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An Autonomous BMP2 Regulatory Element in Mesenchymal Cells

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Abstract

BMP2 is a morphogen that controls mesenchymal cell differentiation and behavior. For example, BMP2 concentration controls the differentiation of mesenchymal precursors into myocytes, adipocytes, chondrocytes, and osteoblasts. Sequences within the 3'untranslated region (UTR) of the *Bmp2* mRNA mediate a post-transcriptional block of protein synthesis. Interaction of cell and developmental stage-specific trans-regulatory factors with the 3'UTR is a nimble and versatile mechanism for modulating this potent morphogen in different cell types. We show here, that an ultra-conserved sequence in the 3'UTR functions independently of promoter, coding region, and 3'UTR context in primary and immortalized tissue culture cells and in transgenic mice. Our findings indicate that the ultra-conserved sequence is an autonomously functioning post-transcriptional element that may be used to modulate the level of BMP2 and other proteins while retaining tissue specific regulatory elements.

Keywords

GENE REGULATION; POST-TRANSCRIPTIONAL; GROWTH FACTOR; PATHOLOGICAL CALCIFICATION; CORONARY VASCULATURE; AORTA

INTRODUCTION

BMP2 IS A MORPHOGEN THAT CONTROLS CELL DIFFERENTIATION AND BEHAVIOR

BMP2 concentration controls the differentiation of mesenchymal precursors into myocytes, adipocytes, chondrocytes, and osteoblasts [Wang et al., 1993; Katagiri et al., 1994; Denker et al., 1999; Sottile and Seuwen, 2000; Cheng et al., 2003; Rosen and MacDougald, 2006]. In the developing heart, BMP2 levels control cardiac mesoderm commitment and differentiation [Schlange et al., 2000; Schultheiss et al., 1997] and proepicardial identity [Kruithof et al., 2006; Schlueter et al., 2006]. As expected for a morphogen, BMP2 dosage is critical throughout fetal and post-natal development. BMP2-deficient mouse embryos die early in embryogenesis [Zhang and Bradley, 1996]. Conditionally null *Bmp2* alleles have shown that BMP2 is required in the developing heart at later stages and in post-natal bone [Ma et al., 2005; Rivera-Feliciano and Tabin, 2006; Tsuji et al., 2006]. Quantitative aspects of BMP2 function in key structures have been addressed using chimeras with *Bmp2* null embryonic stem cells and wild type blastocysts [Castranio and Mishina, 2009], compound heterozygotes of *Bmp2* and *Bmp4* null alleles [Uchimura et al., 2009], and a hypomorphic allele created by appending a neo cassette to the 3'UTR of the *Bmp2* mRNA [Singh et al., 2008]. Genetic background has been shown to influence the severity of defects resulting from BMP2 deficiency, most likely due to differences in *cis* and *trans* regulatory mechanisms that modulate BMP2 synthesis and to differences in downstream signaling [Castranio and Mishina, 2009; Uchimura et al., 2009]. Understanding the regulatory motifs that control the precise level of BMP2 is required to fully understand the diverse roles of this morphogen during embryogenesis.

Aberrant BMP2 levels and function also have been directly implicated in all forms of pathological calcification: atherosclerotic lesions, calcified cardiac valves, and calcified medial arteries [Bostrom et al., 1993; Mohler et al., 2001; Shao et al., 2005; Caira et al., 2006]. The abnormal signaling leading to calcification has been characterized as a reawakening of embryonic signaling paths [Lincoln et al., 2006; Srivastava, 2006] or as ectopic osteogenesis [Vattikuti and Towler, 2004; Hruska et al., 2005; Shao et al., 2006]. Procalcification conditions and signals such as high fat diet, metabolic changes associated with diabetes and other disorders, oxidative stress, mechanical stresses, and tumor necrosis factor (TNF) α , induce *BMP2* gene expression in diverse models of vascular disease [Cheng et al., 2003; Cola et al., 2004; Csiszar et al., 2005, 2006; Nett et al., 2006; Shao et al., 2006; Al-Aly et al., 2007]. Pathologically elevated BMP2 levels have been observed both in endothelial [Csiszar et al., 2005, 2006] and mesenchymal vascular cell types [Towler et al., 1998; Cheng et al., 2003; Shao et al., 2006]. Elucidating the roles of BMP2 in diseases including arthritis, cancer, and pathological calcification requires characterizing the mechanisms that modulate BMP2 levels in mesenchymal cells.

