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Activation of the ERK Pathway in Osteoblastic Cells, Role of Gremlin and BMP-2

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Abstract Gremlin is a glycoprotein that binds and antagonizes the actions of bone morphogenetic proteins (BMPs) -2, -4, and -7. Gremlin appears to activate the extracellular regulated kinase (ERK) pathway in endothelial and tumor cells, and as a consequence to have direct cellular effects. To determine whether gremlin has direct effects in osteoblasts, independent of its BMP binding activity, we examined its effects in ST-2 murine stromal cell lines and in primary cultures of murine calvarial osteoblasts. Gremlin did not activate Signaling mothers against decapentaplegic (Smad), and suppressed the BMP-2 induced Smad 1/5/8 phosphorylation and the transactivation of the BMP/Smad reporter construct 12xSBE-Oc-pGL3, confirming its BMPs antagonizing activity. Neither gremlin nor BMP-2 induced ERK 1/2 activation in ST-2 cells or calvarial osteoblasts. Moreover, slight changes in culture conditions induced the phosphorylation of ERK independent from BMP or gremlin exposure. In conclusion, gremlin inhibits BMP-2 signaling and activity, and does not have independent actions on ERK signaling in osteoblasts. Consequently, gremlin activity in osteoblasts can be attributed only to its BMP antagonizing effects. J. Cell. Biochem. 104: 1421–1426, 2008.

Key words: ERK; gremlin; BMP-2; osteoblasts

Gremlin, the product of *grem1*, is a 20 kiloDalton (kDa) secreted glycoprotein originally identified as a dorsalizing agent in Xenopus [Hsu et al., 1998]. Gremlin binds bone morphogenetic proteins (BMPs) -2, -4, and -7, and antagonizes their actions [Topol et al., 2000; Canalis et al., 2003]. Gremlin plays a central role in limb bud patterning and development [Khokha et al., 2003], in skeletal homeostasis and kidney development [Michos et al., 2004]. In addition, gremlin is expressed by stromal cells surrounding certain neoplasms [Sneddon et al., 2006], where it is considered to play a role in cell survival and possibly tumorigenesis [Topol et al., 1997]. Targeted overexpression of gremlin in the murine bone environment leads to osteopenia [Gazzerro et al., 2005], whereas the conditional deletion of *grem1* in bone tissue causes a transient increase in bone formation and trabecular bone volume [Gazzerro et al., 2007]. Overexpression of gremlin in skeletal cells opposes BMP activity, whereas, downregulation of gremlin expression by RNA interference sensitizes osteoblasts to BMP signaling and activity [Gazzerro et al., 2007]. These observations suggest that the effects of gremlin can be explained by its ability to bind and oppose BMP activity. However, activities independent of BMP binding, involving modulation of phosphorylated extracellular regulated kinase (ERK), have been postulated to play a role in angiogenesis [Stabile et al., 2007].

BMP-2 is a member of the transforming growth factor β family of growth factors [Wozney et al., 1988]. BMP-2 induces osteoblastic differentiation of mesenchymal cells in vitro, and enhances osteoblastic function [Canalis et al., 2003]. BMP-2 is dispensable for skeletal development in vivo, but it is necessary for bone fracture healing [Tsuji et al., 2006]. BMP-2 binding to pre-dimerized BMP receptor (BMPR) complexes induces Signaling mothers against decapentaplegic (Smad) 1/5/8

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phosphorylation on carboxyl-terminal serine residues [Kretzschmar et al., 1997b]. Activated Smad 1/5/8 form a complex with Smad 4 [Chen et al., 1997; Miyazono, 1999] and translocate to the nucleus [Massague, 1998], where they promote the transcription of BMP-2 target genes [Miyazono, 1999]. In addition, BMP-2 binding to type II receptors induces activation of type I receptors and dimerization, and as a result activation of mitogen activated protein kinases (MAPK) [Nohe et al., 2002]. BMP-2 induces the phosphorylation of the MAPK ERK 1/2 in human osteoblasts [Lai and Cheng, 2002] and C3H10T1/2 murine mesenchymal progenitor cells [Lou et al., 2000].

