Chapter 7
Identification of kinases involved in BMP signalling

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Abstract

Bone Morphogenetic Proteins (BMPs) play an important role in a variety of processes, such as bone formation and angiogenesis. In cancer, BMP-Smad signaling is often deregulated. In this study, we sought to identify protein kinases, which affect BMP signaling. Using an overexpression approach, we were able to identify known players in BMP signaling, such as ALK1, ActRIIA and ActRIIB . In addition, we identified two novel regulators of BMP signaling: ribosomal protein S6 kinase, 90kDa, polypeptide 4 (RPS6KA4) and Cyclin Dependent Kinase 8 (CDK8). RPS6KA4 enhanced BMP signaling, whereas CDK8 inhibited BMP signaling. Considering their involvement in cancer progression these protein kinases may play an important role in oncogenic BMP-Smad signalling.

Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)-β superfamily of growth factors and are involved in a wide variety of processes, including bone formation, angiogenesis and cardiac development. A dozen of BMPs have been identified up to now, which all signal in a similar manner [1]. Ligand binding induces the formation of a heteromeric complex, consisting of a type I receptor and a type II receptor. BMPs are able to bind to the type I receptors activin receptor-like kinase (ALK) 1, 2, 3 and 6 and to the type II receptors BMP Receptor type II (BMPRII), Activin Receptor II (ActRII) A and B. Within this ligand-receptor complex, the type II receptor phosphorylates the type I receptor, which in turn phosphorylates Smad1, 5 and 8. This phosphorylation causes a conformational change in these Smads, which allows them to interact with Smad4 to form heteromeric complexes. This complex translocates into nucleus, where it affects transcription of target genes [1,2]. Besides, the induction of Smad signalling, BMPs may also induce non-Smad signalling pathways. For example, the BMP receptors activate TGF-β activated kinase-1 (TAK-1), which mediates the activation of p38 and JNK [1].

BMPs are necessary for tissue homeostasis and deregulation of BMP signalling leads to vascular disorders, such as primary pulmonary hypertension (PAH) and skeletal disorders, such as fibrodysplasia ossificans progressiva (FOP) [1]. In addition, disturbed BMP signalling has been implicated in cancer. Mutations in the *SMAD4* and *ALK3* genes result in juvenile polyposis, a hereditary form of colorectal cancer [3]. Furthermore, the BMP pathway is also inactivated by mutations in the BMP receptors in the majority of sporadic colorectal cancers during progression from adenoma to carcinoma [4,5]. In glioblastoma, BMP-4 treatment inhibited tumor growth [6]. However, in other types of cancers BMPs may either promote or inhibit tumorigenesis and metastasis. For example, loss of BMPRII has been correlated with poor prognosis in prostate cancer, whereas BMP-6 expression has been correlated with metastasis in prostate cancer [7]. The interplay with other growth factors or hormones may affect the outcome. For example, the effect of BMP-7 on prostate cancer cells relies on their hormone dependency. In androgen sensitive cell lines, BMP-7 inhibits proliferation, whereas it has no effect on androgen insensitive cell lines [8]. BMP-4 only stimulates cell growth in the presence of growth factors, such as Hepatocyte Growth Factor, Epidermal Growth Factor and [9]. Thus, the role of BMPs in cancer is highly context-dependent.

Interplay between the BMP-Smad signals and signals from other growth factors can be regulated by phosphorylation. For example, extracellular regulated kinase (ERK) phosphorylates Smad1 in the linker regions in response to growth factors, which results in exclusion of Smad1 from the nucleus [10]. Smad1 is also phosporylated by Glycogen synthase kinase 3 (GSK3), which results in poly- ubiquitination and subsequent degradation of Smad1. Stimulation with Wnt decreases the levels of GSK3, with a concomitant increase in Smad1 protein levels and a an increase in BMP signalling activity [11]. Thus, kinases play an important role in regulating BMP signalling.

In this study, we sought to identify novel kinases which modulate BMP-Smad signalling in cancer. To this end, we made use of a human kinome overexpression library [12]. Compared to a loss of function approach, use of this gain of function approach allows for identification of kinases that are not expressed by the cell type, act redundantly or

activate pathways only under pathological conditions. Using this library, we found known and novel regulators of BMP signalling. Further validation identified two novel kinases which regulate BMP-Smad signalling.

Materials and Methods

Cell culture

C2C12 mouse myoblast and human embryonic kidney (HEK) 293T transformed cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 50 μg/ml streptomycin (Gibco). C2C12 cells were not allowed to grow beyond 70-80% confluency. All cell lines were grown in a humidified incubator at 37 °C and 5 % CO_2.

