Inhibition of signaling cascades in osteoblast differentiation and fibrosis

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Identification of a key residue mediating bone morphogenetic protein (BMP)-6 resistance to Noggin inhibition allows for engineered BMPs with superior agonist activity

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Chapter 3

Identification of a key residue mediating bone morphogenetic protein (BMP)-6 resistance to Noggin inhibition allows for engineered BMPs with superior agonist activity

3.1 Abstract

Bone morphogenetic proteins (BMPs) are used clinically to induce new bone formation in spinal fusions and long bone non-union fractures. However, large amounts of BMPs are needed to achieve these effects. BMPs were found to increase the expression of antagonists, which potentially limit their therapeutic efficacy. Yet, the relative susceptibility of osteoinductive BMPs to different antagonists is not well characterized. Here we show that BMP-6 is more resistant to Noggin inhibition, and more potent in promoting osteoblast differentiation in vitro and inducing bone regeneration in vivo, when compared to its closely related BMP-7 paralog. Noggin was found to play a critical role as a negative feedback regulator of BMP-7 but not BMP-6 induced biological responses. Using BMP- 6/7 chimeras, we identified lysine 60 as a key residue conferring Noggin resistance within the BMP-6 protein. A remarkable correlation was found between the presence of a lysine at this position and Noggin-resistance among a panel of osteoinductive BMPs. Introduction of a lysine residue at the corresponding positions of BMP-2 and BMP-7 allowed for molecular engineering of recombinant BMPs with increased resistance to Noggin antagonism.

3.2 Introduction

Bone morphogenetic proteins (BMPs) are dimeric secreted cytokines that were discovered based on their ability to induce ectopic bone and cartilage formation *in vivo* [34, 37, 26, 22]. BMPs belong to the transforming growth factor-β (TGF-β) superfamily, which also includes TGF-βs and activins. Over 15 distinct BMP family members have been identified that signal via specific BMP type I and type II serine/threonine kinase receptors [19]. Three BMP type II receptors, i.e. BMP type II

receptor (BMPR-II), activin type II receptor (ActR-II) and ActR-IIB and four distinct BMP type I receptors, i.e. activin receptor-like kinase (ALK)1, ALK-2, ALK-3 and ALK-6, have been described [19, 32, 25, 38]. Cell surface binding of BMPs to their receptors result in heteromeric complex formation upon which the constitutively active type II receptor phosphorylates the type I receptor on specific serine and threonine residues in the juxtamembrane region. Different BMPs bind with different affinities and specificities to different BMPR complexes [32, 25, 38, 29, 17]. The activated BMP type I receptor initiates intracellular signaling by phosphorylating specific receptor regulated (R-)Smad proteins, i.e. Smad-1, Smad-5 and Smad-8. Activated R-Smads form heteromeric complexes with Smad-4, which translocate to the nucleus and regulate, in cooperation with transcriptional co-activators and co-repressors, the transcription of target genes [19]. BMP signaling is controlled at different levels by both positive and negative regulators. At the extracellular level BMP antagonists bind BMPs and interfere with their binding to BMP receptors. An important extracellular BMP antagonist of the osteogenic activity of BMPs is Noggin. The crystal structure of the Noggin-BMP-7 complex demonstrated that binding of Noggin to BMPs resembles that of BMP receptors, and thereby prevents the binding of the BMP- binding epitopes to both the type I and type II receptors [12]. Noggin expression is potently induced by BMP activity, and may thus contribute to the negative feed-back loop mechanism controlling BMP action *in vivo* [8]. Whereas mice deficient in Noggin display failure of joint specification and formation of excessive cartilage, transgenic mice that overexpress Noggin demonstrate impaired osteoblastic function with osteopenia and fractures [33, 6]. Noggin mutations in humans have been linked to proximal symphalangism and multiple synostoses syndrome [10]. The relative sensitivity of different BMPs to Noggin antagonism has not been clearly and systematically characterized. BMPs promote bone formation by stimulating the proliferation and differentiation of mesenchymal stem cells (MSC) and preosteoblasts [35]. In physiological settings, decreased levels of BMP activity have been correlated with non- unions and impaired healing [18, 14]. BMP-2 and BMP-4 expression decreases with aging, possibly leading to a decrease in osteoblast number and activity [20]. In contrast, constitutive activity of the BMP type I receptor, ALK-2, has been linked to fibrodysplasia ossificans progressiva, a disease characterized by heterotopic bone formation [28, 13]. Elevated BMP activity has been found in the ossification of the posterior longitudinal ligament [39]. BMP-2 and BMP-7 have been shown to be efficient in stimulating bone regeneration in defects of the femur in rats and sheep, and of mandible and calvariae in dogs and baboons [9, 4, 23]. However, relatively high amounts of BMP are needed to demonstrate clinical benefits in patients [36]. One reason why large amounts of BMPs may be required could be the presence of BMP antagonists, such as Noggin, that limit the effects of surgically implanted BMPs [31]. Here we have characterized in detail the differential interactions of various BMPs with Noggin, and through the use of domain swapping and point mutations mapped the key residue in BMP-2 and BMP-7 mediating sensitivity to Noggin inhibition, thereby generating BMPs with superior agonistic activity.

3.3 Experimental Procedures

Materials

HEK293T, C2C12, COS and A549 cells were obtained from ATCC (Manassas, VA). ROS 17/2.8 cells were kindly provided by Majeska RJ and Rodan GA (University of Connecticut, Farmington CT). Recombinant human BMP-2, BMP-6 and BMP-7 were produced in Chinese hamster ovary cells. Human BMP-4, BMP-5, BMP-9 and Noggin-Fc were purchased from R&D Systems (Minneapolis, MN). Tissue culture media, sera, Geneticin, and pre-cast NuPAGE gels were purchased from Invitrogen (Carlsbad, CA). Bright-Glo luciferase assay reagent was purchased from Promega (Madison WI). IRDye labeled secondary antibodies and molecular weight markers were from Li-Cor Biosciences (Lincoln, NE). FuGENE 6 and HD were purchased from Roche diagnostics (Indianapolis, IN). Polyclonal anti-human BMP-7 antibodies against the mature BMP-7 region were raised in rabbit. BCA protein assay kit was from Thermo /Fisher (Pittsburgh, PA). KOD hot start DNA polymerase and custom oligos were purchased from EMD (Gibbstown, NJ) and IDT (Coralville, IA), respectively.

Cell culture

COS, C2C12 and HEK293T cells were maintained in DMED supplemented with 10% FBS and Pen/Strep. A549 and A549-BRE cells were maintained in F12K medium supplemented with 10% wtw. ROS 17/2.8 cells were maintained in F12 medium supplemented with 5% FBS, 2 mM L-glutamine and 0.8 mM $CaCl_2$. Cells were grown at 37°C in a humidified incubator under 5%CO_2.

Plasmids

Wild type full length BMP- 7 expression plasmid, BMP-7 WT, was constructed by inserting the XmaI/BamHI fragment encoding the full length human BMP-7 (Genbank accession: NM_001719) open reading frame (ORF) into a proprietary mammalian expression vector. BMP-7 mutants were generated by site-directed mutagenesis of the BMP-7 WT construct, using the QuikChange protocol, under the conditions suggested by the manufacturer (Stratagene La Jolla, CA). DNA segments encoding residues 1-40, 45-80, and 90-120 of the mature BMP-7 domain were replaced by their corresponding regions in BMP-6 to generate three chimeras, 40-1, 80-1, and 90-1, respectively. A PCR based method was used to generate these chimeras. Briefly, for each chimera, overlapping 5' and 3' BMP-7 DNA fragments as well as the middle BMP-6 region were amplified by PCR. DNA plasmids containing the wild type human BMP-7 and BMP-6 open reading frames were used as templates for these PCR reactions (DNA primers used are available upon request). For each chimera, the three PCR fragments generated in this fashion were subsequently used as template to amplify a chimeric region spanning the entire mature domain. The assembled chimeric DNA was subsequently substituted for its corresponding BMP-7 wild type sequence, downstream of the pro-domain encoding region, in the BMP- 7 WT construct described above. Four

copies of BMP response element (BRE) as previously described by Korchynskyi and ten Dijke [16], were cloned into TA-Luc (Panomics Fremont, CA) at the NheI site to generate the BMP reporter construct, 'BRE 4xR-luc'. Human ALK-2, ALK-6 (HA-tagged), BMPRII and ActR-II have been previously described [25, 38].

