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NEPHROBLASTOMA OVEREXPRESSED (NOV) INDUCES GREMLIN IN ST-2 STROMAL CELL LINES BY POST-TRANSCRIPTIONAL MECHANISMS

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Abstract

Nephroblastoma overexpressed (Nov) inhibits osteoblastogenesis in part because it binds bone morphogenetic protein (BMP)-2. In the present study, we investigated whether Nov regulated the expression of the BMP antagonist gremlin. Overexpression of Nov increased gremlin mRNA levels in ST-2 cells, and its downregulation by RNA interference decreased gremlin mRNA. Nov did not affect *Grem1* transcription, but prolonged the half-life of gremlin mRNA in ST-2 cells, demonstrating that Nov acts by post-transcriptional mechanisms. This was confirmed by demonstrating that downregulation of Nov destabilizes gremlin transcripts. To assess whether the 3′-untranslated region (UTR) of gremlin mRNA mediated the effect of Nov, the decay of a chimeric *cfos gremlin 3′-UTR* construct was compared to that of *cfos* in ST-2 cells. The presence of the gremlin 3′-UTR prolonged the half-life of *cfos* and was responsible for the effect of Nov. To examine the binding of the gremlin 3′-UTR to ribonucleoproteins, radiolabeled gremlin RNA fragments were incubated with cytosolic extracts from Nov overexpressing and control cells. RNA electrophoretic mobility analysis revealed that Nov enhanced the binding of cytosolic proteins to the fragments spanning the 3′-UTR of gremlin between bases 1358–1557 and 1158–1357 from the transcriptional start. Mutations of AU-rich elements in these two RNA fragments prevented the formation of RNA-protein complexes induced by Nov. Nov did not alter the binding of cytosolic extracts to sequences present in the 5′-UTR or coding region of gremlin. In conclusion, Nov stabilizes gremlin transcripts, and this effect is possibly mediated by AU-rich elements present in the 3′-UTR of gremlin.

Keywords

bone morphogenetic proteins; gremlin; Nov; osteoblasts; post-transcriptional control

INTRODUCTION

The fate of mesenchymal cells and their differentiation toward cells of the osteoblastic lineage is tightly controlled by extracellular and intracellular signals (Canalis et al., 2003). Bone morphogenetic proteins (BMPs) are important determinants of cell fate, and regulate osteoblastogenesis and endochondral bone formation (Canalis et al., 2003; Miyazono, 1999; Thies et al., 1992). The effects of BMPs are controlled by a large group of secreted antagonists that can prevent BMP signaling by binding to BMPs to preclude ligand-receptor interactions (Canalis et al., 2003; Gazzerro and Canalis, 2006; Miyazono, 1999).

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Members of the CCN family of cysteine-rich secreted proteins include cysteine-rich 61 (Cyr 61), connective tissue growth factor (CTGF), nephroblastoma overexpressed (Nov), and Wnt inducible secreted proteins (WISP) 1, 2, and 3 (Brigstock et al., 2003; Brigstock, 2003). CCN proteins are structurally related to certain BMP antagonists, and CTGF and Nov can bind to BMPs (Abreu et al., 2002; Garcia et al., 2002; Rydziel et al., 2007). Recently, we demonstrated that transgenic mice overexpressing Nov under the control of the osteocalcin promoter exhibit decreased bone formation leading to osteopenia (Rydziel et al., 2007). Overexpression of Nov in bone marrow stromal cells decreases the effect of BMP-2 on osteoblastogenesis and impairs BMP signaling, whereas *Nov* inactivation sensitizes osteoblasts to the effects of BMP-2 (Canalis et al., 2010; Rydziel et al., 2007). Co-immunoprecipitation experiments and plasmon surface resonance were used to demonstrate direct interactions between Nov and BMP-2, explaining the inhibitory actions of Nov on BMP signaling (Canalis et al., 2010). However, alternate mechanisms of Nov action in skeletal cells were not excluded and could involve the induction of BMP antagonists.

Gremlin and Noggin are classic secreted BMP antagonists, and were originally identified as dorsalizing agents (Hsu et al., 1998; Smith and Harland, 1992; Topol et al., 1997; Topol et al., 2000). *Gremlin* is a member of the *Differentially screening-selected gene aberrative in neuroblastoma (Dan)/Cerberus* family of genes and Noggin is a component of the Spemann organizer (Canalis et al., 2003; Gazzerro and Canalis, 2006). Two related *Gremlin* genes have been described, *Gremlin1* (*Grem1*) and *Gremlin2* or *Protein related to Dan and Cerberus (Prdc)*. Gremlin1 (subsequently termed gremlin) and Noggin oppose the effects of BMP on osteoblastogenesis, and transgenic mice overexpressing these BMP antagonists in the skeletal environment exhibit severe osteopenia secondary to decreased bone formation (Deregowski et al., 2006; Gazzerro et al., 2003; Devlin et al., 2003; Gazzerro et al., 2005). Accordingly, inactivation of *Grem1* results in a transient increase in bone formation (Gazzerro et al., 2007). Previous work from our laboratory has demonstrated that BMP-2 can induce the transcription of *Noggin* and *Grem1* in osteoblasts (Gazzerro et al., 1998; Pereira et al., 2000). This may be a feedback mechanism utilized by BMPs to temper their activity in the bone microenvironment. Although other growth factors can induce gremlin and noggin expression, there is no knowledge regarding the regulation of BMP antagonists by post-transcriptional mechanisms or by proteins with BMP antagonizing activity.