Constructs

The cDNA kinase expression library [12] and BMP Responsive Element (BRE)-Luc reporter construct [13] have been described before. The *Renilla* luciferase construct pRL-SV40 was purchased from Promega. Constructs encoding human Ribosomal protein S6 kinase, 70kDa, polypeptide 1 (RPS6KB1), Mitogen Activated Kinase Kinase Kinase 10 MAP3K10, ribosomal protein S6 kinase, 90kDa, polypeptide 4 (RPS6KA4), ephrin type-A receptor 2 (EPHA2), ephrin type-B receptor 2 (EPHB2) and EPHB3 in pDNOR221 [12] were cloned into pcDNA DEST40 (Invitrogen) using the Gateway system (Invitrogen). Mouse Cyclin Dependent Kinase 8 (CDK8) shRNA constructs were from the TRC1 shRNA library (Sigma), namely constructs constructs TRCN0000023107 and TRCN0000023104. Construct SHC002, expressing a non-targeting shRNA, was used as a negative control.

Kinase screen and data analysis

The kinase screen was performed in a 96-well plate format. Luciferase-reporter screening was performed in C2C12 cells, using 50 ng of protein kinase expression constructs and 50 ng of reporters (1:19 mix of control to pathway reporter) per well. The *Renilla* luciferase construct pRL-SV40 (Promega) was used as a control reporter; BRE-luc was used as a specific reporter for the BMP pathway. One day after transfection using FuGENE HD (Roche),

cells were serum starved over night and subsequently stimulated with BMP-6 (100 ng/ml; a kind gift of K. Sampath, Creative Biomolecules, Inc, Hoptinton, USA) in medium containing 0.5% serum. After 8 hr of stimulation, firefly and *Renilla* luciferase activities were determined using the dual-Luciferase kit (Promega), followed by subtraction of background luminescence counts from untransfected cells. Relative luciferase activities were calculated by dividing the pathway-specific firefly luciferase counts by the control *Renilla* luciferase counts separately for all replicates, and sample mean and standard deviation (SD) were calculated from these values (n = 3). Constructs that caused a severe drop in *Renilla* luciferase counts (below 15000 counts) were classified as toxic and were not analyzed further. Data was analyzed using GraphPad Prism.

Luciferase assays

C2C12 cells were seeded into a 24 wells plate and transfected the next day for 4 hr with 100 ng BRE-Luc and 100 ng of the kinase overexpression or knockdown construct using Lipofectamine (Invitrogen). An expression plasmid for β-galactosidase (200 ng) was co-transfected to correct for transfection efficiency. The next day, cells were serum starved overnight and stimulated with BMP-6 (100 ng/ml) for 8 hr. Luciferase and β-galactosidase acitivity were determined as previously described [14]. Each transfection was carried out in triplicate and representative experiments are shown.

Western blot

293T cells were seeded into a 6 wells plate and were transfected with 500 ng kinase overexpression construct using polyethylenimine (Sigma-Aldrich) and harvested two days after transfection in Laemmli buffer. C2C12 cells were transfected using Lipofectamine (Invitrogen), harvested 3 days after transfection in Laemmli buffer without bromophenol blue and β-mercaptoethanol. 10 μg of protein was used for SDS-PAGE & Western blotting. V5-antibody was from Invitrogen and CDK8 antibody was from SantaCruz. Staining for β-actin (Sigma) was used as a loading control.

Results

Kinase screening identifies putative positive regulators of BMP-Smad signalling

To identify new players in BMP-Smad signalling, we made use of a kinome cDNA expression library. We used activation of the transcriptional reporter BRE as a readout in the highly BMP responsive myoblast cell line C2C12. Without ligand stimulation, ectopic overexpression of protein kinases previously implicated in BMP-Smad signalling, namely ActRIIA, ActRIIB and ALK1, strongly induced promotor activity (figure 1A). Interestingly, ALK4 and ALK7, receptors for the TGF-β superfamily ligands Activin and Nodal, also enhanced basal BRE-reporter activity, 2.6 fold and 5.5 fold respectively. This suggests positive crosstalk between Activin/Nodal and BMP-Smad pathways. When cells were stimulated with the ligand BMP-6, overexpression of ActRIIA further enhanced reporter activity (figure 1B). The positive identification of known BMP-Smad pathway regulators proved that our high throughput system is suited for the search for new kinases involved in the control of BMP signaling

Further analysis of the results revealed a number of kinases which enhanced BMP-6 induced reporter activity more strongly than ActRIIA (Figure 1C, Figure 2A). Especially mitogen- activated protein kinase kinase kinase 10 (MAP3K10) had a strong effect on Smad1 transcriptional activity, whereas ribosomal protein S6 kinase 90kDa polypeptide 4 (RPS6KA4) enhanced reporter activity to a lesser extent. Interestingly, three members of ephrin receptor subfamily (EPH) of receptor protein tyrosine kinases, namely EPHA2, EPHA3 and EPHB2, enhanced reporter activity to the same extent as ActRIIA. Our results suggest that the kinases MAP3K10, RPS6KA4, EPHA2, EPHA3 and EPHB2 are potential positive regulators of BMP-Smad signalling.