Expression of recombinant BMPs in HEK293 T cells

HEK293T cells were seeded in DMEM supplemented with 10% FBS. The next day cells were transfected with the appropriate expression construct, using FuGENE 6 or FuGENE HD, according to the manufacturer's recommendations. 24 hours post-transfection, the culture medium was replaced with fresh DMEM supplemented with 0.5% FBS. Conditioned media were collected 48 to 72 hrs post transfection. Small scale expression of recombinant BMPs was carried out on 6-well plates whereas medium/large scale expression was performed in 150 and $225cm^2$ flasks.

Purification of the 80-1 and BMP-7 E60K proteins

HEK293T cells were seeded in $225cm^2$ flasks and transfected with plasmids encoding either the 80-1 (20 flasks) or the BMP-7 E60K (30 flasks) proteins. Recombinant proteins secreted in the conditioned medium (0.8 to 1.2 liters) were first precipitated with ammonium phosphate (50% saturation) over night at 4 °C. Precipitated proteins were subsequently reconstituted in 20 mM HEPES buffer containing 0.5 M NaCl and 10 mM imidazol (pH 7.2). Proteins were then loaded onto a nickel-IMAC (GE healthcare, Piscataway NJ) column. Bound proteins were eluted with 20 mM HEPES buffer, containing 0.5 M NaCl and 6 M urea (pH 7.2). The fractions containing BMP activity were pooled and further purified by SD. HPLC fractions were analyzed by SDS-PAGE and silver staining and by western blot. Fractions containing the BMP of interest were pooled, lyophilized and stored until further use.

siRNA-mediated silencing of Noggin expression

Knockdown of Noggin was performed using siRNAs from Dharmacon. Transfections were performed according to manufacturer's instructions using Dharmafect 3 as transfection reagent.

BMP Reporter cell line

An A549 reporting line was generated by stably transfecting A549 cells with the reporter construct, BRE 4xR-Luc, which contains the firefly luciferase gene driven by 4 copies of BRE (see above). Clones were selected in the presence of Geneticin and tested for BMP- induced luciferase activity. A clone, A549- BRE, showing high signal to noise ratio was selected, expanded and used for future studies.

Reporter Gene Assays using A549- BRE cells

BMP activity and susceptibility to Noggin inhibition was assessed using a reporter gene assay, in the presence or absence of Noggin. In brief, A549-BRE cells (see above) were seeded onto 96-well plates in F12K medium supplemented with 1% FBS. Reporter gene expression was induced upon addition of BMPs, in the presence or absence of Noggin, in a 25 μL volume. Cells were incubated at 37$decC$ for 24 hours and luciferase activity was measured using the Bright-Glo reagent (Promega) following the manufacturer's recommendations. Luciferase activity was subsequently normalized to total protein using a BCA assay (Thermo/fisher) following manufacturer's protocol and data were analyzed using the SoftMax Pro software (Molecular Devices). Alternatively, BRE-luc assays were performed as previously described [16]. The effect of Noggin on BMP activity was quantified by calculating either the percent (%) inhibition or the Residual BMP activity. The % inhibition was determined using the following formula: % inhibition $= ((A - B)/A) \times 100$ Whereas 'A' represents the activity in the absence of Noggin and 'B' the activity in the presence of Noggin. The Residual BMP Activity was determined by calculating the ratio of the normalized luciferase activity (RLU/mg of total protein) measured in the presence of Noggin to that in the absence of Noggin.

Western blot analysis

Protein samples were resolved on precast NuPAGE 4-12% Bis-Tris mini-gel and transferred to nitrocellulose membranes with a semi-dry transfer cell, Trans-Blot SD (Bio Rad). Membranes were blocked in Odyssey blocking reagent (Li-Cor) for 3 hours and then incubated in primary antibody in blocking reagent over night at room temperature. After washing in TBST, membranes were incubated in IRdye labeled secondary antibody for 45 minutes, washed, then scanned and analyzed with the Odyssey Infrared Imaging System (Li-Cor Biosciences). Smad phosphorylation was detected using PS-1 antibody recognizing phosphorylated Smad-1, phosphorylated Smad-5 and phosphorylated Smad-8 [21]. Total Smad-1 was detected using anti-Smad-1 antibody (Zymed).

RNA isolation and quantitative real- time PCR

Total DNA-free cellular RNA was extracted with the RNeasy kit (Macherery- Nagel). Reverse transcriptase-polymerase chain reaction was performed using the RevertAid H Minus First strand cDNA synthesis kit (Fermentas) according to manufactures instructions. The oligonucleotide primers for PCR were designed using Primer Express Software (Applied Biosystems). The sequences of the PCR primers used in this study are available upon request. Taqman PCR reactions were performed using the StepOne Plus Real-Time PCR System (Applied Biosystems). All samples were plated in duplicate. Gene expressions were determined with the comparative $\Delta\Delta$Ct method using *GAPDH* as reference and the non-stimulated condition was set to 1.

Alkaline phosphatase assays (ALP)

ROS 17/2.8 cells were seeded at 3.7 x 104 cells per well on 96-well plates in 200 μL of F12 medium, supplemented with 0.2% BSA and incubated for 24 hours at 37°C under 5% CO^2 in a humidified incubator. Alkaline phosphatase activity was induced upon addition of BMPs, in the presence or absence of Noggin, in a 50μL volume. Following a 24 hour incubation, 150μL of medium was removed and cells were lysed by adding 100μL of 2% Triton X-100. The lysate was cleared by centrifugation and 20μL (supernatant) was transferred to a clear plate. 100μL of 1:5 pNPP was added to the lysate and incubated for 10 min at 37°C. 75μL of 0.5 N NaOH was then added to each well to stop the reaction. The plates were scanned and analyzed using a molecular device plate reader. Alternatively, ALP assay was performed as previously described [15].

Analysis of BMP binding to Noggin

Fc derivatized sensor surfaces by Surface Plasmon Resonance (BIAcore) - Sensor surface CM4 was selected and derivatized as follows: Anti-mouse immunoglobulins were diluted to 30$\mu g/mL$ in 10 mM acetate pH 5.0 coupling buffer. The surfaces of both control and active flow cells were activated for 7 minutes with a 1:1 mixture of 0.1 M NHS and 0.4 M EDC at flow rate of 10$\mu L/min$. Anti- mouse immunoglobulins were then injected for 7 minutes at a flow-rate of 10$\mu L/min$ and this was followed by an injection of 1 M ethanolamine, pH 8.5 for 7 minutes at a flow- rate of 10$\mu L/min$. The surface containing immobilized anti-mouse immunoglobulins was then conditioned with three 2 minute injections of 10mM Glycine-HCl pH 1.7. Kinetic measurements were performed at 25°C in a buffer containing 50mM HEPES pH 7.4, 150mM NaCl, 1mg/mL BSA and 0.05% Surfactant P20. Each sample cycle consisted of the following steps: Noggin-Fc was injected at 0.5$\mu g/mL$ for 45 seconds at 10$\mu L/min$ over the active flowcell which was followed by a 30 second stabilization phase. The BMP sample was then injected at 75$\mu L/min$ for 180 seconds (over both the active and control flowcells), followed by a 300 second undisturbed dissociation phase. For at least one concentration of each sample the dissociation phase was extended to 1200 seconds to ensure accurate measurement of slow dissociation constants.