The purpose of this study was to investigate whether Nov, a CCN protein capable of binding BMPs, could induce the expression of gremlin or noggin in ST-2 stromal cells and examine the mechanisms involved.

MATERIALS AND METHODS

Cell Culture, Transient Transfections and RNA Interference (RNAi)

ST-2 cells, cloned stromal cells isolated from bone marrow of BC8 mice, were grown in a humidified 5% CO_2 incubator at 37°C in α-minimum essential medium (α-MEM, Life Technologies Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) (Otsuka et al., 1999). Transduced ST-2 cells overexpressing Nov under the control of the cytomegalovirus (CMV) promoter were created as previously described (Rydziel et al., 2007). Cells transduced with the pLPCX vector (Clontech, Palo Alto, CA) were used as controls. Cells were plated at a density of 10^4 cells/cm^2, and cultured in α-MEM supplemented with 10% FBS until reaching confluence (2–4 days). Transient transfections were conducted in cells cultured to 70% confluence using FuGene6 (3μl FuGene6/2μg DNA), according to manufacturer's instructions (Roche, Indianapolis, IN). Cells were exposed to the FuGENE6-DNA mix for 16 h, transferred to fresh medium for 24 h and either harvested or processed for subsequent experiments. To downregulate Nov expression *in vitro*, a 19-mer double stranded small interfering (si)RNA, was obtained

commercially (siRNA id# 156559, Life Technologies). A scrambled 19-mer siRNA with no homology to known mouse or rat sequences was used as a control (Life Technologies) (Elbashir et al., 2001; Sharp, 2001). Nov or scrambled siRNA at 20 nM were transfected into 60 to 70% confluent ST-2 cells using siLentFect lipid reagent, in accordance with manufacturer's instructions (BioRad, Hercules, CA).

Luciferase Assays

The activity of a 2.1 kilobase (kb) fragment of the *Grem1* promoter (A. Economides, Tarrytown, NY) cloned into pGL4 upstream of *luciferase* was examined. To study the effect of Nov overexpression on *Grem1* transcription, the *Grem1* promoter fragment was transfected into wild type ST-2 cells and into transduced ST-2 cells overexpressing Nov, or in controls. Wild type ST-2 cells were co-transfected with a vector, where the coding sequence of *Nov* was cloned into pcDNA 3.1 downstream of a CMV promoter, or with pcDNA 3.1 as control. In selected experiments, wild type ST-2 cells were serum deprived for 6 h and treated with recombinant human (rh)NOV protein (Peprotech, Rocky Hill, NJ) and harvested. To study the effect of Nov downregulation on *Grem1* transcription, the *Grem1* promoter fragment was transfected into wild type ST-2 cells transfected with a Nov siRNA or with a scrambled siRNA control. A CMV directed β-galactosidase expression construct (Clontech) was co-transfected to assess transfection efficiency. Luciferase and β-galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β-galactosidase activity.

mRNA Decay Experiments

The stability of the gremlin transcript was investigated in ST-2 cells transduced with pLPCX-Nov or pLPCX vector control cells, and in wild type ST-2 cells either in the context, or not, of Nov downregulation by RNAi. In these experiments, levels of gremlin mRNA were determined following transcriptional arrest. In addition, to determine whether sequences present in the 3′-UTR of the gremlin RNA were responsible for transcript stabilization, wild type *cfos* and *cfos/Grem1 3′-UTR* chimeric mRNA constructs were created and tested in transient transfection experiments. For this purpose, a 2.1-kb rat *cfos* cDNA (F. Curran, Memphis, TN) was digested with EcoRI and subcloned into pcDNA 3.1 to create pCMV*cfos* (Curran et al., 1987) (Figure 1). To create a *cfos/Grem1* chimeric construct, the 960 base pair (bp) *Grem1 3′-UTR* sequence was amplified by PCR using a mouse *Grem1* template (R. Harland, Berkley, CA). The *Grem1 3′-UTR* was sequenced and ligated to a construct containing the 1.27-kb 5′-UTR and coding region (CR) of *cfos*, cloned into pcDNA3.1, creating pCMV*cfos*/*Grem13′-UTR* (Figure 1). To test the decay of cfos mRNA, wild type ST-2 cells were transiently transfected with pCMV*cfos* and pCMV*cfos*/*Grem1 3′-UTR*. To test the effect of Nov on the decay of the chimeric construct, ST-2 cells transduced with pLPCX-Nov or pLPCX vector were transfected with pCMV*cfos*/*Grem1 3′-UTR*. To study gremlin transcript decay, transcription was arrested by exposing ST-2 cells to 5, 6 dichloro-1-β-ribofuranosyl benzimidazole (DRB) (Biomol, Plymouth MA), dissolved in dimethyl sulfoxide and diluted in culture medium 1:200, or to actinomycin D (Sigma-Aldrich, St. Louis, MO), dissolved in absolute ethanol. Equal amounts of vehicle were added to control cultures. At selected times, cells were collected and total RNA was extracted and subjected to real time RT-PCR analysis for the detection of gremlin or cfos transcripts.

Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted and mRNA levels determined by real-time RT-PCR (Nazarenko et al., 2002b; Nazarenko et al., 2002a). For this purpose, RNA was reverse-transcribed using SuperScript III Platinum Two-Step qRT-PCR kit (Life Technologies), according to manufacturer's instructions and amplified in the presence of specific primers for noggin,

gremlin, Nov, cfos, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L38 (RPL38) (Table 1). PCR reactions were carried out in the presence of Platinum Quantitative PCR SuperMix-UDG (Life Technologies) at 54–60°C for 45 cycles. Copy number was estimated by comparison with a standard curve constructed using *Nov* (American Type Culture Collection, Manassas, VA), *Noggin* (Regeneron Pharmaceuticals, Tarrytown, NY), *Grem1* (R. Harland) or *cfos* (T. Curran, Memphis, TN) cDNAs, and corrected for *Gapdh* (R. Wu, Ithaca, NY) or for *Rpl38* copy number (Hsu et al., 1998; Curran et al., 1987; Valenzuela et al., 1995; Tso et al., 1985). Reactions were conducted in a 96-well spectrofluorometric thermal iCycler (Bio-Rad Laboratories, Hercules, CA), and fluorescence was monitored during every PCR cycle.

RNA Electrophoretic Mobility Shift Assay (REMSA)

To generate RNA probes for testing in REMSA, 9 plasmids were constructed containing 81–200 bp sequences spanning the entire 1.5 kb mouse *Grem1* transcript (Hsu et al., 1998; Topol et al., 1997) (Figure 1). PCR amplification of a *Grem1* gene template (R. Harland) was performed with *Pfu* polymerase (Stratagene, La Jolla, CA), in the presence of 5′- and 3′-gremlin specific primers, and the PCR products were subcloned into pcDNA3.1(-) (Life Technologies). Point mutations in the 3′-UTR region were generated by site-directed mutagenesis (Franchimont et al., 1997; Horton, 1993; Rydziel et al., 2004). The identity of each construct was verified by DNA sequencing prior to use. DNA templates were linearized with HindIII and transcribed with T7 RNA polymerase (Roche Applied Science, Indianapolis, IN) in the presence of [^{32}P]uridine 5′-triphosphate (UTP) (800 Ci/mmol; PerkinElmer, Boston, MA) to produce transcripts with a specific activity of 3.5×10^7 disintegrations per minute (dpm)/μg of RNA. Unlabeled RNA was used in competition reactions. The DNA template was removed with DNase I, and unincorporated ribonucleotides removed by ethanol precipitation with 0.5 M ammonium acetate.

Cytosolic extracts were obtained from confluent ST-2 cells transduced with pLPCX-Nov or pLPCX vector. The cell layer was rinsed with ice-cold phosphate-buffered saline, collected, and resuspended in HEPES-binding buffer, consisting of 10 mM HEPES buffer, pH 7.1, 3 mM $MgCl_2$,14 mM KCl, 5% glycerol (all from Sigma-Aldrich), 1 mM dithiothreitol (Life Technologies), and protease inhibitors (Sigma-Aldrich), and frozen at −80°C. Cell lysis was accomplished by 4 cycles of thawing at 37°C for 5 min, followed by snap-freezing in acetone on dry ice for 5 min. Lysates were cleared by centrifugation, and protein concentrations were determined by the DC protein assay (BioRad). 20 μg of cytosolic extract were incubated with 10^5 dpm [^{32}P]RNA sequences at 25°C for 30 min in HEPES binding buffer followed by the addition of 1 unit of RNase T1 (Roche Applied Science) and heparin (Sigma-Aldrich) at 5 mg/ml for 10 min each (Leedman et al., 1995; Leibold and Munro, 1988). Samples were subjected to electrophoresis on a 6% native acrylamide gel (acrylamide/bisacrylamide ratio of 29:1; Sigma-Aldrich) at 4°C, and radiolabeled complexes were detected by autoradiography on Kodak X-AR film (Kodak, Rochester, NY). In RNA competition assays, unlabeled homologous RNA sequences were added in excess (500-fold) to the binding reactions prior to the addition of radiolabeled RNA probes.

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by Student's *t* test or ANOVA. Slopes of RNA decay experiments were analyzed by analysis of covariance (ANCOVA).