HUMAN BMP2 GENE VARIATION

The importance of sequence polymorphisms in controlling human morphological variation and the onset and progression of disease is profound. Not surprisingly, studies have begun to link variation in the *BMP2* gene and its regulatory elements to various human conditions: genetic hemochromatosis [Milet et al., 2007, 2010], bone quality and osteoporosis

[Styrkarsdottir et al., 2003; Reneland et al., 2005; Choi et al., 2006; McGuigan et al., 2007; Tranah et al., 2008], osteoarthritis [Valdes et al., 2004, 2006], and the human limb malformation, autosomal-dominant brachydactyly type A2 (BDA2, [Dathe et al., 2009]). Several of these examples involve tissues derived mainly from mesenchymal cell types whose differentiation is directly influenced by BMP2 concentration [Wang et al., 1993; Katagiri et al., 1994; Denker et al., 1999; Sottile and Seuwen, 2000; Cheng et al., 2003; Rosen and MacDougald, 2006].

A single nucleotide polymorphism (SNP) found in an osteoporosis-linked haplotype [Styrkarsdottir et al., 2003] alters an ultra-conserved sequence (83 to 446 nt downstream of the stop codon) within the *BMP2* 3'untranslated region (UTR) that has been conserved between mammals and fishes [Abrams et al., 2004]. This SNP (rs15705) was significantly associated with skeletal muscle, subcutaneous fat, and bone variation in a cohort of healthy young adults [Devaney et al., 2009]. The rs15705 SNP also altered the in vitro decay rates and specific protein affinities for RNAs bearing the ultra-conserved sequence [Fritz et al., 2006] and the expression of luciferase reporter genes [Devaney et al., 2009; Jiang et al., 2010b]. This SNP also would alter the hybridization of a microRNA (miR-568) to the *BMP2* mRNA. The ultra-conserved sequence and these *trans*-regulatory factors may control *BMP2* levels and thus influence embryogenesis and multifactorial diseases influenced by *BMP2*.

We previously used cell culture models and transgenic mice to show that the ultra-conserved sequence is a post-transcriptional regulatory switch that mediates *BMP2* down-regulation in normal lung cells and up-regulation in transformed lung cells [Jiang et al., 2010b]. We now demonstrate that the ultra-conserved sequence is an autonomously acting repressor that functions independently of promoter, coding sequence, and 3'UTR in several mesenchymal cell types in vitro and in vivo.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

C3H10T½ cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, D5796 Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were grown in 5% CO₂ at 37°C.

Mouse primary calvarial cells—Calvaria from 4-day-old mouse pups were digested in digestion solution (0.024 g of collagenase, 6 ml of 0.25% trypsin, 6 ml of PBS, 12 ml of DMEM) at 37°C, collected and pooled, then washed with complete medium (DMEM D6546, 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine) and cultured at 37°C in a humidified incubator supplied with 5% CO₂. After reaching confluence, 5×10^5 cells were plated into 10 cm culture dishes and used for transfection.

PLASMIDS AND TRANSFECTIONS

Cells were transfected using FuGene6 Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. Twenty-four hours (C3H10T½ cells) or 48 h (primary calvarial cells) after transfection, cells were lysed with 1× Passive Lysis Buffer

(Promega, Madison, WI) and luciferase activities were measured using the Luciferase Assay System (Promega, Madison, WI) following the manufacturer's protocol.