Iodination of BMP ligands and affinity labeling of BMP receptors

Iodination of BMP-6 or BMP-7 was performed according to the choramine T method, and subsequently transfected COS cells were affinity-labeled with the radioactive ligand as previously described [38]. In brief, cells were incubated on ice for 3 hours with the radioactive ligand in the absence or presence of Noggin. After incubation, cells were washed and crosslinking was performed with 54 mM disuccinimidyl suberate (DSS) and 3 mM bis (sulphosuccimidyl suberate (BS3, Pierce) for 15 min. Cells were washed, scraped and lysed. Lysates were incubated with the respective antisera overnight and immune complexes were precipitated by adding Protein A Sepharose

(Amersham). Samples were washed, boiled in SDS sample buffer and subjected to SDS-PAGE. Gels were dried and scanned with the STORM imaging system.

Critical size defect of rabbit ulna

An ulnar critical size defect model was used to evaluate the efficacy of BMP-6 and BMP-7 in a model of bone healing of adult male New Zealand White rabbits (3 to 4 kg weight) as previously described [5]. BMP-6 and BMP-7 ($100\mu g$ and $500\mu g$) were added on to the bovine collagen hemostatic sponge (Helistat) and left on room temperature for 2 hours. Animals were divided into five groups: A/ control, defect filled with Helistat only (n=10); B/ defect filled with Helistat and BMP-6 ($100\mu g; n = 11$); C/ defect filled with Helistat and BMP-6 ($500\mu g; n = 9$); D/ defect filled with Helistat and BMP-7 ($100\mu g; n = 10$); E/ defect filled with Helistat and BMP-7 ($500\mu g; n = 8$). X-rays were taken bi- weekly and scored as described [30]. The protocol was approved by the institutional Ethics and Animal Committee.

μ-CT

The microcomputerized tomography apparatus (μCT 40) and the analyzing software used in these experiments were obtained from SCANCO Medical AG (Bassersdorf, Switzerland). The bone was scanned in the dorsoventral direction as described [24].

Statistical Analysis

Values are expressed as mean ±S.E. For statistical comparison of two samples, a two-tailed Student's t-test was used. $P < 0.05$ was considered significant. One way analysis of variance (ANOVA, Dunnet test) was performed to determine the effect of BMP-6 on *in vivo* regeneration of bone defects by μCT.

3.4 Results

3.4.1 Comparative Analysis of the Osteogenic Activity of a BMP Panel Revealed a Significant Difference in Activity between BMP-6 and BMP-7

To compare the potency of different BMPs to promote osteoblast differentiation, we tested a panel of BMPs for their ability to induce an early marker of osteoblast differentiation, alkaline phosphatase (ALP) activity, in the rat osteosarcoma cell line, ROS 17/2.8 (Figure 1A). Among the growth factors tested, BMP-6 showed the highest potency (EC50=14 ng/mL), followed by, in a descending order, BMP-7, BMP-4, BMP-2 and BMP-5. GDF-5 and GDF-6/BMP-13 showed only marginal activity in this assay. Interestingly, the dose response curves for BMP-2 and BMP-4 had shallower slopes compared to those for BMP- 5, BMP-6 and BMP-7, suggesting a potentially

more pronounced negative regulatory mechanism for BMP-2 and BMP-4. The difference in potency observed between the highly related BMP-6 and BMP-7 (73% amino acid identity) triggered our interest initially. We confirmed this difference in C2C12 myoblasts using both, alkaline phosphatase activity as well as BMP response element (BRE)-driven luciferase reporter gene expression as read outs (Figure 1B). This difference in potency was also confirmed in BMP-treated C3H10T1/2 mesenchymal osteoprogenitor cells (data not shown).

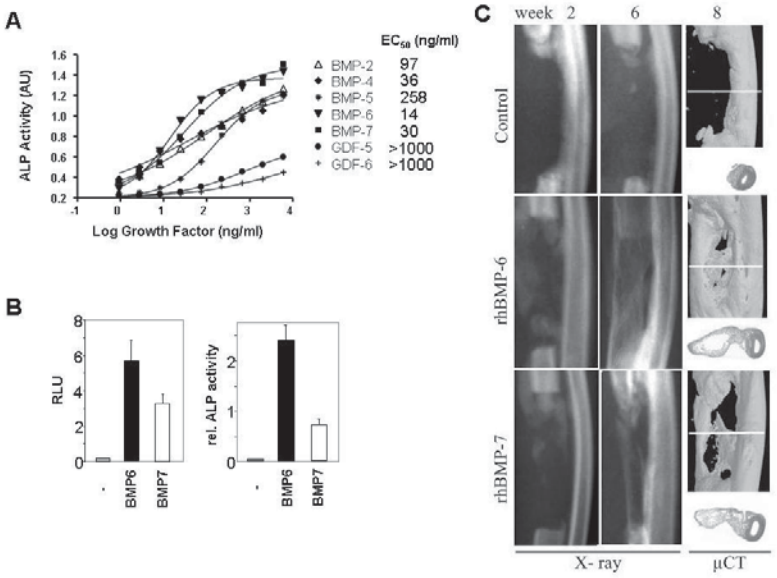


Figure 1: Differential potency of BMP-6 and BMP-7 is determined by their difference in sensitivity to Noggin inhibition. (a) A panel of BMPs, i.e. BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, growth and differentiation factor (GDF)5/BMP-14 and GDF6/BMP-13 were tested for their ability to induce alkaline phosphatase (ALP) activity in the rat osteosarcoma cell line, ROS 17/2.8. Each growth factor response was examined in a nine point dose response in triplicates. Potency was assessed based on the EC50 derived from the non linear regression of the mean result (see Supplementary Table 1). (b) BMP-6 is more potent than BMP-7 in stimulating BRE- transcriptional activity and alkaline phosphatase activity. Serum starved C2C12 cells were transfected with BRE-luc reporter and stimulated with 25 ng/mL BMP-6, 25 ng/ml BMP-7 or vehicle control and luciferase activity was analyzed 24 hrs post transfection. Right panel: Serum starved C2C12 cells were stimulated with 100 ng/mL BMP-6, 100 ng/mL BMP-7 or vehicle control and alkaline phosphatase activity was analyzed 48 hrs post transfection. (c) BMP-6 is more potent than BMP-7 in ectopic bone formation. X-ray of critical size defects of rabbit ulnae treated with a collagen sponge (Helistat) alone (control; top panel) or with rhBMP-6 (100μg; middle panel) and with rhBMP-7 (100μg; lower panel) *in vivo* for 2 and 6 weeks after surgery. μCT analyses of critical size defects were performed *in vivo* at 8 weeks after surgery (crosssections were horizontal and vertical). a $P<0.05$ vc C; b $P<0.05$ vs A (ANOVA; Dunnett test).

Therapy	X-ray score			μ-CT BV/TV[a] 8 weeks
	2 weeks		6 weeks ($100\mu g$)	
	$100\mu g$	$500\mu g$		
A: Control	0.3±0.2	0	0	0.5±0.2
B: BMP-6	$3 \pm 0.3^{b,c}$	4 ± 0.5	5 ± 0.5	$26.7 \pm 6.4^{b,c}$
C: BMP-7	2±0.5	3±0.4	4±0.1	14.5±2.1

Table 1: *In vivo* X ray and *ex vivo* μCT analysis of critical size defects of rabbit ulna treated with (A) bovine collagen alone, (B) bovine collagen + rhBMP-6 and (C) bovine collagen + rhBMP-7. At 2 week period representative X rays of both BMP doses of 100 and 500 μg are shown. Healing efficacy was analyzed by radiographic grading scores (0-6) and BV/TV values were determined by μCT ($100\mu g$ ligand) (in %). Results are presented as mean ±SD. [a] Bone volume/tissue volume [b] $p < 0.05$ versus BMP-7 (analysis of variances, Dunnett's test) [c] $p < 0.01$ versus control (analysis of variances, Dunnett's test)

Importantly, using a critical size defect model of rabbit ulna, we showed that implantation of $100\mu g$ of BMP-6, on a bovine collagen sponge, induced faster and more robust bone regeneration as compared to a similar dose of BMP-7 (Figure 1C). Two weeks following implantation of BMP-6, partial defect bridging with material of non-uniform radiodensity was apparent (Figure 1C). At the same time point, only flocculent radiodensity with flecks of calcification and no defect bridging was observed in rabbits treated with the $100\mu g$ dose of BMP-7. Bone formation, comparable to that induced with BMP-6 ($100\mu g$), was found in animals treated with a 5- fold higher dose ($500\mu g$) of BMP-7 (Table 1). At 6 weeks post-surgery, BMP-6 induced significantly more bone repair as compared to BMP-7; and by 8 weeks, μCT analyses revealed that almost two times more bone was formed with BMP-6 as compared with the same amount of BMP-7 (Figure 1C and Table 1).