RESULTS

To determine whether Nov regulates noggin or gremlin expression, ST-2 cells were transduced with pLPCX-Nov or pLPCX control retroviral vectors. Gremlin mRNA levels were increased in ST-2 stromal cells transduced with pLPCX-Nov, when compared to pLPCX control cells (Figure 2). Similarly, addition of rhNOV protein to wild type ST-2 cells increased gremlin mRNA levels by ~2-fold. Conversely, downregulation of Nov by RNA interference (RNAi) caused a decrease in gremlin mRNA levels in wild type ST-2 cells. These results indicate that Nov induces gremlin expression, and it is required for its basal expression (Figure 2). The effect of Nov was selective to gremlin, since the expression of noggin mRNA was not modified in ST-2 stromal cells transduced with pLPCX-Nov. *Noggin* copy number corrected for *Gapdh* was (means ± SEM; n = 4) 88±3 in control cells transduced with pLPCX, and 80±1 in cells transduced with pLPCX-Nov. Noggin mRNA levels did not change following the addition of rhNOV protein (not shown).

To explore mechanisms of gremlin induction by Nov, a 2.1 kb fragment of the *Grem1* promoter cloned into pGL4 was transfected into ST-2 cells transduced with pLPCX-Nov or pLPCX control or co-transfected with pcDNA-Nov or pcDNA control vector into wild type ST-2 cells. In addition, wild type ST-2 cells transfected with the *Grem1* promoter fragment were exposed to control medium or rhNOV protein. *Grem1* promoter activity was not altered by Nov under any of these 3 conditions, indicating lack of transcriptional control of gremlin by Nov (not shown). To confirm these observations, the *Grem1* promoter fragment was transfected into wild type ST-2 cells, where Nov was downregulated by RNAi. Transfection of Nov siRNA decreased Nov expression from (means of *Nov* copy number corrected for *Rpl38* ± SEM; n = 4) 1.9±0.3 to 0.5±0.3, but did not alter the activity of the *Grem1* promoter, indicating that Nov is not required for the basal transcription of *Grem1* (not shown).

To determine whether post-transcriptional mechanisms were operational in the regulation of gremlin by Nov, confluent ST-2 cells transduced with pLPCX or pLPCX-Nov were transcriptionally arrested with DRB and the decay of gremlin transcripts determined. Gremlin mRNA half-life was prolonged from ~12 h in control pLPCX transduced cultures to ~48 h in pLPCX-Nov cells (Figure 3, left panel). Conversely, downregulation of Nov by RNAi in ST-2 cells destabilized gremlin transcripts shortening the half-life of gremlin mRNA from ~11 h in control to ~2.7 h in Nov downregulated ST-2 cells (Figure 3, right panel).

Although various mRNA regions can be responsible for changes in transcript stability, the 3′-UTR is most frequently responsible for this event (Anant and Davidson, 2000; Balmer et al., 2001; Dean et al., 2001; Rydziel et al., 2004). To determine whether the 3′-UTR of the gremlin RNA was responsible for transcript stabilization and for the effects of Nov, the rate of transcript decay was examined in ST-2 cells transiently transfected with a CMV-driven *cfos* expression plasmid (pCMV*cfos*)or a chimeric construct in which the CMV promoter drives the expression of the *cfos* coding region linked to the 3′-UTR of gremlin *(*pCMV*cfos/Grem1 3′-UTR)*. The *cfos* gene was chosen because of the short half-life of its mRNA (Shyu et al., 1989). Wild type ST-2 cells were transiently transfected with the described constructs, grown to confluence and treated with actinomycin D to arrest transcription. cfos mRNA was detected by real time RT-PCR. The half-life of native cfos transcripts following transcriptional arrest was ~10 min, whereas transcripts generated from pCMV*cfos/Grem1 3′-UTR* did not decay throughout a 20 min period, confirming that the gremlin 3′-UTR contained transcript stabilizing sequences (Figure 4, left panel). In a subsequent experiment, the half-life of transcripts generated from pCMV*cfos/Grem1 3′-UTR* was determined to be2

h, and overexpression of Nov further stabilized these transcripts, prolonging their half-life to 3.75 h (Figure 4, right panel).

Examination of the gremlin mRNA reveals a 158-nucleotide 5′-UTR, a 552-nucleotide protein coding region, and a 900 bp 3′-UTR(Hsu et al., 1998; Topol et al., 1997) (Figure 1). To determine regions of the gremlin RNA interacting with Nov-dependent proteins, the binding of cytosolic proteins with RNA sequences spanning the entire transcript were examined. For this purpose, cytosolic extracts prepared from ST-2 cells transduced with pLPCX-Nov or pLPCX control were incubated with 150–200 bp fragments of *in vitro* transcribed radiolabeled RNA sequences, and the complexes resolved by polyacrylamide gel electrophoresis. RNA sequences from the 5′-UTR failed to form RNA-protein complexes; and although sequences from the coding region formed complexes, their intensity was not different in cell extracts from control or Nov overexpressing cells (data not shown).

