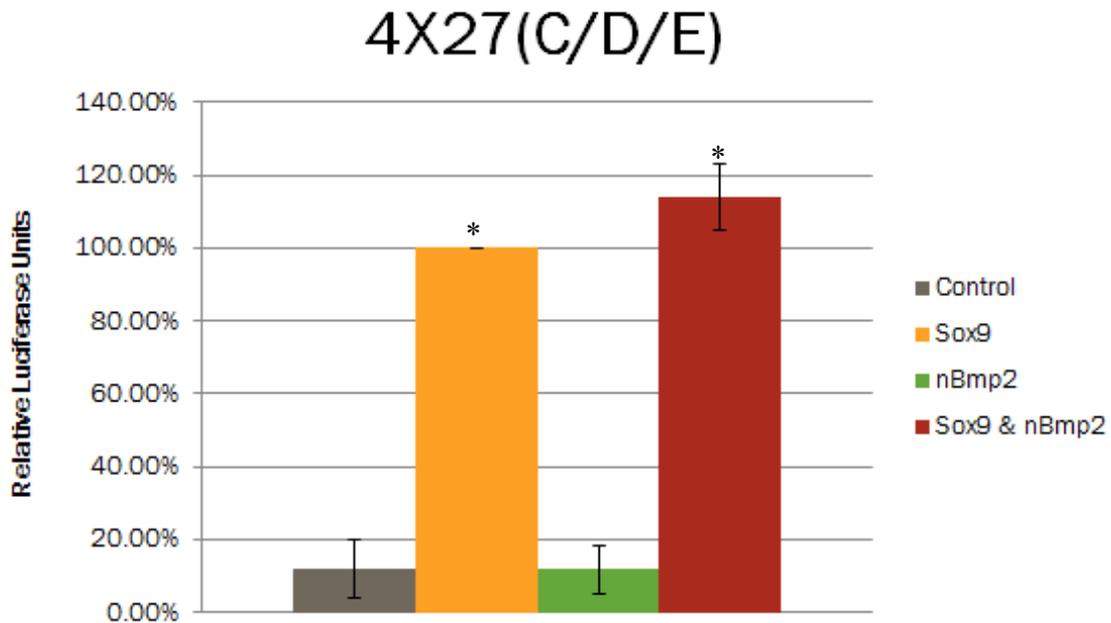
Figure 5. nBmp2 increases SOX9 activation of the *Col27a1* C/D/E enhancer element. The 4x27(C/D/E) enhancer/reporter plasmid was transfected into HEK293 cells in combination with Sox9 and/or nBmp2 expression plasmids as shown. The pRL4 renilla luciferase expression plasmid was included in each reaction as an internal control for transfection efficiency. This figure includes data from six independent experiments, each performed in duplicate or triplicate. The results of each independent experiment were normalized to 100% activity for Sox9 (yellow). The control included reporter plasmid with empty pcDNA3.1 vector, without Sox9 or nBmp2 (gray). *(p= 0.021)

22
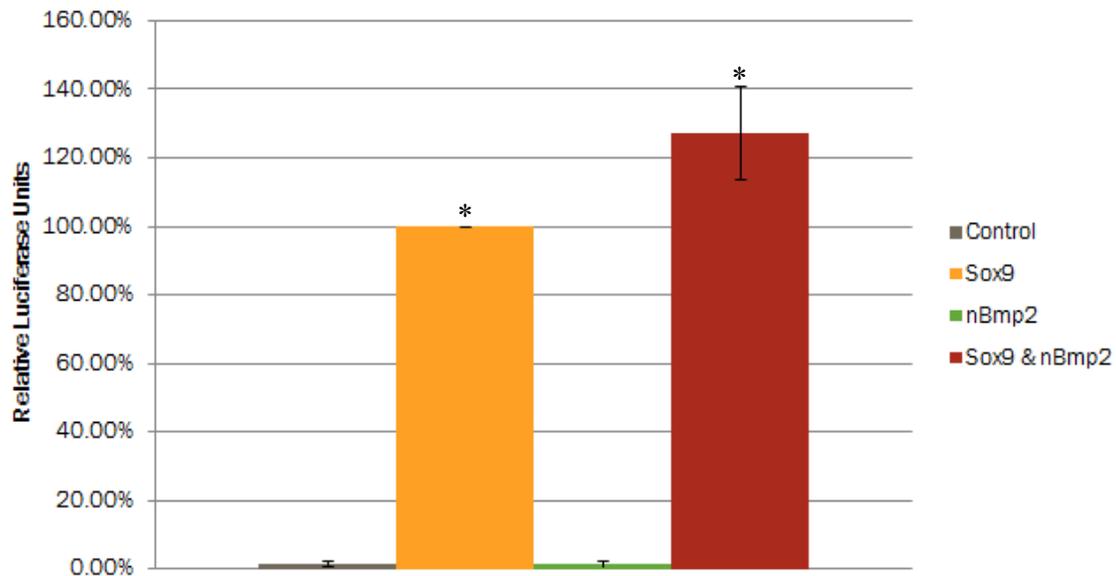
Figure 6. nBmp2 increases SOX9 activation of the *Col27a1* F/G enhancer element. The 4x27(F/G) enhancer/reporter plasmid was transfected into HEK293 cells in combination with Sox9 and/or nBmp2 expression plasmids as shown. The pRL4 renilla luciferase expression plasmid was included in each reaction as an internal control for transfection efficiency. This figure includes data