3.4.2 BMP-6 and BMP-7 Induce Noggin Expression with Different Potencies

We next set out to determine the mechanism underlying the differential *in vitro* and *in vivo* potency between BMP-6 and BMP-7. Noggin, a natural extracellular BMP inhibitor, has been shown to be induced by BMPs and to antagonize their activity by direct binding and interfering with BMP-BMP receptor interaction [12]. To investigate a potential difference in Noggin negative feedback regulation of BMP-6 and BMP-7, we initially examined the effect of both BMPs on Noggin expression. In C2C12 cells, BMP-7 induced Noggin mRNA (Figure 2A) and protein (Figure 2B) expression more potently than BMP-6. As a result, we found a more sustained Smad-1 phosphorylation in BMP-6 treated C2C12 cells (Figure 2C) and MC3T3 pre-osteoblasts (data not shown) compared to BMP-7. Upon siRNA-mediated Noggin knockdown, BMP-7-induced Smad-1 phosphorylation became more sustained and mimicked the BMP-6-induced response that was also slightly elevated under these conditions (Figure 2D). Consistent with this result, Noggin knockdown also increased BMP-7 effects on down-

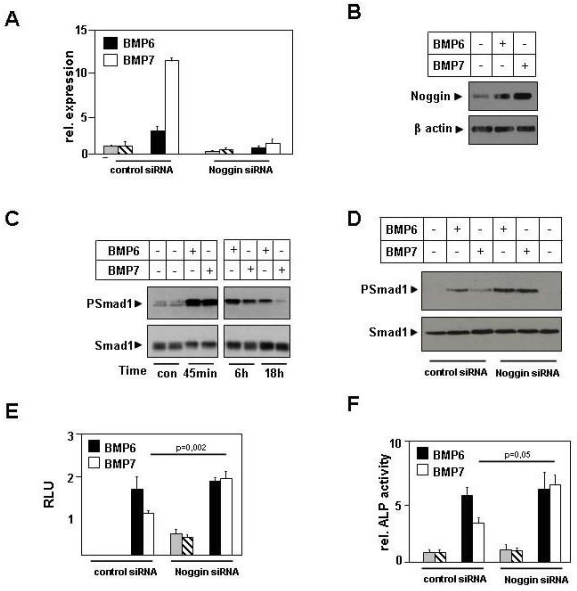


Figure 2: BMP-7 is more potent in inducing Noggin mRNA expression than BMP-6. (a,b) Serum starved C2C12 cells were stimulated with BMP-6 (50 ng/mL) or BMP-7 (50 ng/mL) or vehicle control for 18 hrs. Total RNA was extracted and cDNA prepared and expression of *Noggin* was determined using qPCR with expression being normalized to *GAPDH* and expressed as fold change relative to control. Non-stimulated controls are indicated with gray bars for BMP-6 and hatched bars for BMP-7.(c) BMP-6-induced Smad-11 phosphorylation is more sustained than BMP-7-induced Smad-1 phosphorylation. Serum starved C2C12 cells were stimulated with BMP- 6 (50 ng/mL) or BMP-7 (50 ng/mL) for the indicated times and cell lysates were analysed by immunoblotting with phosphoSmad-1/-5 and total Smad-1 antibodies. Upon Noggin knockdown BMP-7-induced responses are comparable to BMP-6-induced levels of Smad-1 phosphorylation (d), BRE-luc activity (e) and ALP activity (f). C2C12 cells transfected with control siRNA or Noggin siRNA were stimulated with BMP-6, BMP-7 or vehicle control. For Western blotting cell lysates were analyzed by immunoblotting with phosphorylated Smad-1/-5 or total Smad-1 antibody (e). For BRE-luc response C2C12 cells were transfected with BRE-luc and analyzed for luciferase activity (e). For ALP activity cell extracts were analyzed (f). Non-stimulated controls are indicated with gray bars for BMP-6 and hatched bars for BMP-7.

stream effectors such as BRE-luciferase reporter gene expression and ALP activity to BMP-6 levels (Figure 2E and 2F). Taken together, these results indicate that the differential induction of Noggin expression by BMPs is a key component of the negative feedback loop regulation of their biological activities.

3.4.3 BMP-6 is more resistant to Noggin inhibition than BMP-7

In order to compare the susceptibility of BMP-6 and BMP-7 to inhibition by exogenous Noggin, both BMPs were tested, along with BMP-2 and BMP-4, for their ability to promote ALP activity in ROS 17/2.8 cells, in the presence of increasing concentrations of Noggin. Of the BMPs tested, BMP-4 was the most sensitive to Noggin inhibition, followed by BMP-2, then BMP-7 (Figure 3A). BMP-6 demonstrated the highest resistance (IC50>5,000 ng/mL) to Noggin inhibition (Figure 3A), even compared to its closest homolog BMP-7 (IC50=800 ng/mL). This differential sensitivity was confirmed using BRE-reporter gene assay in C2C12 cells (Figure 3B) and A549-BRE cells (data not shown). Similarly, in primary bone marrow-derived human mesenchymal stem cells (MSCs), QPCR analysis showed that BMP-6-induction of the osteoblast marker genes Id-1, Dlx-5 and Msx-2, in addition to Noggin, was less susceptible to Noggin inhibition compared to BMP-7 (Figure 3C). Therefore, the relative BMP-6 resistance to Noggin inhibition is not restricted to a particular cell or assay system, but is rather an intrinsic characteristic of BMP-6.

3.4.4 BMP-6 and BMP-7 have comparable binding characteristics to immobilized Fc- Noggin using a Biosensor assay

One possible explanation for the lower susceptibility to Noggin inhibition is that affinity of Noggin for BMP-6 is significantly lower than for BMP-7. To test this hypothesis, we profiled the affinity of recombinant Noggin on a panel of BMPs, using surface plasmon resonance (BIAcore). Recombinant Fc-Noggin protein was immobilized on the Bioacore chip surface and free BMP-2, 4, 6 or 7 was added at various concentrations. BMP binding to Fc-Noggin was observed for all four BMPs and was dose dependent. Noggin bound with the highest affinity to BMP-4, followed by BMP-2 (Figure 4). Surprisingly, in this experimental paradigm, BMP-6 apparent affinity to Noggin (KD = 1.3 x 10-11 M) was higher than that of BMP-7 (KD = $2x10^{-}10$ M) (Figure 4).

This paradoxical result was in conflict with the functional data presented in Figure 3. Interestingly, close analysis of the biosensor data showed that this apparent higher affinity for BMP-6 is driven mainly by its apparent low dissociation rate (Kd of $6x10^{-}51/s$ for BMP-6 compared to $5.6x10^{-}31/s$ for BMP-7). In contrast, the on-rate for BMP-6 was almost half that of BMP-7 (Ka of $7.4e^{6}1/Ms$ for BMP-6 compared to $4.7e^{6}1/Ms$ for BMP-7). Moreover, at comparable concentrations, more BMP-7 was bound to Fc-Noggin compared to BMP-6, as judged by the response units measured by the instrument (Figure 4). These results suggest that the binding characteristics of BMP-6 and BMP-7 to immobilized Fc-Noggin fusion protein are significantly different.