The role of the MAPK signaling network in osteogenesis and bone homeostasis is controversial. In vitro studies have shown that MAPK signaling can both promote or prevent osteoblastic differentiation [Schindeler and Little, 2006]. MAPK signaling can induce activated Smad 1 nuclear export via phosphorylation of specific serines and, as a consequence, antagonize BMP/Smad signaling [Kretzschmar et al., 1997a]. Since Smad signaling promotes osteoblastic differentiation [Ryoo et al., 2006], these results point toward a negative effect of MAPK activation on osteoblastogenesis. In support of this possibility, the transduction of a dominant negative MAP/ERK kinase (MEK) construct or the use of a chemical MEK inhibitor favor osteogenic differentiation in cultured osteoblasts [Higuchi et al., 2002; Nakayama et al., 2003]. However, subsequent studies have demonstrated that the expression of constitutively active MEK increases osteogenesis in vivo and in vitro, whereas expression of dominant-negative MEK inhibits osteogenesis, demonstrating that the ERK pathway stimulates osteoblastic differentiation [Ge et al., 2007].

The purpose of the current studies was to determine whether gremlin has direct effects on ERK signaling in osteoblasts. To this end, we tested the effects of gremlin in the presence and absence of BMP-2 on ST-2 murine stromal cells and in primary cultures of mouse calvarial osteoblasts.

MATERIALS AND METHODS

Cell Culture

Osteoblasts were isolated from parietal bones of 3- to 5-day-old FVB mice by sequential collagenase digestion, as previously described [McCarthy et al., 1990]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Grand Island, NY) supplemented with non essential amino acids, 20 mM HEPES, 100 μg/ml ascorbic acid and 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). ST-2 cells, cloned stromal cells isolated from bone marrow of BC8 mice, were plated at a density of 10^4 cells/cm^2 in α-Minimum Essential Medium (α-MEM), supplemented with 20 mM HEPES and 10% FBS. Osteoblasts and ST-2 cells were cultured in a humidified 5% CO_2 incubator at 37°C. To test for effects on BMP signaling, 70% confluent cells were transfected with a BMP/Smad reporter construct and treated with BMP-2 (Wyeth Research, Collegeville, PA) or gremlin (R&D Systems, Minneapolis, MN), as described under transient transfections. Alternatively, primary osteoblasts or ST-2 cells were grown to confluence, serum deprived overnight and treated with BMP-2 or gremlin, alone or in combination, to test for effects on Smad 1/5/8 or ERK 1/2 phosphorylation. In one experiment, confluent ST-2 cells were treated with gremlin or BMP-2 in the continuous presence of serum.

Transient Transfections

To determine changes in BMP-2/Smad signaling, a construct containing 12 copies of a Smad 1/5 consensus sequence linked to an osteocalcin minimal promoter and a luciferase reporter gene (12xSBE-Oc-pGL3; M. Zhao, Antonio, TX) was tested in transient transfection experiments [Zhao et al., 2004]. ST-2 cells were transiently transfected with 12xSBE-Oc-pGL3 reporter construct using FuGENE6 (3 μl FuGENE:2 μg DNA), according to manufacturer's instructions (Roche, Indianapolis, IN) [Deregowski et al., 2006]. A cytomegalovirus (CMV) directed β-galactosidase expression construct (Clontech, San Jose, CA) was used to control for transfection efficiency. Cells were exposed to the FuGENE–DNA mix for 16 h and transferred to serum free medium for 6 h. Cells were then treated with BMP-2 or gremlin alone or in combination for 24 h, and harvested. Luciferase and β-galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β-galactosidase activity.

Western Blot Analysis

To determine the level of Smad 1/5/8 or ERK 1/2 phosphorylation, the cell layer of osteoblasts or ST-2 cells was washed with cold phosphate

buffered saline (PBS) and extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) in the presence of protease and phosphatase inhibitors, as described [Gazzerro et al., 2005]. Protein concentrations were determined using a DC protein assay kit (BioRad Hercules, CA), and 20 μg of total cellular protein were fractionated by electrophoresis in 10% polyacrylamide gels under reducing conditions. Proteins were transferred to Immobilon P membranes (Millipore, Billerica, MA), which were blocked with 3% bovine serum albumin in PBS. For Smad phosphorylation, membranes were exposed to a rabbit polyclonal antibody, which recognizes Smad 1/5/8 phosphorylated at carboxyl-terminal serine residues (Cell Signaling Technology) or exposed to a mouse monoclonal antibody to unphosphorylated Smad 1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1,000 dilution. For ERK phosphorylation, membranes were exposed to a rabbit polyclonal antibody, which recognizes ERK 1 and 2 phosphorylated on an activating threonine residue (Cell Signaling Technology) or exposed to a rabbit polyclonal antibody to unphosphorylated ERK 1 and 2 (Cell Signaling Technology) at a 1:5,000 dilution. Blots were then exposed to anti-rabbit or anti-mouse IgG antiserum conjugated to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO) and developed with a chemiluminescence detection reagent (Perkin Elmer Life Sciences, Shelton, CT).