from four independent experiments, each performed in duplicate or triplicate. The results of each independent experiment were normalized to 100% activity for Sox9 (yellow). The control included reporter plasmid with empty pcDNA3.1 vector, without Sox9 or nBmp2 (gray). *(p= 0.007)
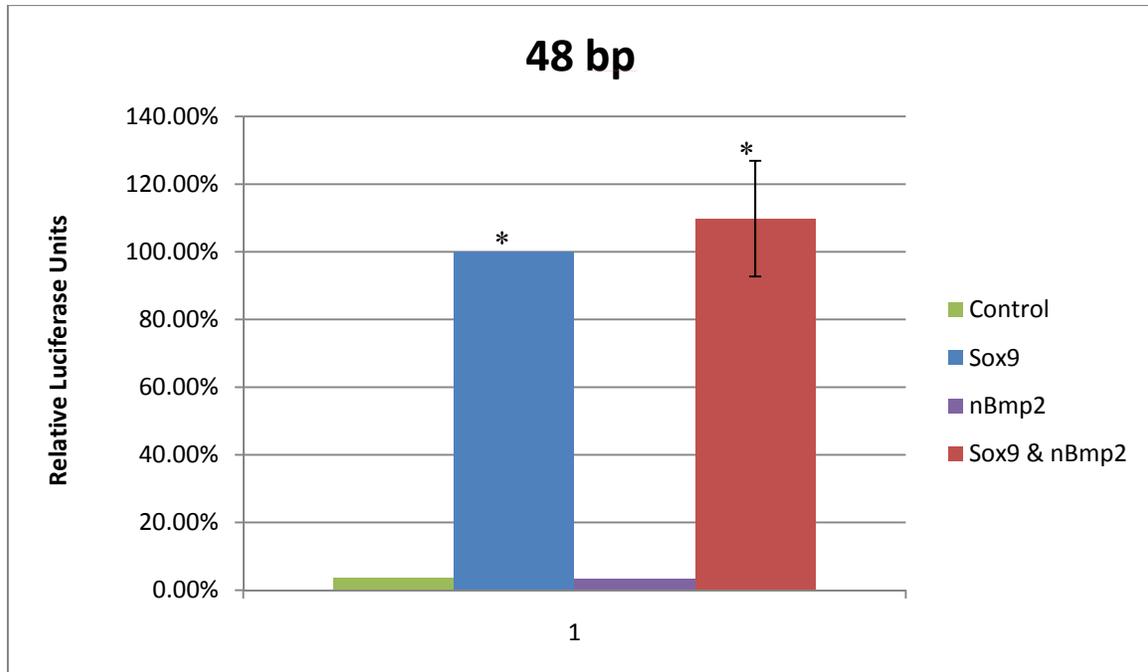
Figure 7. nBmp2 has no effect on SOX9 activation of the *Col2a1* 48-bp enhancer element. The 4x48-bp enhancer/reporter plasmid was transfected into HEK293 cells in combination with Sox9 and/or nBmp2 expression plasmids as shown. The pRL4 renilla luciferase expression plasmid was included in each reaction as an internal control for transfection efficiency. This figure includes data from six independent experiments, each performed in duplicate or triplicate. The results of each independent experiment were normalized to 100% activity for Sox9 (blue). The control included reporter plasmid with empty pcDNA3.1 vector, rather than Sox9 or nBmp2 (green). *(p=0.189)
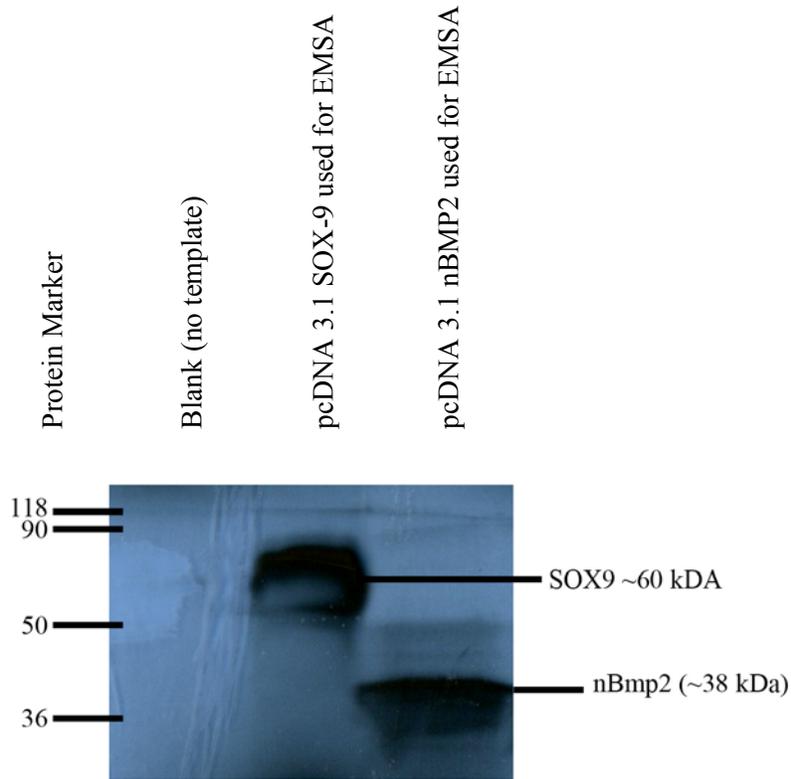