Kinase screening identifies possible negative regulators of BMP-Smad signalling

A large number of kinases inhibited BMP-6 induced reporter activity. Among these were different splice variants of TGF-β activated kinase (TAK)-1 (Figure 1B), which has been shown previously to negatively effect BMP-Smad signalling by interfering with the DNA

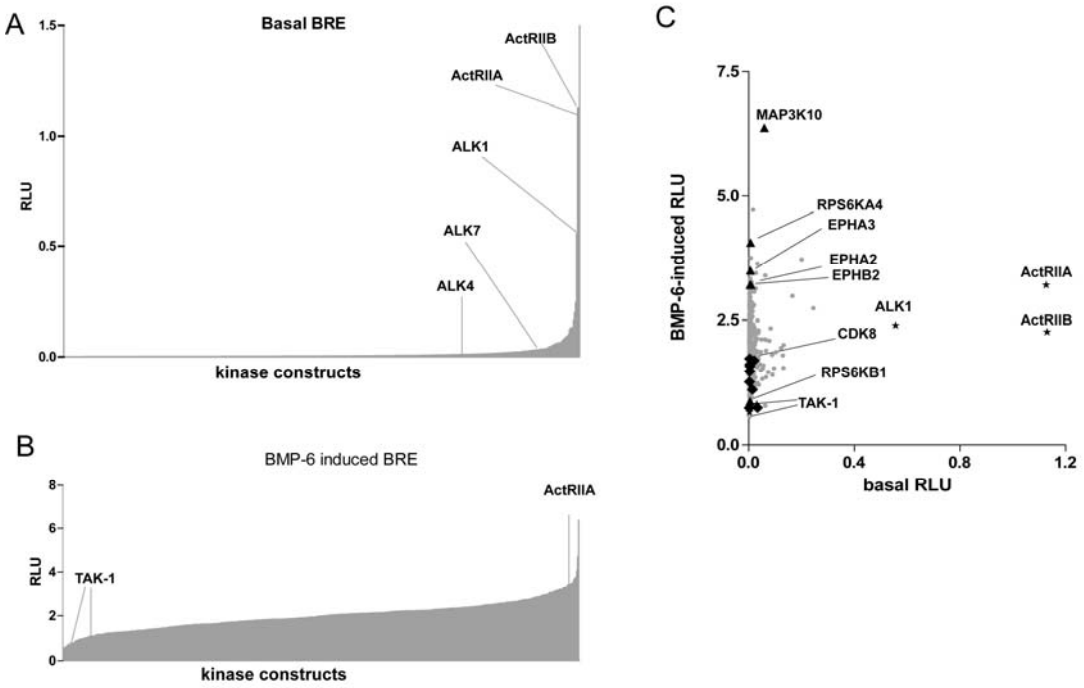

Figure 1 *Kinase cDNA screen for novel BMP pathway regulators.* (A and B) Effect of kinase expression on the BMP reporter BRE-Luc activity in C2C12 in the absence (A) or presence (B) of BMP-6 (100 ng/ml). The kinome overexpression constructs were co-transfected with the BRE-Luc reporter in C2C12 cells. Cells were starved and mock treated or BMP-6 (100 ng/ml) stimulated for 8 hours. The RLU of the kinases were plotted after sorting for RLU value. (C) Several kinases affect the BMP pathway. Basal RLU is plotted against BMP-6-induced BRE. Kinases for follow-up are indicated with triangles, kinases with a known function in the BMP pathway are indicated with stars and CDKs are indicated with diamonds. Relative luciferase activity (RLU) is expressed as mean of triplicate cultures.

binding domain of Smads [15]. One kinase negatively affecting BMP-6-induced reporter activity was ribosomal protein S6 kinase 70kDa polypeptide 1 (RPS6KB1) (Figure 1C, Figure 2B). Interestingly, cyclin dependent kinases, namely CDK2,4, 5, 8 and 9 all had an inhibitory effect on BMP-6-induced reporter activity (Figure 2B). These kinases are essential for cell cycle progression and they are frequently overexpressed in cancer [16]. Taken together, RPS6KB1, CDK2, 4, 5, 8 and 9 are possible negative regulators of BMP-Smad signalling.