Statistical Analysis

Data are expressed as means ± SEM. Statistical significance was determined by ANOVA.

RESULTS

To test for the effect of BMP-2 and gremlin alone or in combination on Smad 1/5/8 phosphorylation, confluent ST-2 stromal cells and calvarial osteoblasts were treated with BMP-2 or gremlin for 20 min, a period of exposure previously shown to have a maximal effect of BMP-2 on Smad 1/5/8 phosphorylation [Gazzerro et al., 2005]. BMP-2 induced Smad 1/5/8 phosphorylation compared to control vehicle; gremlin alone had no effect, but the addition of gremlin to BMP-2 abolished the effect of BMP-2 on Smad 1/5/8 phosphorylation in ST-2 cells (Fig. 1A) and calvarial osteoblasts (Fig. 1B). To test for effects on Smad transactivation, ST-2 cells were transfected with a

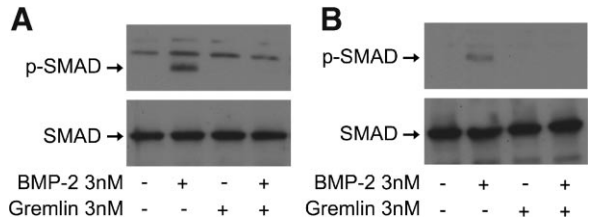

Fig. 1. Effects of BMP-2 and gremlin on Smad phosphorylation. ST-2 stromal cells (**panel A**) or mouse calvarial osteoblasts (**panel B**) were cultured to confluence, serum deprived overnight and exposed to control vehicle, BMP-2, gremlin or BMP-2 and gremlin at the indicated doses for 20 min. Cell lysates were examined for the presence of phosphorylated Smad 1/5/8 or unphosphorylated Smad 1 by Western blot analysis.

12xSBE-Oc-pGL3 reporter construct, which contains 12 repeats of a Smad binding element upstream of *luciferase*, and were treated with BMP-2 or gremlin for 24 h. BMP-2 induced the transactivation of the reporter construct; gremlin alone had no effect, but when added to BMP-2 gremlin reduced the effect of BMP-2 on the transactivation of the reporter construct (Fig. 2). These results indicate that gremlin does not have a BMP-2 independent effect on Smad phosphorylation and that it decreases the effects of BMP-2 on Smad signaling.

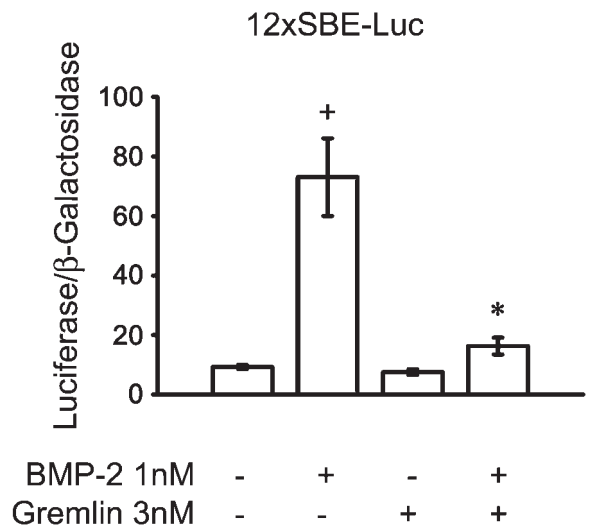

Fig. 2. Effects of BMP-2 and gremlin on Smad transactivating activity. Subconfluent ST-2 cells were transiently transfected with a 12xSBE-Oc-pGL3 and a CMV/β-galactosidase reporter construct. After 16 h, cells were switched to serum free medium for 6 h and treated with BMP-2 for 24 h with or without gremlin at the indicated doses. $^{+}$Significantly different between control and cells treated with BMP-2, $P < 0.001$. *Significantly different between cells treated with BMP-2 and cells treated with BMP-2 and gremlin, $P < 0.01$.