Figure 8. *In vitro* transcription/translation of the SOX9 and nBmp2. Proteins for EMSAs were synthesized in vitro from the SOX9-pcDNA-5'-UT SOX9 expression plasmid and the nBmp2-pcDNA3.1 expression plasmids using the TNT Coupled Reticulocyte Lysate System (Promega). Radiolabeled methionine was incorporated into a small parallel reaction with each plasmid to confirm protein synthesis by visualization of the protein after SDS-PAGE separation. Both SOX9 (~60 kDa) and nBmp2 (~38 kDa) were synthesized successfully.

Sox 9 Ab:			+				+	
BMP2 Ab:					+			+
Protein:	blank	SOX9	SOX9	nBMP2	nBMP2	SOX9+nBMP2	SOX9+nBMP2	SOX9+nBMP2
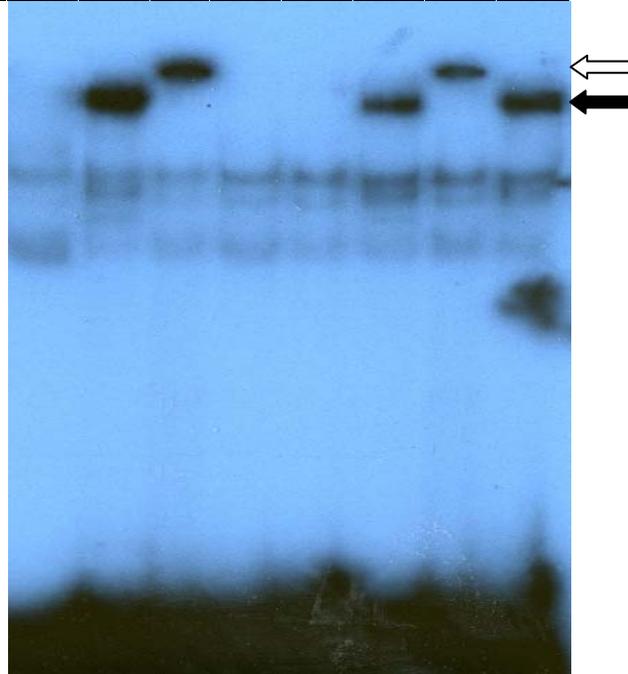
Figure 9. nBmp2 does not bind to the *Col9a1* M1/M2/D1/D2 enhancer in EMSA. The *Col9a1* M1/M2/D1/D2 enhancer was radiolabeled by end-filling and incubated with proteins as indicated. Incubation with SOX9 produced a shifted band (black arrow) that was supershifted by addition of the anti-SOX9 antibody (white arrow). Incubation with nBmp2, however, produced no shifted band. Incubation of both SOX9 and nBmp2 with the enhancer produced the same DNA-protein complexes that formed with SOX9 alone, indicating that nBmp2 does not bind to the M1/M2/D1/D2 enhancer.