Luciferase plasmids—RPL10-Luc (pSGG_3UTR_empty) and RPL10-Luc-3'UTR (pSGG_3UTR_BMP2, catalog #S208437) were purchased from Switch Gear Genomics (Menlo Park, CA). The BMP2 plasmid was fully sequenced, compared to the human BMP2 reference sequence NM_001200.2, and shown to bear 1,254 nt of BMP2 sequence with the full-length BMP2 3'UTR including both polyadenylation signals [Fritz et al., 2006; Liu et al., 2008]. BmpLuc (mouse nt -1,237 to 471 relative to the distal promoter, pGL1.7XX) and BmpLucUCS (mouse nt -1,237 to 471 and mouse 9,574 to 9,938 nt relative to the distal promoter or +83 to 446 relative to the stop codon, pGLB2-5'mouseCNS) were described previously [Fritz et al., 2004]. CMVLUCBmp (pCβSLucmHCNSPvAcGH) containing the mouse (9,574–9,938 nt) ultra-conserved sequences inserted downstream of luciferase in CMVLUC (pCβSLuc) was described in [Devaney et al., 2009].

BMP2 expression plasmids—CMV-BMP2-UCS (pCβs hBMP2 hCNS) was generated by replacing the luciferase gene from pCβSLuchHCNSHcAcGH containing the human ultra-conserved sequence nt 11,488 to 11,877 relative to the distal promoter [Jiang et al., 2010b] with a T4 DNA polymerase filled-in *EcoRV* and *XbaI* BMP2 coding fragment from pcDNA31huBMP2 (provided by J. Patrick O'Connor, UMDNJ-NJMS). CMV-BMP2 (pCβshBMP2 GH) was made by inserting the blunted BMP2 fragment into pCβsBGHpA [Natalizio et al., 2002].

ANIMALS

All animals were handled in accordance with the Guidelines for Care and Use of Experimental Animals and approved by the NJMS IACUC (protocols #04086, #00100). The transgene construct pGLB25'3'LacZloxpcNS and the production of transgenic mice by oocyte microinjection was described previously [Fritz et al., 2006]. The β-gal expression pattern of the reporter gene in transgenic mice was detected by whole-mount X-Gal staining [Agah et al., 1997]. The mice that constitutively express Cre-recombinase under the control of the CMV promoter (CMV-Cre) were described previously [Schwenk et al., 1995].

PCR AND PRIMERS

Genomic DNA isolated from Cre-negative or Cre-positive β-gal transgene littermates was genotyped for the presence of the intact or recombined transgene by semi-quantitative PCR as follows: 95°C, 5 min; followed by 24–30 cycles of 94°C, 30 s; 57°C, 30 s; 72°C, 1.5 min; ending with a final longer extension cycle of 2 min at 94°C, 2 min at 57°C, and 10 min at 72°C. PCR primers and amplicon lengths are in Table I. Real time PCR was performed on an Applied Biosystems 7500 Real Time PCR machine with version 2.0.1 of the associated software. The ABI TaqMan probe for the mouse *β-actin* gene (Mm00607939_s1, ABI FAM-labeled TaqMan MGB probe) was the endogenous reference. The number of transgene copies was determined by the comparative Ct method, where 2^{-Ct} yields the relative copy number of *β-actin* or the *lacZ* bearing transgene.

Genomic DNA and total RNA was collected from the tails of the BAC transgenic weanlings using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) and an Aurum Total RNA Fatty and Fibrous Kit (BioRad, Hercules, CA), respectively. Approximately 1 µg of total RNA was converted to cDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA), and an equal amount was used for a control reaction where reverse-transcriptase was omitted. The three resulting samples were then measured by quantitative PCR on a single-color iCycler (BioRad) with SYBR GreenER qPCR SuperMix for iCycler (Invitrogen). The samples were measured in triplicate and the resulting Ct values were transformed, averaged, and normalized to the values obtained from genomic DNA.

IMMUNOHISTOCHEMISTRY

Whole-mount tissues stained for βgal were embedded in paraffin and sectioned (6 mm). Sections were boiled for 20 min in 0.01 M citrate buffer for antigen retrieval, blocked with 5% donkey serum in 0.1% Tween-20/PBS, incubated with primary antibodies against α-SMA (Dako North America, Inc., Carpinteria, CA), followed by Alexa-conjugated secondary antibody (Molecular Probes/Invitrogen, Carlsbad, CA) incubation to visualize the antibody binding.