An RNA segment from the *Grem1 3′-UTR* sequence between base 1358 and 1557 from the start of transcription, bound cytosolic proteins from transduced ST-2 cells, and the intensity of the binding was increased in the context of Nov overexpression (Figure 5, left panel). A second segment from the *Grem1 3′-UTR* sequence, spanning base 1158 to 1357 from the start of transcription, formed a complex with cytosolic proteins from cells overexpressing Nov, but no complex was detected in control cells (Figure 5, right panel). Mutation of the AUUUA sequence (Mut 1413) in the RNA fragment spanning the *Grem13′-UTR* sequence from base 1358 to 1557, prevented the stimulatory effects of Nov on the formation of the cytosolic protein-RNA complex, whereas mutation of other AU-rich sequences (Mut 1377 and Mut 1443) did not modify the effect of Nov on the RNA-protein complex (Figure 5, left panel). Mutation of the UAAAUUAAUU sequence (Mut 1191) present in the RNA fragment spanning the *Grem1 3′-UTR* sequence from base 1158 to 1357, precluded the formation of the cytosolic protein-RNA complex in Nov overexpressing cells (Figure 5, right panel). Incubation of the cytosolic extracts with excess unlabeled homologous sequences prevented the formation of the radiolabeled RNA-protein complex, confirming the specificity of the binding reactions (Figure 5). These results demonstrate that the 3′-UTR of gremlin contains sequences that are responsible for the binding of gremlin mRNA to cytosolic proteins, and that the RNA-cytosolic protein interaction is modulated by Nov.

DISCUSSION

In the present study, we examined the regulation of gremlin expression by Nov and the mechanisms involved. We demonstrate that Nov induces gremlin mRNA levels by post-transcriptional mechanisms. The effect of Nov seemed to be selective to gremlin, since the expression of noggin, another BMP antagonist, was not modified. This study also demonstrates that Nov is required for basal gremlin expression since Nov downregulation destabilized gremlin transcripts.

Transgenic overexpression of Nov results in decreased bone formation and osteopenia, whereas *Nov* inactivation causes a modest skeletal phenotype (Canalis et al., 2010; Rydziel et al., 2007). This would suggest that when in excess Nov can have detrimental effects in bone. These may be explained by the fact that Nov binds BMP-2 and has the potential to act directly as a BMP antagonist. In the present study, we demonstrate that the induction of gremlin by Nov may be a secondary, but potentially important, regulatory mechanism to temper the actions of BMP in cells of the osteoblastic lineage (Canalis et al., 2010). Previous work from our laboratory has demonstrated that noggin and gremlin expression is induced by BMP utilizing transcriptional mechanisms; however, knowledge regarding post-transcriptional control of BMP antagonists has been limited (Gazzerro et al., 1998; Pereira et al., 2000). Gremlin has the potential to antagonize BMP and Wnt signaling and its

transgenic overexpression impairs bone formation, and as a consequence causes osteopenia (Gazzerro et al., 2005). Conversely, the conditional inactivation of *Grem1* in osteoblasts *in vivo* causes a transient increase in bone formation (Gazzerro et al., 2007). Therefore, the induction of gremlin by Nov may play a significant role in the control of BMP action and of skeletal homeostasis.

It is not unusual for CCN proteins to act on target cells by multiple mechanisms and to regulate various signaling proteins (Abreu et al., Nat. Cell Biol. 4:599, 2002; Mercurio et al., Development 131:2137, 2004). For example, CTGF can bind to BMP, insulin-like growth factor I and Wnt co-receptors; and as a consequence, it has the potential to decrease their signaling activity (Abreu et al., 2002; Mercurio et al., 2004; Smerdel-Ramoya et al., 2008b). In addition, both CTGF and Nov regulate Notch signaling in cells of the osteoblastic lineage, explaining some of their effects on osteoblastic cells (Rydziel et al., 2007; Smerdel-Ramoya et al., 2008a). These actions have implied a more direct interaction of a CCN protein with a regulatory signaling molecule, and the current study reveals an additional level of regulation, namely the induction of a growth factor antagonist by a post-transcriptional mechanism.

Our data demonstrated that the 3′-UTR is the region of the gremlin mRNA responsible for the transcript stabilization by Nov, and RNA mobility shift assays revealed that specific sequences of the gremlin 3′-UTR interact with RNA-binding proteins and the expression of these proteins or their interactions with gremlin RNA sequences are regulated by Nov. Classical and non-classical AU-rich elements, respectively located at bases 1411 to 1415 and 1184 to 1194 of the gremlin mRNA are necessary to observe the effect of Nov on the formation of RNA-protein complexes. These results confirm previous observations revealing that classical as well as non-classical AU-rich motifs can function as important RNA regulatory elements (Chen and Shyu, 1994). Transcript stabilization provides an additional level of regulation for the expression of secreted proteins, and this mechanism is utilized to control the expression of matrix proteins, such as collagenase 3, growth factors, such as granulocyte macrophage colony stimulating factor and transforming growth factor-β and hormones, such as parathyroid hormone (Rydziel et al., 2004; Esnault et al., 2006; Shen et al., 2008; Nechama et al., 2009). However, there are no prior reports to document that transcript stabilization is responsible for the regulation of secreted growth factor antagonists. Future studies should address the isolation and characterization of RNA binding proteins responsible for the regulation of gremlin mRNA stabilization since they could prove to be an important target of gene expression control in cells of the osteoblastic lineage.