3.4.5 Noggin inhibition of BMP binding to cell surface BMP receptors is more pronounced in the case of BMP-6 compared to BMP-7

In order to further validate the functional data presented in Figure 3, and to investigate Noggin interactions with BMP-6 and BMP-7 using an alternative method to

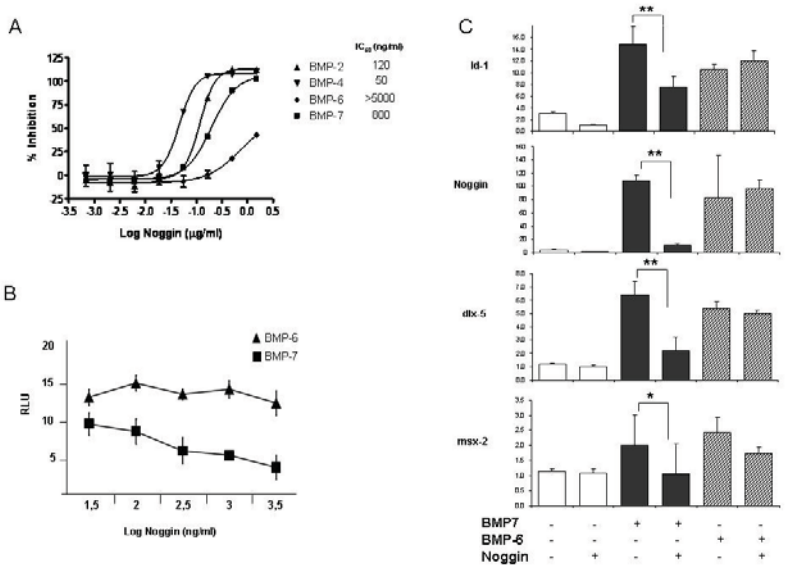
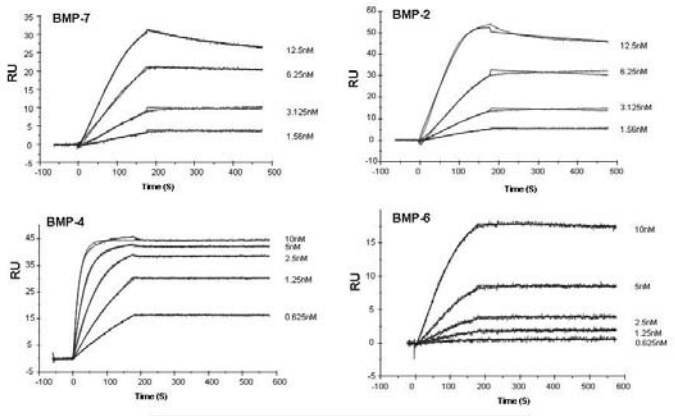


Figure 3: BMP-6 induction of downstream genes in primary human bone marrow-derived mesenchymal stem cells (hMSCs) is less susceptible to Noggin inhibition than BMP-7. (A) In order to compare BMP susceptibility to Noggin inhibition, BMP-6, BMP-7 and BMP-2 and BMP-4 were tested by an ALP assay in the ROS cells, in the presence of increasing concentrations of Noggin. Each condition was tested in triplicates, and Noggin dose response curves were derived by fitting the data to a non-linear regression. The IC50 for Noggin corresponding to each BMP was then calculated. These results indicate that the four BMPs tested are affected differently by Noggin. BMP-4 is the most susceptible, followed by BMP-2, BMP-7 and then BMP-6. While BMP-4, BMP-2 and BMP-7 are clearly sensitive to Noggin inhibition, BMP-6 demonstrated marked resistance to Noggin inhibition, with an IC50 four to sixteen times higher than that of other BMPs tested. (B) BMP-6, but not BMP-7, is resistant to Noggin mediated inhibition. C2C12 cells transfected with BRE-luc reporter were challenged with BMP-6 or BMP-7 in the absence or presence of the indicated amounts of Noggin and analyzed for luciferase activity. (C) HMSCs were stimulated with BMP-6 or BMP-7 and effects on target genes including Id-1, Dlx-5, Msx-2, and Noggin was measured by qPCR. Induction of these target genes by BMP-7 was significantly inhibited by Noggin in all cases. In particular, Noggin inhibits its own induction by BMP-7 in a greater degree than other genes. In contrast, Noggin did not affect induction of Id-1, Dlx-5, and Noggin transcription induced by BMP-6. Noggin only marginally decreased the induction of Msx-2 by BMP-6.

BIAcore, we measured the effect of Noggin on the binding of BMP-6 or BMP-7 to cell surface BMP type I and type II receptors. To accomplish this, we overexpressed BMP receptor II (BMPRII) on the surface of COS cells, in conjunction with either BMP type I receptors ALK-2 or ALK-6. The effect of Noggin on the binding of radiolabelled BMP-6 or BMP-7 to these cell surface BMP receptors was assessed by immunoprecipitation



	K_a 1/uMs	K_d/s	K_D 10^{-9} M
BMP-2	7.4	4.4×10^{-31}	0.06
BMP-4	7.0	4×10^{-41}	0.0006
BMP-6	4.7	6×10^{-21}	0.013
BMP-7	5.6	5.6×10^{-21}	0.2

Figure 4: Affinity measurements of Noggin for BMPs using surface plasmon resonance. The affinity of recombinant Noggin to BMPs was determined using surface plasmon resonance (BIAcore). BMP-6, BMP-7, BMP-2 or BMP-4 were immobilized on the BIAcore chip and Noggin-Fc was used as a free ligand. Each BMP was evaluated in the presence of four concentrations of Noggin. Zero-concentration samples or blank buffer samples were included as negative controls and later used to double-reference the data sets during analysis. The affinity to Noggin-Fc for each BMP was determined by Kd/Ka. As expected and as shown previously, BMP-4 demonstrated the highest affinity to Noggin with a KD of $6x10^{-}13M$. Interestingly, in this assay system, BMP-6 affinity to Noggin (KD = $1.3x10^{-}11M$) was higher than that of BMP-2 (KD = $6x10^{-}11M$) and BMP-7 (KD = $2x10^{-}10M$). Based on these data, it does not appear that the reduced susceptibility of BMP-6 to Noggin inhibition in functional assays is due to a low affinity of the BMP-6 to Noggin.

followed by autoradiography. Consistent with our functional data, Noggin effectively interfered with BMP-7, but not BMP-6 binding to BMP receptors (Figure 5). This effect was observed both in the case of BMPRII-ALK-2 and BMPRII-ALK-6 complexes. Thus, using cell based assays we found that Noggin consistently interferes with BMP-7, but not with BMP-6 receptor binding.

3.4.6 A central region of the mature domain of BMP-6 confers Noggin resistance

We subsequently sought to map the region conferring the functional resistance to Noggin in the BMP-6 protein. To this end, we compared the amino acid sequences of BMP- 6 and its closest paralog BMP-7, and identified residues that are different between these two molecules (Figure 6A). Based on this analysis, we generated three

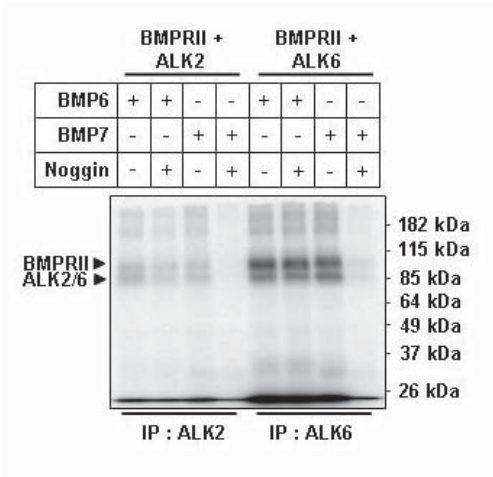


Figure 5: Noggin inhibits BMP-7 but not BMP-6 binding to BMP receptors. Indicated BMP receptors were transfected into COS cells and affinity labeled by crosslinking with radio-labeled BMP-6 or BMP-7 in the absence or presence of Noggin. Affinity labeled BMP-BMP receptors were immunoprecipitated from cell extracts with ALK-2 or ALK-6 specific antibodies. Samples were washed, boiled in SDS sample buffer and subjected to SDS-PAGE. Gels were dried and scanned with the STORM imaging system (Amersham). BMPR-II represents BMPR-II-BMP crosslinked complex and ALK-2 and ALK-6 represent ALK-2-BMP and ALK-6-BMP crosslinked complexes, respectively.