ALKALINE PHOSPHATASE STAINING

Cells were rinsed in PBS, fixed in 100% methanol for 1 min at room temperature, rinsed with PBS, then overlaid with ALP buffer (100 mM Tris-HCl, pH9; 150 mM NaCl, 1 mM MgCl₂) with freshly added 0.15 mg/ml BCIP and 0.3 mg/ml NBT (BCIP/NBT Color Development Substrate, Promega, Madison, WI).

RESULTS

ULTRA-CONSERVED SEQUENCE-MEDIATED REPRESSION IS NOT INFLUENCED BY VECTOR CONTEXT

A messenger RNA molecule can fold into numerous complex structures. The post-transcriptional regulatory proteins that control mRNA processing, nuclear export, translation efficiency, and stability recognize both primary sequence and secondary structure. Consequently, the function of any post-transcriptional regulatory element is often influenced by the flanking mRNA sequence. We previously tested the effect of the ultra-conserved sequence on vectors driven by the *Bmp2* promoter (BmpLUC) or a constitutive cytomegalovirus (CMV) promoter (CMVLuc) in transiently transfected C3H10T½ cells. The luciferase activity of each vector was repressed by 62% and 76% respectively [Devaney et al., 2009]. We further tested if vector context altered ultra-conserved sequence-mediated repression by testing the effect of a 1,254 bp fragment bearing the full-length 3'UTR in a vector driven by the ribosomal protein L10 promoter (RPL10). Like the *Bmp2* and CMV-driven vectors, the RPL10 vector was significantly repressed by the 3'UTR-bearing fragment (Fig. 1A, compare RPL10-Luc to RPL10-Luc-3'UTR). In addition to different promoters, these three vectors had highly distinct 3'UTRs. The RPL10 vector had the full-length *Bmp2* 3'UTR including the two *Bmp2* poly(A) signals located 879 and 1,175 nt downstream of the *Bmp2* stop codon [Fritz et al., 2004; Liu et al., 2008] followed by an SV40 poly(A) signal. The *Bmp2*-driven vectors had the isolated ultra-conserved sequence

followed by an SV40 poly(A) signal. The CMV vectors had the isolated ultra-conserved sequence followed by a bovine growth hormone poly(A) signal. The strong repression observed in all three contexts indicates that promoter, 5'UTR, and other 3'UTR elements did not influence ultra-conserved sequence-mediated repression in C3H10T½ cells.

THE ULTRA-CONSERVED SEQUENCE REPRESSES GENE EXPRESSION IN PRIMARY MESENCHYMAL CELLS

C3H10T½ mesenchymal cells have been widely studied in the contexts of BMP2-regulated myoblast, adipocyte, and osteoblast differentiation and calcification [Katagiri et al., 1990; Wang et al., 1993; Cheng et al., 2003; Shao et al., 2005; Rosen and MacDougald, 2006]. However, they are immortalized cells that may have acquired altered regulatory properties. Like C3H10T½ cells, mouse calvarial cells are mesenchymal cells that can differentiate into osteoblasts and adipose cells. Consequently, we compared the luciferase activity of primary mouse calvarial cells transfected with CMVLuc or BMPLuc to cells transfected with CMVLucUCS or BMPLucUCS. Figure 1B shows that, as in C3H10T½ cells, the ultra-conserved sequence significantly repressed luciferase expression generated from either vector in primary calvarial cells.