In conclusion, Nov induces the expression of gremlin mRNA by a post-transcriptional mechanism, and this effect appears to be mediated by AU-rich motifs present in the 3′-UTR of the gremlin transcript.

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ABBREVIATIONS

The abbreviations used are

BMPs	Bone morphogenetic proteins
bp	base pair
BSA	Bovine serum albumin
CCN	Cyr61, CTGF, Nov
CMV	cytomegalovirus
CR	coding region
CTGF	connective tissue growth factor
Cyr 61	cysteine-rich 61
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
kb	kilobase
MEM	minimum essential medium
Nov	Nephroblastoma overexpressed
REMSA	RNA electrophoretic mobility shift assay
RNAi	RNA interference
RPL38	ribosomal protein L38
RT-PCR	Reverse Transcription Polymerase Chain Reaction
UTR	untranslated region

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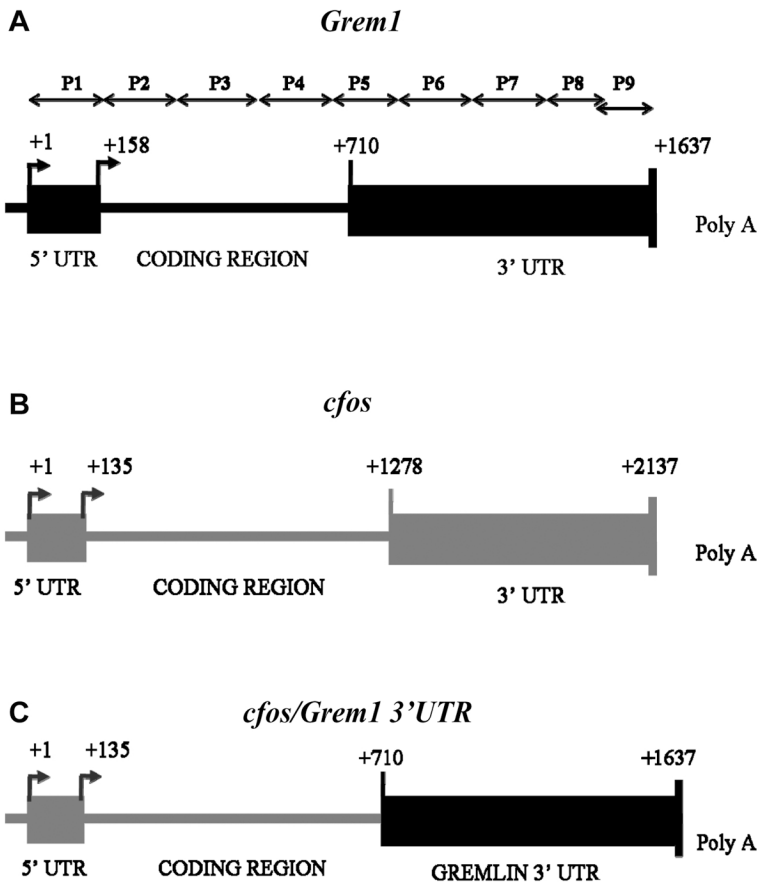

Figure 1.

Diagrams representing the *Grem1* and *cfos* mRNA and the *cfos*/*gremlin* chimeric construct. A, diagram of the *Grem1* mRNA, where +1 is the start of transcription, and +158 the start of translation. B, diagram of the *cfos* mRNA, where +1 is the start of transcription +135 the start of translation and from +1278–2137 the 3′-UTR. C, diagram representing the *cfos*/*Grem1* chimeric construct, where the 3′-UTR of *cfos* is exchanged with the 3′-UTR of *Grem1*.

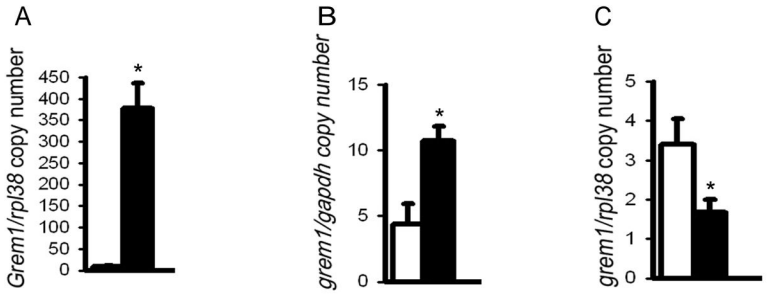

Figure 2.