	blank	SOX9	SOX9	nBMP2	nBMP2	SOX9+nBMP2	SOX9+nBMP2	SOX9+nBMP2
Sox 9 Ab:			+				+	
BMP2 Ab:					+			+
Protein:								

Figure 10. nBmp2 does not bind to the *Col27a1* 27(C/D/E) enhancer in EMSA. The *Col27a1* 27(C/D/E) enhancer was radiolabeled by end-filling and incubated with proteins as indicated. Incubation with SOX9 produced a shifted band (black arrow) that was supershifted by addition of the anti-SOX9 antibody (white arrow). Incubation with nBmp2, however, produced no shifted band. Incubation of both SOX9 and nBmp2 with the enhancer produced the same DNA-protein complexes that formed with SOX9 alone, indicating that nBmp2 does not bind to the 27(C/D/E) enhancer.

Sox 9 Ab:			+				+	
BMP2 Ab:					+			+
Protein:	blank	SOX9	SOX9	nBMP2	nBMP2	SOX9+nBMP2	SOX9+nBMP2	SOX9+nBMP2
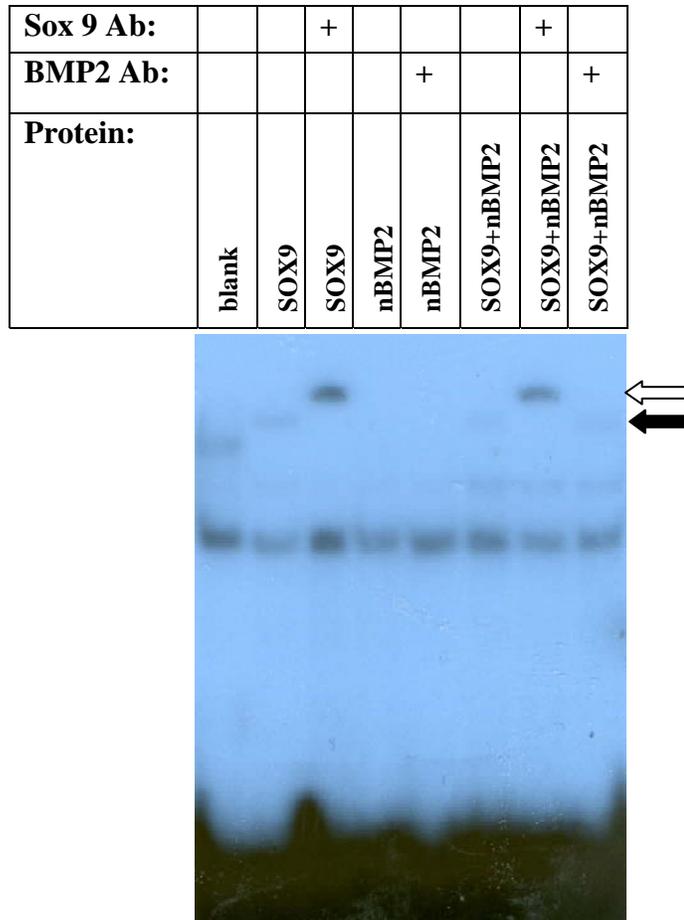
Figure 11. nBmp2 does not bind to the *Col27a1* F/G enhancers in EMSA.
The *Col27a1* 27(F/G) enhancer was radiolabeled by end-filling and incubated with proteins as indicated. Incubation with SOX9 produced a shifted band (black arrow) that was supershifted by inclusion of the anti-SOX9 antibody (white arrow). Incubation of the enhancer sequence with nBmp2, however, produced no shifted band. Incubation of both SOX9 and nBmp2 with the enhancer produced the same DNA-protein complexes that formed with SOX9 alone, indicating that nBmp2 does not bind to the 27(F/G) enhancer.

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