ULTRA-CONSERVED SEQUENCE-MEDIATED REPRESSION IN MESENCHYMAL CELLS OF THE AORTA AND CORONARY VASCULATURE IN TRANSGENIC MICE

To understand the function of the ultra-conserved sequence *in vivo*, we previously generated two lines of transgenic mice bearing a *lacZ* transgene driven by the mouse *Bmp2* promoter region, the entire *Bmp2* 3'UTR and 934 nt of downstream sequence [Fritz et al., 2006]. Two loxP sites were inserted flanking the ultra-conserved sequence in the reporter gene to allow its *in vivo* excision from 3'UTR by Cre-recombinase (Fig. 2). If the ultra-conserved sequence represses *Bmp2*, then deleting the ultra-conserved sequence will up-regulate the reporter gene. We mated mice with the floxed *Bmp2* reporter transgene to mice that ubiquitously express Cre-recombinase under control of the CMV promoter (CMV-Cre, [Schwenk et al., 1995]). The amplified products from the PCR reaction shown in Figure 2 demonstrates that Cre-recombinase caused complete deletion of the ultra-conserved sequence from the reporter transgene.

We stained isolated calvarial cells from transgenic mice that possessed either the intact (no Cre) reporter vector or the recombined vector (+Cre) for βgal activity. A few βgal-positive cells were observed in the +Cre plates only. However, these cells were rare and were very faintly stained (data not shown). This was expected because a potent osteoblast enhancer of *Bmp2* expression (ECR1) is located 156 kb downstream of the *Bmp2* gene [Chandler et al., 2007; Dathe et al., 2009; Jiang et al., 2010a]. As a post-transcriptional regulatory element, the function of the ultra-conserved sequence can only be detected in cells where the reporter gene is transcribed. Without the ECR1 enhancer, the low copy number transgene would be poorly transcribed in calvarial cells. The absence of an mRNA makes it impossible for this transgene to query the post-transcriptional effect of the ultra-conserved sequence.

However, the recombined transgene lacking the ultra-conserved sequence (+Cre) was strongly expressed in the coronary vasculature and aorta (Fig. 3A). The recombined *Bmp2*

reporter gene was robustly expressed in all smooth muscle cells of both the ascending and descending aorta of +Cre mice. In contrast, few cells in the heart or aorta expressed the intact transgene (no Cre). Immuno-histochemical analyses using α -smooth muscle actin (SMA) established that β gal occurred in smooth muscle cells (SMC) which have a mesenchymal origin (Fig. 3B,C). Interestingly, isolated cells within the aorta (Fig. 3C) occasionally expressed the intact transgene in mice lacking Cre-recombinase (no Cre). The potential significance of this apparently “leaky” expression will be discussed.

An independent transgene observation corroborates the hypothesis that the ultra-conserved sequence inhibits *Bmp2* expression in coronary vascular cells at the post-transcriptional level. Like our *Bmp2* transgene in +Cre mice, a bacterial artificial chromosome (BAC) *Bmp2* reporter transgene [Chandler et al., 2007] was ectopically expressed in coronary vessels (Fig. 4). In this transgene, a strong SV40 polyadenylation (pA) signal is between the *βgeo* coding sequence and the *Bmp2* 3'UTR. This SV40 pA site was originally used in transgenes to block transcription of the natural 3'UTR [Mountford et al., 1994]. We used RT-PCR to directly confirm that this BAC reporter gene (Fig. 4C) and other *Bmp2* reporter genes with this SV40 pA site [Jiang et al., 2010b] yield mRNAs lacking the ultra-conserved sequence. Thus, Cre-lox mediated deletion (Fig. 3) or premature polyadenylation (Fig. 4) relieve gene repression mediated by the inclusion of the ultra-conserved sequence in the reporter mRNAs.

THE ULTRA-CONSERVED SEQUENCE REPRESSES BMP2 PROTEIN SYNTHESIS

We tested if the ultra-conserved sequence could repress the synthesis of BMP2 itself by inserting the human BMP2 coding sequence with or without the ultra-conserved sequence into a strong expression vector [Natalizio et al., 2002]. Non-transfected or mock transfected C3H10T $\frac{1}{2}$ cells do not synthesize detectable levels of BMP2 [Jiang et al., 2007]. However, cells transfected with the vector bearing only the BMP2 coding region secreted large quantities of BMP2 into the culture media. In contrast, cells transfected with the vector bearing the BMP2 coding region and the ultra-conserved sequence secreted over 80% less BMP2 (Fig. 5A).