Effect of Nov on gremlin mRNA levels. In panel A, ST-2 cells transduced with pLPCX vector (white bars) or pLPCX-Nov (black bars) were cultured to confluence. In panel B, wild type ST-2 cells were cultured to confluence, and treated (black bars) or not (white bars) with rhNOV at 1 μg/ml for 24 h. In panel C, subconfluent ST-2 cells were transfected with Nov (black bars) or scrambled (white bars) small interfering (si) RNA. Total RNA was extracted and gremlin transcripts were detected by real time RT-PCR. Data are expressed as *Grem1* copy number corrected for *Rpl38* or *Gapdh* expression. Nov expression was reduced from (means ± SEM; n = 4) 2.3 ± 0.2 in control to 0.9 ± 0.2 *Nov* copy number corrected for *Gapdh* in cells transfected with Nov siRNA ($p < 0.05$). *Significantly different between control and Nov and between control and Nov downregulated cells, $p < 0.05$.

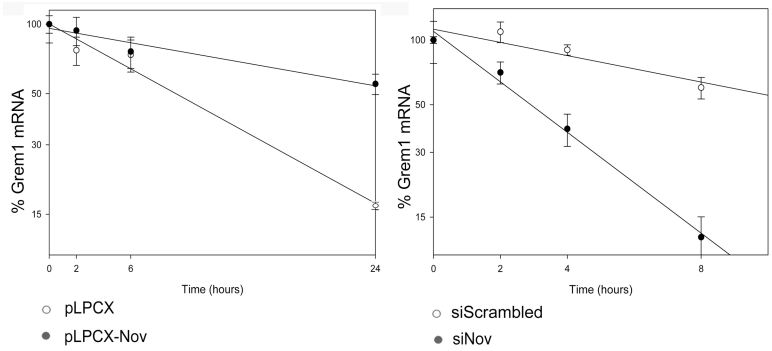

Figure 3.

Effect of Nov on the decay of gremlin mRNA in transcriptionally arrested cells. In the left panel, ST-2 cells were transduced with pLPCX vector (open circles) or pLPCX-Nov (black circles). In the right panel, subconfluent ST-2 cells were transfected with Nov (black circles) or scrambled (open circles) small interfering (si) RNA. At confluence (time 0), transcription was arrested (in both panels) by the addition of DRB at 200 ug/ml. RNA was extracted at the indicated times after the initial DRB treatment (time 0), and gremlin transcripts were determined by real time RT-PCR and corrected for Rpl38. Values are means ± SEM, n=4, and are expressed as percent gremlin mRNA levels relative to the time of the initial DRB addition. Nov expression was reduced from (means ± SEM) 3.4±0.1 in control to 0.4±0.1 *Nov* copy number corrected for *Rpl38* in cells transfected with Nov siRNA. Slopes of decay between pLPCX and pLPCX-Nov, and between siNov and siScrambled RNA are significantly different by ANCOVA, $p < 0.05$.

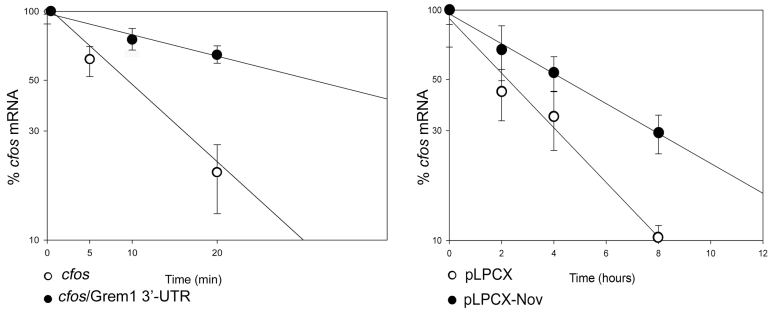

Figure 4.

Effect of gremlin 3′-UTR on the decay of cFos mRNA in transcriptionally arrested ST-2 cells. In the left panel, subconfluent ST-2 cells were transfected with either pCMV*cfos* (open circles) or pCMV*cfos*/*Grem1 3′-UTR* plasmid (black dots), where the *cfos* 3′-UTR was substituted with the *Grem1 3′-UTR*. In the right panel, ST-2 cells transduced with pLPCX (open circles) or pLPCX-Nov (black dots) were transfected with pCMV*cfos/Greml 3′-UTR*. ST-2 cells were cultured to confluence and transcription was arrested (in both panels) by the addition of actinomycin D at 10 μg/ml at time 0; actinomycin D was added as a pulse at 4 h in cultures where transcriptional arrest was continued for 8 h. RNA was extracted at the indicated times after the initial actinomycin D treatment (time 0), and cfos transcripts were determined by real time RT-PCR and corrected for *Rpl38*. Values are means ± S.E.; n = 4, and are expressed as percent cFos mRNA levels relative to the time of initial actinomycin D addition. Slopes of decay between *cfos* and *cfos/Grem1 3′-UTR* and between pLPCX and pLPCX-Nov are significantly different by ANCOVA, $p < 0.05$.