To test for the effect of BMP-2 and gremlin on the phosphorylation of ERK, ST-2 cells and osteoblasts were treated with vehicle, BMP-2 or gremlin. Due to the immediate and transient nature of the activation of the ERK pathway [Raman et al., 2007], cells were treated for different periods of time for up to 10 min. No differences in p-ERK levels between added gremlin, BMP-2 or vehicle were detected in either ST-2 cells or osteoblasts treated for 2, 5, or 10 min (Fig. 3A,B). It is important to note that in ST-2 cells, the levels of p-ERK were higher at 2 and 5 min, when compared to the basal levels of p-ERK (0 min) in vehicle, gremlin and BMP-2 exposed cultures (Fig. 3A). In osteoblasts, the levels of p-ERK in vehicle control, BMP-2 and gremlin treated cultures were higher at 2, 5, and 10 min, when compared to the basal levels of p-ERK (Fig. 3B).

Treatment with BMP-2, gremlin or vehicle of ST-2 cells and osteoblasts for 1 h did not cause a change in p-ERK levels, when compared to basal levels. Furthermore, p-ERK levels were not different between cultures treated with BMP-2 or gremlin and vehicle control after 1 h of exposure (Fig. 4A,B).

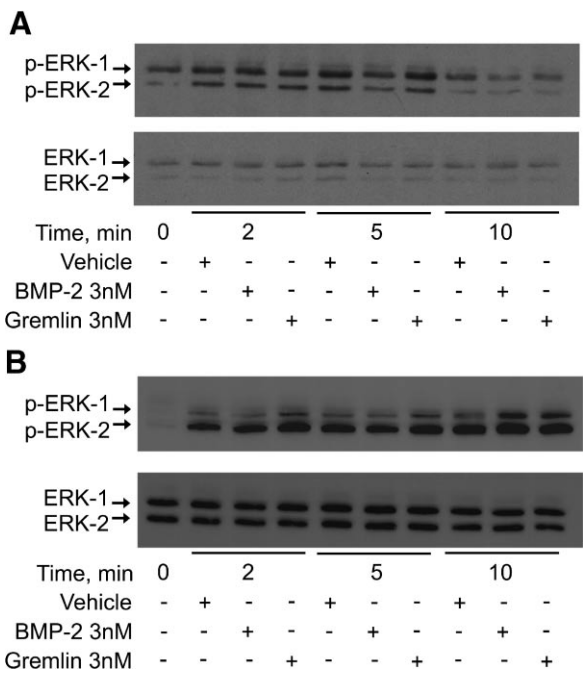

Fig. 3. Effects of BMP-2 and gremlin on ERK activation. ST-2 stromal cells (**panel A**) or mouse calvarial osteoblasts (**panel B**) were cultured to confluence, serum deprived overnight and exposed to control vehicle, BMP-2 or gremlin at the indicated doses for 2, 5, and 10 min. Cell lysates were examined for the presence of phosphorylated ERK 1/2 or unphosphorylated ERK 1/2 by Western blot analysis.

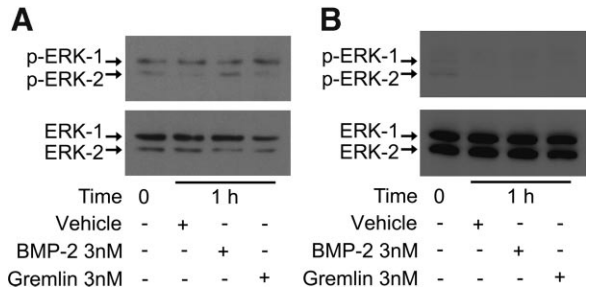

Fig. 4. Late effects of BMP-2 and gremlin on ERK activation. ST-2 stromal cells (**panel A**) or mouse calvarial osteoblasts (**panel B**) were cultured to confluence, serum deprived overnight, and exposed to control vehicle, BMP-2 or gremlin at the indicated doses for 1 h. Cell lysates were examined for the presence of phosphorylated ERK 1/2 or unphosphorylated ERK 1/2 by Western blot analysis.

Since the presence of serum can influence the activation of ERK in its response to BMP-2 [Osyczka and Leboy, 2005], albeit in non-skeletal cells, we tested the effects of BMP-2 and gremlin in ST-2 cells cultured in the continuous presence of serum. Similar to the effects observed in serum deprived cells, BMP-2 and gremlin had no effect on p-ERK, when compared to vehicle control cultures, but p-ERK levels were higher in treated and control cultures when compared to basal levels (Fig. 5).