C3H10T $\frac{1}{2}$ cells differentiate into alkaline phosphatase-positive osteoblasts in response to BMP2 [Katagiri et al., 1990]. The BMP2 secreted in plates transfected with either the promoter-only plasmid (CMV-BMP2) or the plasmid bearing the ultra-conserved sequence (CMV-BMP2-UCS) was fully functional as assessed by the presence of alkaline phosphatase positive nodules. However, consistent with the lower level of secreted BMP2, plates transfected with the vector that included the ultra-conserved sequence developed fewer nodules (Fig. 5).

DISCUSSION

The ability of the 3'UTR to interact with *trans*-regulatory factors is a nimble and versatile mechanism for modulating the synthesis of a potent morphogen in different cell types. We have shown that the *Bmp2* ultra-conserved sequence represses gene expression in C3H10T $\frac{1}{2}$ mesenchymal cells and primary mouse calvarial cells (Fig. 1), and vascular smooth muscle cells (Fig. 3). Thus, these cells share a common post-transcriptional *Bmp2* regulatory

mechanism. Because the concentration of BMP2 controls the differentiation programs of these pluripotent mesenchymal cells in vitro and in vivo [Katagiri et al., 1990; Vattikuti and Towler, 2004; Hruska et al., 2005; Lincoln et al., 2006; Shao et al., 2006; Srivastava, 2006], this regulatory mechanism is a potent influence on their function and behavior (Fig. 5).

The ultra-conserved sequence functions independently of promoter context, because it repressed transiently transfected luciferase genes driven by three different promoters (*Bmp2*, *RPL10*, CMV; Fig. 1). The ultra-conserved sequence functions independently of 3'UTR context, because it repressed either as an isolated element (Figs. 1 and 5) or within the intact *Bmp2* 3'UTR (Figs. 1 and 3). Additionally, the ultra-conserved sequence repressed reporter genes bearing three different poly(A) signals (endogenous *Bmp2*, bovine growth hormone (bGH), and SV40; Figs. 1 and 3). The ultra-conserved sequence functions independently of coding sequence, because it repressed luciferase (Fig. 1), β galactosidase (Fig. 3), and BMP2 itself (Fig. 5).

The strongly autonomous function of the *Bmp2* ultra-conserved sequence may have a practical benefit. Understanding the function of BMP2 and other important proteins requires reliable strategies to alter protein levels. However, the expression of randomly inserted transgenes often fails to correlate with the number of inserted copies owing to position effects at the integration site. The Smithies group developed a method to alter transgene expression by modifying the 3'UTR of the transgenes [Kakoki et al., 2004]. This post-transcriptional approach avoids chromosomal influences on transcription and successfully modulated gene expression by 100-fold. However, although two RNA elements functioned out of context, others did not. For example, two well-characterized stabilizing elements (the β globin C-rich stabilizing element and a part of the bovine growth hormone 3'UTR) failed to increase expression [Kakoki et al., 2004]. Our data indicates that the ultra-conserved sequence is an autonomously functioning 3'UTR element that can modulate the level of BMP2 and other proteins while retaining tissue specific regulatory elements. Conditional removal of this regulatory block in specific tissues, for example by Cre-mediated excision in transgenic mice, would be a valuable and unique addition to the transgenic tool kit.

Manipulation of the *Bmp2* ultra-conserved sequence in mice and in cultured cells revealed that the *Bmp2* gene is active in many more cells than actually synthesize protein. The establishment of an initial broad field of gene expression followed by spatial restriction of protein synthesis is a common theme in embryogenesis. We occasionally observed isolated blue nuclei in the aorta of adult animals lacking the Cre gene (Fig. 3C). This suggests that these cells are poised to express *Bmp2*, but that BMP2 synthesis is post-transcriptionally repressed. Local stimuli such as inflammation or oxidative stress may relieve this repression. The retention of a mechanism whereby the *Bmp2* gene is broadly active, but synthesis is post-transcriptionally blocked has ramifications to pathological calcification in humans.