RPS6KA4 is a positive regulator of BMP-Smad signalling

To further validate the effect of the different kinases on BMP-Smad signalling MAP3K10, RPS6KA4, EPHA2, EPHB2, EPHA3, and RPS6KB1 cDNAs were cloned into pcDNA DEST40 containing a C-terminal triple V5 tag. The expression levels of these constructs was analyzed in 293T cells by Western blotting following transfection of the different constructs (Figure 3A). In order to analyze their effects on BMP induced transcriptional activity using the BRE reporter construct. V5-Tagged MAP3K10, RPS6KA4, EPHA2, EPHB2, EPHA3, and RPS6KB1 were overexpressed in C2C12 cells together with the BRE reporter construct. As shown in Figure 3B, only the stimulatory effect of RPS6KA4 was reproducible, whereas ectopic expression of MAP3K10, RPS6KB1, EPHA2, EPHA3 and EPHB2 had no effect on BMP-6-induced BRE-Luc activity. CDK2 and CDK4 were the strongest inhibitors. These two CDKs were already known to phosporylate Smad2 and 3, which are homologous to Smad1, 5 and 8, resulting in inhibition of their transciptional activity [17]. Since CDK8 is also a colorectal oncoprotein [18] and BMP signalling is often inactivated in colorectal cancer [3], we decided to further characterise the effects of CDK8. We chose to analyze if knockdown of CDK8 had a stimulating effect on BMP-Smad signalling in C2C12 cells. Knockdown efficiency was determined by Western blot. Two constructs gave sufficient knockdown in C2C12 cells (Figure 3A). These constructs strongly enhanced BMP-6-induced BRE-luc activity, thus indicating that CDK8 is a negative regulator of BMP-Smad signalling.

Discussion

In this study, we performed a protein kinase overexpression screen to identify novel players in BMP-Smad signalling. The reliability of the screen was reinforced by the identification of kinases already known to be involved in BMP-Smad signalling. The receptors ALK1, ActRIIA and ActRIIB enhanced BMP reporter activity, whereas TAK-1 inhibited BMP signalling. The first ones are receptors for BMPs [1,2] , whereas the latter has been reported to bind to the DNA binding domain of Smads to prevent transcriptional transactivation [15]. However, TAK-1 was shown to be essential for activation of BMP-Smad signalling during chondrogenesis [19,20]. The previous study showing negative effects of TAK-1 in BMP-