DISCUSSION

In the present study we examined the role of gremlin in the regulation of ERK activation in osteoblasts. Assessing the effects of BMP-2 and gremlin on Smad signaling confirmed the stimulatory effects of BMP-2 and the inhibitory

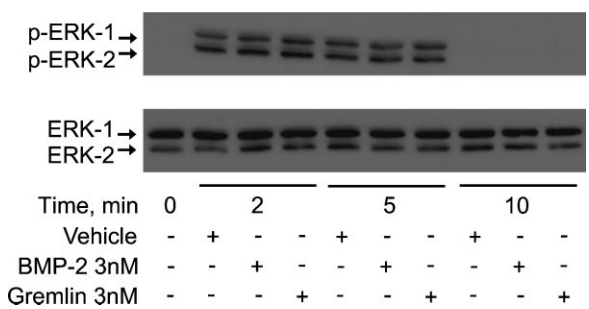

Fig. 5. Effects of BMP-2 and gremlin on ERK activation in the presence of serum. ST-2 stromal cells were cultured to confluence and exposed to control vehicle, BMP-2 or gremlin at the indicated doses for 2, 5, and 10 min. Cell lysates were examined for the presence of phosphorylated ERK 1/2 or unphosphorylated ERK 1/2 by Western blot analysis.

activity of gremlin on BMP-2 signaling. Moreover, the results also confirm our previous observations demonstrating that gremlin does not have an independent effect on Smad signaling [Canalis et al., 2003]. These results are in agreement with our previous studies on forced overexpression of gremlin [Gazzerro et al., 2005], in vivo and in vitro, demonstrating that gremlin causes osteopenia and reduces osteoblastic differentiation, which is associated with diminished responsiveness to BMP signaling. The present results also confirm our previous work demonstrating that the conditional deletion of *grem1* in the bone microenvironment in vivo, and its downregulation by RNA interference in vitro causes a transient increase in bone formation and bone mass, and enhanced BMP activity and signaling in the presence of gremlin [Gazzerro et al., 2007]. Taken together, these findings indicate that gremlin is a BMP antagonist able to impair bone formation.

In contrast to reports in human osteoblasts [Lai and Cheng, 2002] and C3H10T1/2 murine mesenchymal cells [Lou et al., 2000], we failed to detect either transient or sustained effects of BMP-2 on ERK activation in ST-2 cells and primary osteoblasts [Lou et al., 2000]. The discrepancy in the results obtained can be explained by the use of different cell lines and diverse experimental protocols, to test for changes in ERK activation. In contrast to reports in endothelial cells, gremlin did not induce ERK phosphorylation in cells of the osteoblastic lineage [Stabile et al., 2007]. The differences between our results and those reported in endothelial cells could be explained by different responsiveness of endothelial cells to extracellular stimuli. However, it should be noted that the experiments reported in endothelial cells compared the effect of gremlin to basal levels of ERK phosphorylation, and did not compare the effect of gremlin to parallel cultures exposed to vehicle alone for equal periods of time [Stabile et al., 2007]. Consequently, it is possible that the effect reported in endothelial cells is due to an acute change in culture conditions and it is not due to a direct effect of gremlin on cell signaling. It is possible that gremlin has direct effects on endothelial cell migration, and this activity may or may not be related to the postulated induction of ERK [Stabile et al., 2007].

In the present studies we found acute ERK activation, compared to basal levels following cellular exposure to vehicle alone. This indicates that ERK phosphorylation in osteoblastic cells can be triggered by slight changes in culture conditions, and this occurs both in the presence and absence of serum. This is not surprising considering the multiple and diverse nature of extracellular signals capable of influencing the activity of the MAPK network [Raman et al., 2007]. At present, we cannot ascribe any specific effect on ERK activation to either BMP-2 or gremlin in cells of the osteoblastic lineage.

Recently, an endogenous inhibitor of the ERK pathway, the mitogen activated protein kinase phosphatase-1, has been shown to mediate the glucocorticoid inhibition of osteoblast proliferation [Horsch et al., 2007]; thus implicating that the activation of the ERK pathway has a positive effect on osteoblast proliferation. This is consistent with our results showing that BMP-2 does not induce ERK signaling, since BMP-2 is primarily involved in osteoblast differentiation, rather then proliferation [Canalis et al., 2003].

An intracellular effect of gremlin on BMP-2 secretion and activity, based on an interaction between the two proteins in the endoplasmic reticulum, has been reported in transfected COS-1 cells [Sun et al., 2006]; however, our results using the addition of gremlin protein to the cultures confirm that gremlin can act as an extracellular inhibitor of BMP-2 activity in osteoblasts [Canalis et al., 2003].

In conclusion, gremlin inhibits BMP-2/Smad signaling and activity, and has no independent effects on ERK 1/2 phosphorylation in ST-2 stromal cells and osteoblasts.

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