BMP2 may stimulate three clinically important forms of calcium deposition: cardiac valve, medial artery, and atherosclerotic/fibrotic calcification [Bostrom et al., 1993; Mohler et al., 2001; Shao et al., 2005; Caira et al., 2006]. Our transgenic and tissue culture data suggest that, normally, *Bmp2* is repressed post-transcriptionally in vascular mesenchymal cells. Pathological impairment of this repression by proinflammatory signals, oxidative stress,

metabolic changes, and mechanical stresses may contribute to aberrant BMP2 synthesis in calcifying tissues.

Blocking synthesis at a late step enables a nimble and versatile response to local developmental signals. In an embryo, this is particularly relevant for multi-potential mesenchymal cells that have varied embryonic origins. Furthermore, retention of this post-transcriptional block in the adult aorta and coronary vessels explains the propensity of these tissues to reinitiate BMP2 synthesis leading to pathological calcification. Understanding the molecules that sustain or release this braking mechanism is relevant to pathologies involving mesenchymal cells and abnormal BMP2 production.

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Fig. 1.

Ultra-conserved sequence (UCS) mediated repression is context-independent in immortalized and primary mesenchymal cells. Luciferase reporter genes were driven by the ribosomal protein L10 (RPL10), *Bmp2* (Bmp), or cytomegalovirus (CMV) promoter only upstream of the luciferase gene (RPL10-Luc or BmpLuc or CMVLuc). The effect of 1,254 nt of *BMP2* sequence bearing the full-length 3'UTR with UCS inserted downstream of the luciferase open reading frame (RPL10-Luc-3'UTR) on luciferase activity was assessed in C3H10T½ pluripotent mesenchymal cells (A, n=6). The effect of the isolated UCS (BmpLucUCS, CMVLucUCS, +83 to 446 nt relative to the *Bmp2* stop codon) was assessed in primary mouse calvarial cells (B, n=6). Relative reporter activity is shown $\pm$ SEM. We previously demonstrated in C3H10T½ cells that BmpLucUCS and CMVLucUCS were 62% and 76% less active relative to the corresponding promoter-only plasmids [Devaney et al., 2009]. The *Bmp2*- and RPL10-driven plasmids bear the SV40 late poly(A) signal (SVpA). The RPL10-Luc-3'UTR plasmid also bears the full-length 3'UTR with the two natural *Bmp2* poly(A) signals (B2pA). The CMV plasmids bear the bovine growth hormone (BGHpA) signal.

Fig. 2.

Cre-recombinase mediated excision of the ultra-conserved sequence in mice. Diagrams of a reporter transgene with the murine *Bmp2* promoter (nt –1,237 to 471) and 3'UTR regions (nt 9,392–11,604) flanking the *lacZ* gene. ▽ marks the *loxP* sites flanking the ultra-conserved sequence (UCS, nt 9,392–10,200). The approximate locations of the two natural *Bmp2* poly(A) signals at 10,332 and 10,619 [Fritz et al., 2004; Liu et al., 2008] relative to the promoter (yielding 3'UTRs of ~870 and 1,175 nt, respectively) are indicated by arrows (). Primers that flanked the *loxP* sites were used for PCR to demonstrate complete excision of the ultra-conserved sequence. DNA from two *Bmp2* transgenic littermates were amplified for 24 to 30 cycles as marked. The right series of reactions used DNA from a Cre-positive littermate. Direct sequencing and the size of the amplification products confirmed the predicted sequences of both the intact or recombined transgenes. PCR quantification and sequencing of the junctions between transgene copies indicate that three copies of the intact transgene inserted into the genome in a head to tail orientation. In +Cre mice, recombination yielded one copy of the recombined transgene at this site.

Fig. 3.

The ultra-conserved sequence represses lacZ reporter transgene expression in mesenchymal cells of the aorta and coronary vasculature. A: The aorta and coronary vasculature are strongly β gal-stained in Cre-expressing (+Cre), but not non-expressing (no Cre), adult hearts (8 weeks). B,C: Sections of whole-mount β gal-stained Cre-expressing littermates (ages indicated in panels) were immuno-histochemically stained with an antibody against a smooth muscle actin (α -SMA, red). The merged images illustrate colocalization of β gal activity with α -SMA positive smooth muscle cells (SMC). The enlarged section from the no Cre aorta shown in (C) illustrates that a few blue nuclei (arrows) were present in no Cre mice bearing the intact transgene.