BMP- 6/BMP-7 chimeras (40-1, 80-1 and 90-1) by substituting BMP-6 sequences for their corresponding regions in BMP-7 (Figure 6B). Each chimera was expressed by transient transfection in HEK293T cells and tested for susceptibility to Noggin inhibition using a BRE-luc reporter assay in A549 cells. Wild type BMP-7 and BMP-6 proteins were also expressed and used as controls. The activities of 40-1 and 90-1 were inhibited by Noggin with similar efficiency as that of wild type BMP-7 (Figure 6C).

In contrast, 80-1 resistance to Noggin inhibition was comparable to that of BMP-6 (Figure 6C). To eliminate the possibility that differences between the three chimeras were due to differences in expression levels and/or the presence of other factors in the conditioned media, we purified 80-1 from transfected 293 conditioned medium and tested its activity and susceptibility to Noggin inhibition (Figure 6D). As expected, the susceptibility of purified 80-1 chimera to Noggin inhibition was comparable to that of wt BMP-6. These results suggest that the BMP-6 region extending from residues 45 to 80 in the mature domain is responsible for conferring Noggin resistance.

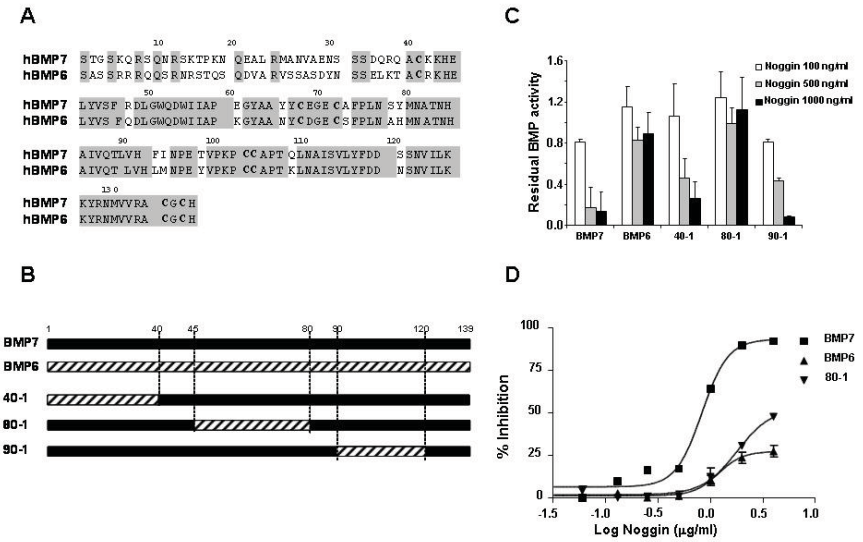


Figure 6: Identification of region in BMP-6 that is responsible for increased resistance to Noggin inhibition (a) Alignment of BMP-6 and BMP-7 mature domains. Shared amino acid residues are shaded. (b) Schematic representation of BMP-6 (shaded), BMP-7 (black) and BMP- 6/7 chimeras. (c) The chimera 80-1 is as resistant to Noggin inhibition as BMP-6. (d) The sensitivity of purified recombinant 80-1 chimera to Noggin inhibition is comparable to that of BMP-6. 80-1 chimera was purified from the conditioned medium of transfected HEK 293 cells and BMP-7 and BMP-6 were purified from stable CHO expressing cell lines. A549-BRE cells were challenged with either BMP-6, BMP-7 or 80-1 proteins, in the presence of increasing concentrations of Noggin. Luciferase activity was measured 24 hrs post BMP treatment and the percent inhibition was calculated for each Noggin concentration.

3.4.7 A single amino acid substitution in BMP-7 yields a protein with increased resistance to Noggin

BMP-6 and BMP-7 differ in seven residues in the region extending from amino acids 45 to 80 of their mature domains (Figure 7A). To identify the residue(s) responsible for Noggin resistance within this region, we first converted each of these seven residues (48, 60, 65, 68, 72, 77 and 78) in the 80-1 chimera back to its native amino acid in wt BMP-7. Revertants (Rev) generated in this fashion were expressed by transient transfection in HEK293T cells and secreted in the conditioned medium (Supplemental Figure 1A). Conditioned media containing each of the revertants was used to induce BRE-Luc reporter gene expression in the A549 BRE cell line (see experimental procedures) in the presence of increasing concentrations of Noggin. Wt BMP-7, wt BMP-6 and 80-1 chimera were used as controls in these experiments. Six of the seven revertants were as resistant to Noggin as 80-1 and wt BMP-6 (Figure 7B, only data

for the highest Noggin concentration is shown), suggesting that these residues are not essential for Noggin resistance. In contrast, reverting lysine 60 back to a glutamic acid (Rev K60E) revealed a marked increase in susceptibility to Noggin as compared with 80-1 (Figure 7B). These results suggest that lysine 60 (K60) is a critical residue contributing to BMP-6 resistance to Noggin inhibition. This was confirmed by generating single amino acid mutants of BMP-7 at positions 60 (E60K), 65 (Y65N) or 78 (Y78H). Each of these mutants was expressed by transient transfection in HEK293T cells and secreted in the conditioned medium (Supplemental Figure 1B). The susceptibility of each of these BMP- 7 mutants to Noggin inhibition was tested as described above for the revertant proteins. Of the BMP-7 mutants tested, E60K was significantly more resistant to Noggin compared to wild type, while Y65N had a marginal effect (Figure 7C, only data for the highest Noggin concentration is shown). A triple mutant BMP-7 protein, R48Q/E60K/Y65N was also generated and was not significantly more resistant to Noggin when compared to E60K (Figure 7C). These results further support the finding that lysine 60 (K60) plays a major role in mediating Noggin resistance in BMP-6.

3.4.8 Mutation of BMP-2 at a position analogous to BMP-7 E60 yields a BMP-2 variant with increased resistance to Noggin

Sequence alignment of BMP-2, 4, 5, 6, 7, and 9 revealed that only BMP-6 and BMP-9 have a lysine residue at the position corresponding to BMP-6 K60 (Figure 8A). Interestingly, side by side comparison of the sensitivity of purified recombinant BMP-2, BMP-6, BMP- 7, BMP-9 and BMP-7 E60K proteins to Noggin (Figure 8B) revealed that BMP-9 is also highly resistant to Noggin inhibition.

This data is in agreement with the recent finding by Rosen V. [24]. Moreover, this data confirm the increased resistance of BMP-7 E60K protein to Noggin inhibition since a purified form of this protein was used rather the conditioned medium from transfected cells. BMP amino acid sequence alignment also showed that a proline residue is found at the corresponding K60 position in BMP-2 and BMP-4 (Figure 8A). This prompted us to test if a proline to lysine mutation at this position in BMP-2 (BMP-2 P36K) could yield a BMP-2 variant with increased Noggin resistance. Such a mutant BMP-2 was generated and tested for Noggin resistance. As expected, this BMP-2 variant demonstrated a significant increase in Noggin resistance compared to its wild type counterpart, although the magnitude of effect was less pronounced than in the case of BMP- 7 (Figure 8C). Taken together, these data suggest that the function of BMP-6 lysine 60 in mediating Noggin resistant can be transferred to other BMPs making them more resistant to this BMP antagonist.