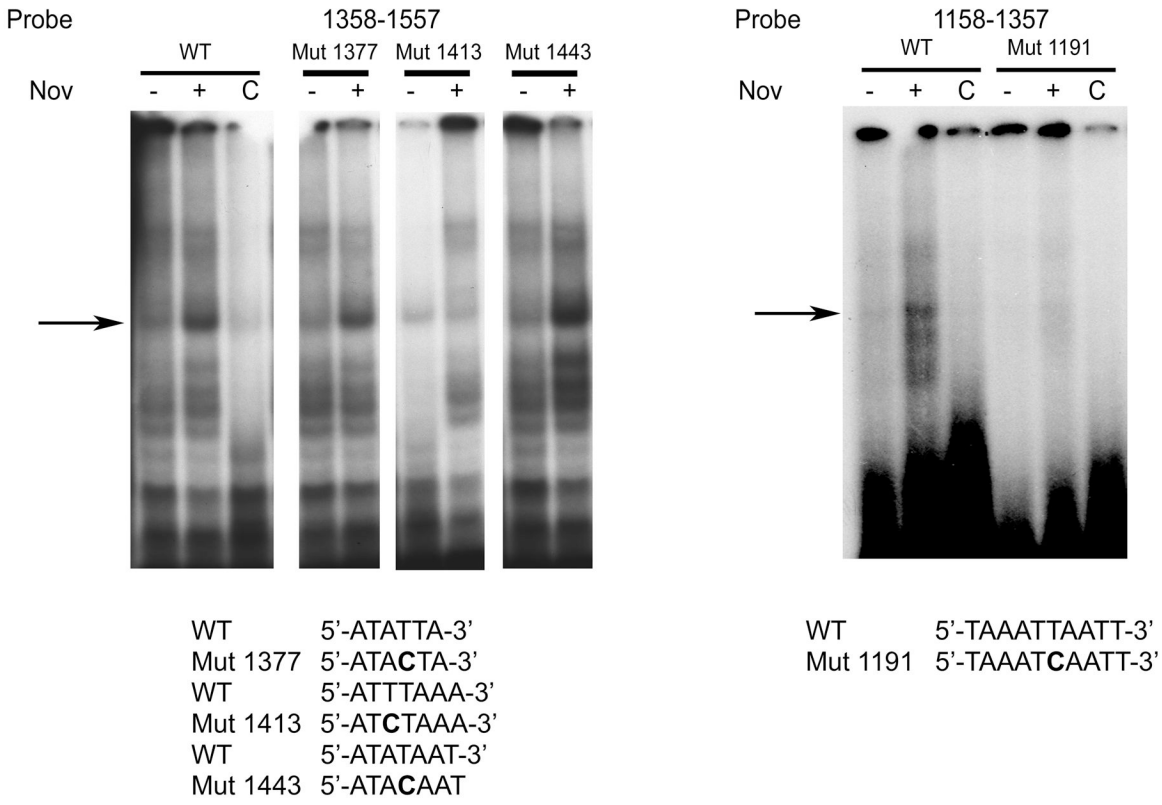

Figure 5.

RNA gel mobility shift assay demonstrating the effect of Nov on cytosolic proteins from transduced ST-2 cells binding to wild type (WT) and mutated (MUT) radiolabeled 3′-UTR sequences of the gremlin RNA. Cytosolic extracts from pLPCX-Nov (+) and pLPCX (−) transduced ST-2 cells grown to confluence were incubated with [^{32}P]-labeled + 1158–1357 (left panel) and [^{32}P]+1358–1557 (right panel) wild type and mutated (MUT) RNA sequences at +1191, +1377, +1413 and +1443, and subjected to electrophoresis on native polyacrylamide gels and developed by autoradiography. Binding following preincubation with 500-fold excess of unlabeled (“cold”) homologous RNA sequence (C), confirmed the specificity of the binding reaction. Specific RNA protein complexes are indicated by the *arrows*.

Table 1

Primers used for real time RT-PCR amplification.

Gene Name	Strand	Sequence (5′–3′)
Nov	F	GAACCACAGACTGGCATTTGCATGG[FAM]G
	R	CAAACTTCTCTCCGTTGCGGTA
Gremlin	F	CGGTTAGCCGCACTATCATCAAC[FAM]G
	R	GTGAACTTCTTGGGCTTGCAGA
Noggin	F	GTCACCCTGTACGCCCTGGT
	R	CGGCTGTGTAGATAGTGCTGGC[FAM]G
C-fos	F	CGGCCTACTACCATTCCCCAGC[FAM]G
	R	GTTGGCACTAGAGACGGACAGA
RPL38	F	CGAACCGGATAATGTGAAGTTCAAGGTT[FAM]G
	R	CTGCTTCAGCTTCTCTGCCTTT
GAPDH	F	CACGCTCTGGAAAGCTGTGGCG[FAM]G
	R	AGCTTCCCGTTCAGCTCTGG