Fig. 4.

A BAC reporter transgene whose β geo transcript lacks the BMP2 3'UTR is ectopically expressed in the coronary vasculature. A: The diagram shows the BAC-based *Bmp2* reporter gene generated previously [Chandler et al., 2007]. A strong SV40 poly(A) signal after the β geo open reading frame prevents inclusion of the *Bmp2* 3'UTR with the ultra-conserved sequence in BAC-derived mRNAs. The approximate positions of the PCR primers used for the data shown in C are indicated. B: β geo activity (blue) in micrographs of sections from BAC-bearing transgenic animals was observed in coronary artery cells. This pattern resembles the ectopic expression observed in +Cre mice (Fig. 3). C: RNA and genomic DNA was collected from mice harboring the BAC transgene and was subjected to reverse transcription-qPCR. The upstream primer hybridized to the β geo coding sequence. The downstream primers hybridized to sequence just upstream (control SVpA) or downstream (past SVpA) of the SV40 poly(A) signal. The signals were normalized to that obtained from genomic DNA. The positive genomic control showed that each primer pair amplified efficiently. The negative control with reverse transcriptase omitted from the cDNA synthesis reaction (-RT) showed that the RNA was not contaminated with genomic DNA. Detection of PCR product only in the reaction using the control SVpA primer, but not the past SVpA primer, indicates that the β geo transcript was efficiently truncated by the SV40 poly(A) signal.

Fig. 5.

The ultra-conserved sequence (UCS) represses a vector encoding functional BMP2 protein.

A: C3H10T $\frac{1}{2}$ cells were transfected with plasmids bearing the human *BMP2* coding region with (CMV-BMP2-UCS) or without (CMV-BMP2) the UCS in the CMV-driven vector described in Figure 1. The amount of BMP2 protein in media conditioned for 2 or 5 days after transfection was measured by ELISA (R&D Systems). The average BMP2 concentration relative to the CMV-BMP2 vector is shown $\pm$ SEM. Day 2, $n=2$; day 5, $n=6$. No BMP2 was detected in untreated or mock-transfected cells or in cells transfected with the empty vector. Cells were then grown for 14 days, stained for alkaline phosphatase positive nodules, and lightly stained with eosin. The graph shows the average number of nodules per square centimeter $\pm$ SEM. No nodules were detected in cells transfected with the empty vector. **B:** Representative plates showing stained C3H10T $\frac{1}{2}$ cells transfected with the empty expression plasmid or plasmids bearing the human *BMP2* coding region with or without the UCS as described in (A).

TABLE I

PCR Primers and Predicted Amplicon Sizes

Target gene	Forward	Reverse	Amplicon (bp)
<i>Bmp2</i> , 3rd exon	CCACAAACGAGAAAAGCGTCAAGCC	CAGTAAAAGGCATGATAGCCCGGAG	146
<i>Actb</i> (semi-quantitative)	GGGAAATCGTGCGTGACATCAAAGAG	GCCATCATCACTTCCTGAC	359
<i>lacZ</i> (real time)	AGGCCACGGCGCTAATCAC	GGCGGGAAGGATCGACAGAT	61
<i>lacZ</i> (semi-quantitative)	CGCTGGATCAAATCTGTCGATCC	AGGTATTCGCTGGTCACTTCGATG	512
<i>loxP</i> spanning reporter probe	GGCGGAAAGTCCAAATTG	GCCATCATCACTTCCTGAC	1,317 (no Cre), 480 (+Cre)
<i>βgeo</i> coding to past SVpA	CGCCTTCTATCGCCTTCTTGACG	CCTCTACAGATGTGATATGGCTG	165
<i>βgeo</i> coding to past SVpA	CGCCTTCTATCGCCTTCTTGACG	CCATCACACTGGCGCTGCACG	506