3.5 Discussion

Recombinant human BMP-2 and BMP-7 proteins have been used clinically for many years to induce new bone formation in spinal fusion [3] and long bone non-union

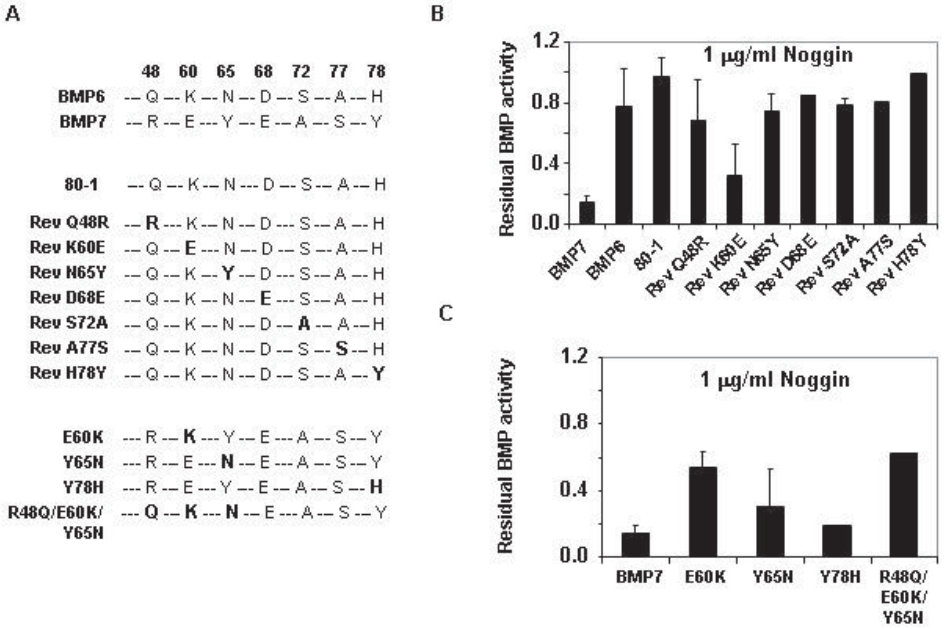


Figure 7: Identification of a single residue in BMP-6 that is responsible for increased resistance to Noggin inhibition (a) Amino acid substitutions (at positions indicated on top) in the 80-1 chimera, in the various revertants (Rev) and in the BMP-7 mutants (E60K, Y65N, Y78H and R48Q/E60K/Y65N). The corresponding amino acids in BMP-6 and BMP-7 are listed on top. Amino acid substitutions are highlighted in bold. (b) Reverting K60 to E in the 80-1 chimera significantly reduces its sensitivity to Noggin ($1\mu g$/mL)-mediated repression of BRE-luc activity in A549-BRE cells. (c) A single BMP-7 mutation at position 60 (E60K) significantly increases the protein resistance to Noggin ($1\mu g$/mL) inhibition. HEK293T cells were transfected with plasmids expressing BMP-7, BMP-6, 80-1 and each of the 80-1 revertants (Rev) (b) or BMP-7 mutants (c) and equal volumes of the conditioned media was used to induce luciferase expression in A549-BRE cells, in the presence or absence of Noggin ($1\mu g$/mL). Residual BMP activity was determined 24 hr post A549-BRE cell treatment.

fractures [36, 7]. Despite their safety and clinical efficacy, these proteins still have significant shortcomings [1]. The most intriguing of these are the relatively high doses of BMPs needed to achieve clinical success. We postulated that the strong negative feedback loop mechanisms built into the biology of these growth factors play a major role in attenuating their function following surgical implantation.

Here we show that while BMP-6 and BMP-7 are highly similar, BMP-6 is more potent in inducing osteoblast differentiation in vitro and bone formation *in vivo*. We postulate that a key underlying mechanism for this differential response could be that Noggin is more effective as a negative regulator for BMP-7 than for BMP-6.

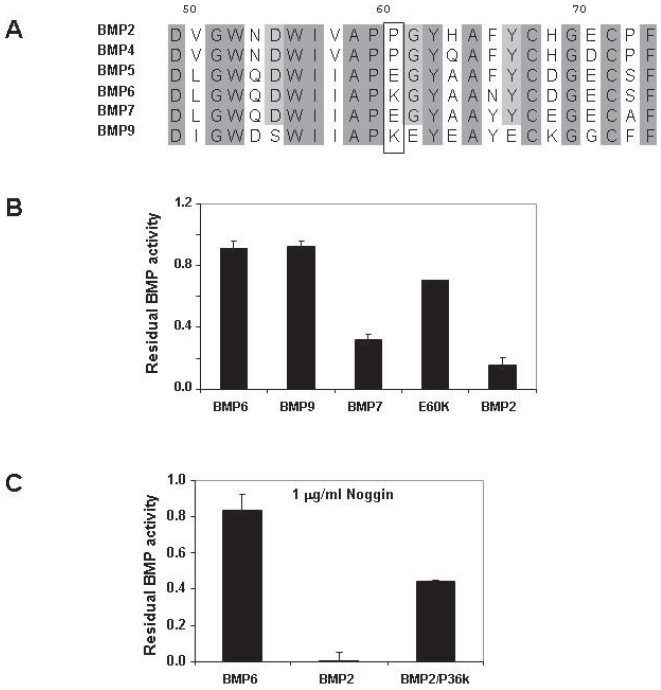


Figure 8: Generation of BMPs with superior agonistic activity (a) Amino acid sequence alignment of the region extending from residues 49 to 73 (BMP-7 numbering) in the mature regions of BMP-2, -4, -5, -6, -7, and -9. (b) The presence of a lysine at position 60 in BMP-6, BMP-9 and BMP-7 E60K is associated with resistance to Noggin inhibition. The activity of purified recombinant BMP-2, BMP-6, BMP-9, BMP-7 and BMP-7 E60K was assessed using the A549 BRE cells. BMP-7 E60K was purified from the conditioned medium following a large scale transfection of HEK 293 cells. BMP-7, BMP-2 and BMP-6 were expressed in CHO cells. BMP-9 was purchased from R&D Systems. A549-BRE cells were challenged with the purified recombinant proteins ($100ng/mL$), in the presence ($1\mu g/mL$) or absence of Noggin. Luciferase activity was measured 24 hrs post BMP treatment and the residual BMP activity was calculated (c) BMP-2 mutant P36K is significantly more sensitive to Noggin ($1\mu g/mL$) inhibition than the wild type BMP-2.

While BMP-7 induces Noggin expression more potently than BMP-6, BMP-6 is more resistant to Noggin inhibition than BMP-7. Moreover, siRNA- mediated depletion of Noggin sustains BMP- 7-induced Smad phosphorylation and cellular differentiation responses to levels similar to BMP-6. Importantly, we identified a single amino acid in BMP-6 that modulates its susceptibility to Noggin inhibition. We show a strong correlation between BMP resistance to Noggin inhibition and the presence of a lysine residue at position 60 of the mature domain (BMP-6 numbering). Amino acid

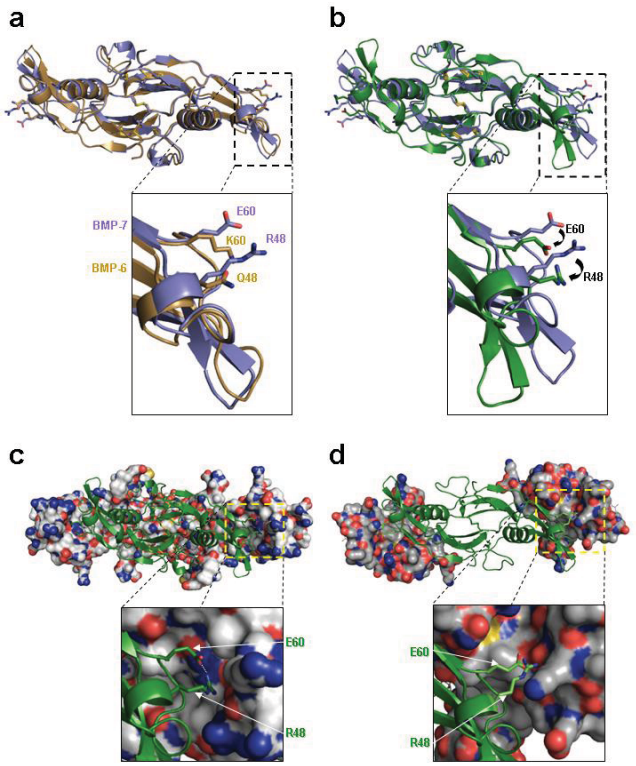


Figure 9: Structural representation of BMP-7 interaction with Noggin and ActR-II. (a) Structural comparison between BMP-6 (brown) and BMP-7 (blue). Images generated using The PyMOL Molecular Graphics System (2002). DeLano Scientific, Palo Alto, CA, USA. `http://www.pymol.org`. Insert shows enlarged view on the location of E60, R48 and Q48 amino acid residues. (b) BMP-7 structure when bound to Noggin (green) or in the free state (blue). Insert shows enlarged view on the location of E60 and R48 amino acid residues. (c) BMP-7/Noggin complex. BMP-7 displayed as a green secondary structure cartoon and Noggin as a molecular surface (carbon – white; oxygen – red; nitrogen – blue; sulfur – yellow). Insert shows enlarged view on the location of E60 and R48 amino acid residues (stick display). (d) BMP-7/ActRII complex. BMP-7 displayed as a green secondary structure cartoon and ActRII as a molecular surface (carbon – white; oxygen – red; nitrogen – blue; sulfur – yellow). Insert shows enlarged view on the locations of E60 and R48 residues (stick display)

sequence alignment of mature BMP-2, 4, 5, 6, 7, 8, 9, 10, 11 and 15 reveals that, in addition to BMP-6 and BMP-9, BMP-11 also has a lysine at this position (data not shown). Our data suggest that it is possible to engineer BMP variants with increased Noggin-resistance by substituting a lysine for the corresponding residue at position 60. This feature, if combined with others that enhance BMP specificity, stability, and

safety could allow the development of more effective recombinant BMPs in the future. The crystal structures of BMP-7 [11] and BMP-6 [2, 27] mature domains have both been solved. Despite their remarkable similarities, the overlay of these two structures reveals significant differences at the tips of finger 1 and finger 2 (Figure 9A). Fingers 1 and 2 mediate BMP binding to both type I and type II BMP receptors. In the case of BMP-7, fingers 1 and 2 are more extended. Moreover, in BMP-6, K60 and N65 form intra-molecular hydrogen bonds between fingers 1 and 2, thus increasing the overall rigidity of the molecule. These interactions are missing in BMP-7, making it more flexible than BMP-6. Interestingly, BMP-7 E60 resides at the tip of finger 1. This region undergoes significant conformational change upon binding to Noggin (Figure 9B). In the Noggin-bound conformation, E60 forms an intra-molecular salt bridge with R48 and interacts with Noggin F54 (Figure 9C). BMP-7 E60 and R48 also engage in direct interactions with type II BMP receptors (Figure 9D). This interaction is preserved in BMP-6 despite the presence of lysine at position 60. Our functional cell-based studies clearly demonstrate that BMP-6 binding to cell surface receptors is less susceptible to Noggin compared to BMP-7. One possible explanation for this difference is that BMP receptors might displace BMP-6 from Noggin more efficiently than in the case of BMP-7- Noggin complex. This is supported by the observation that BMP-6 is more rigid than BMP-7 and thus may not undergo as much of a conformational change when bound to Noggin, making the complex more unstable in the presence of cell surface receptors. Solving the BMP-6-Noggin and BMP-6-BMP receptor ternary complex crystal structures could provide additional insight into the differential mechanisms of BMP-7 and BMP-6 inhibition by Noggin. In conclusion, a key determinant for the increased potency of BMP-6 compared to BMP-7 is the lower sensitivity of BMP-6 to Noggin-mediated inhibition. Identification of Lys 60 in BMP-6 that confers Noggin resistance allowed us to generate BMPs with superior agonistic activity that hold promise for future clinical benefit.

3.6 Acknowledgments

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3.7 Supplementary Data

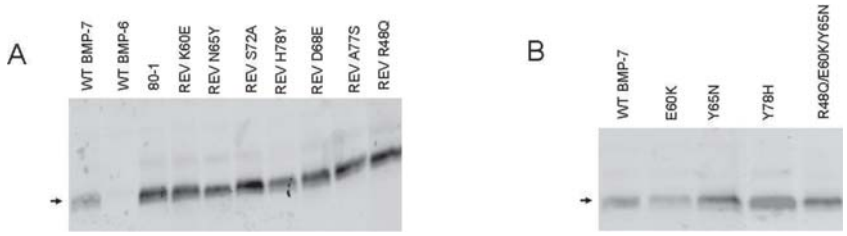

Figure S1: BMP mutant expression analysis by western blot. HEK293T cells were transiently transfected with DNA plasmids encoding each of the revertants (a) or BMP-7 mutants (b) 48 hours post-transfection, the proteins content of the tissue culture conditioned medium was resolved by SDS-PAGE and analyzed by western blot using a rabbit polyclonal anti- BMP-7 antibody.

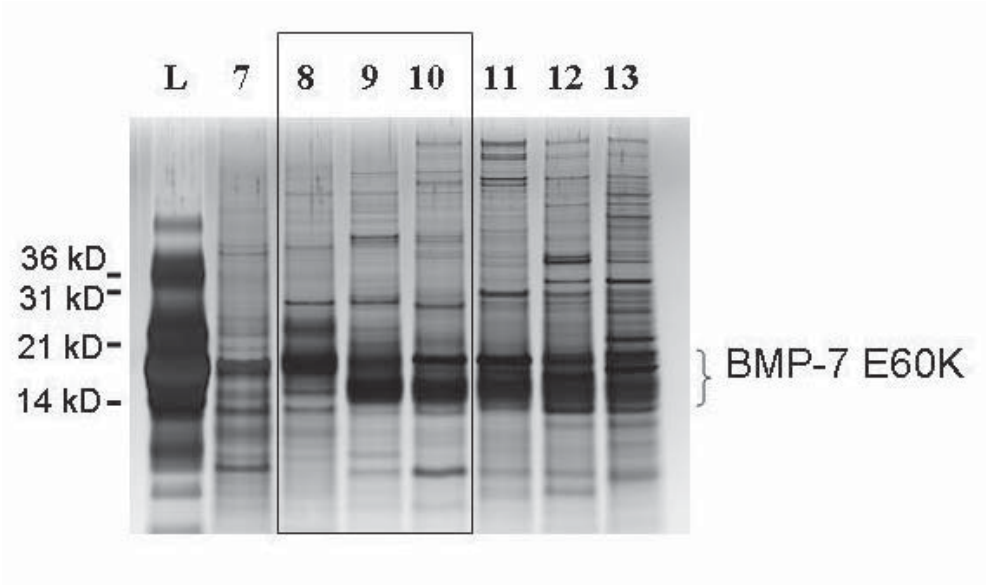

Figure S2: Purification of the BMP-7 E60K mutant protein. HEK293T cells were seeded into $30 \times 225 cm^2$ flasks and transiently transfected with a plasmid encoding the BMP-7 E60K protein. 1.2 L of conditioned medium was collected and used to purify recombinant BMP-7 E60K protein as described in Materials and Methods. HPLC fractions containing the mutant BMP-7 protein were analyzed by SDS-PAGE followed by silver staining. Fractions 8 through 10 were pooled, lyophilized and stored until further use. The position of the BMP-7- E60K protein is indicated on the right. Molecular weight markers are indicated on the left of the gel. L: loaded material on the HPLC column.

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