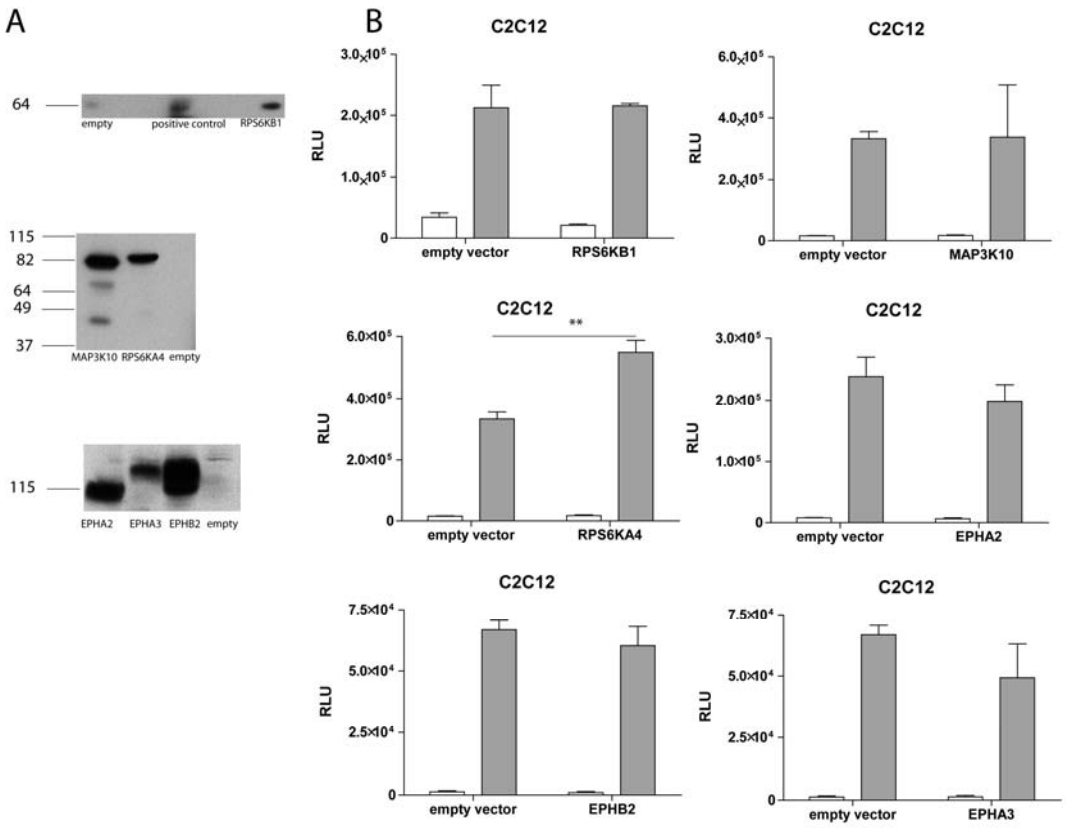

Figure 2 *Validation of the functional role of the identified kinases from the cDNA screen on the BMP signalling.* (A) Potential BMP pathway modifiers MAP3K10, RPS6KA4, EPHA2, EPHB2, EPHA3 and RPS6KB1 were cloned into pcDNA DEST40 vector to generate V5 tagged kinases. These constructs were transfected into 293T cells and expression was verified using a V5 specific antibody. (B) Kinase constructs were co-transfected with the BRE-Luc reporter into C2C12 cells. Cells were starved and mock treated or BMP-6 (100 ng/ml) stimulated for 8 hours. Relative luciferase activity (RLU) is expressed as mean ± S.D. of triplicate cultures. Significance ** $p < 0.01$

Smad signalling was performed in the murine mesenchymal stem cell line C3H10T½ during osteogenesis [15], whereas BMPs also induce osteoblastic differentiation in our C2C12 cells [21]. Thus, it is likely that the effect of TAK-1 is highly dependent on the cellular context.

Interestingly, the type I receptors for Activin and Nodal, ALK4 and ALK7, enhanced basal reporter activity. Such positive effect of the Activin signalling has been also described during chicken development where Activin signalling enhanced BMP signalling through membrane-bound inhibitor (BAMBI) [22].

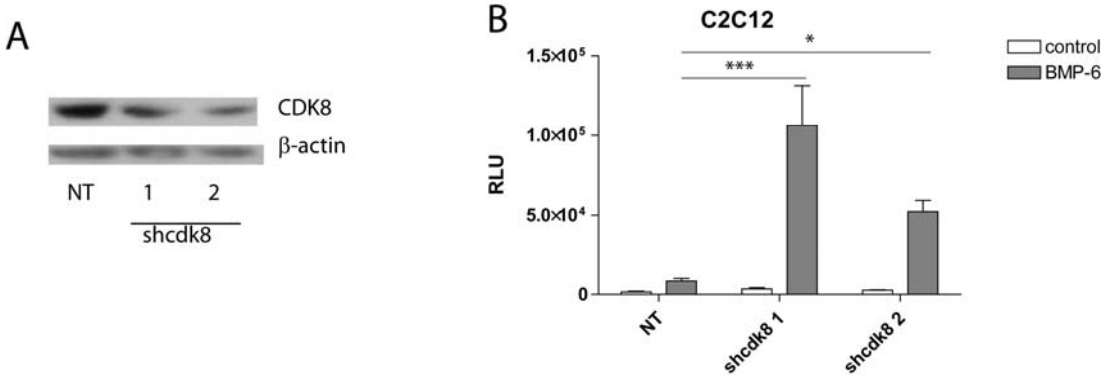

Figure *4 CDK8 as a potential negative regulator of BMP signalling.* (A) C2C12 cells were transfected with the indicated constructs. Knockdown was verified by Western blot. (B) C2C12 cells were transfected with the indicated constructs and the BRE-Luc reporter construct, starved and stimulated with no ligand or BMP-6 (100 ng/ml). Relative luciferase activity (RLU) is expressed as mean ± S.D. of triplicate. Significance * $p < 0.05$ *** $p < 0.001$

A large number of kinases affected BMP signalling in the initial kinome overexpression screen. These included MAP3K10, RPS6KA4, EPHA2, EPHB2, EPHA3, RPS6KB1 and CDK8. However, the effect of most kinases could not be reproduced. This might be due to differences in assay set-up. Most notably, fold activation in the follow-up experiments was much lower than in the original screen. Another possibility is that the tag might influence the kinase activity, despite being rather small.

Validation identified RPS6KA4 as a positive regulator of BMP signalling. RPS6KA4, also known as Mitogen and Stress-activated Kinase 2 (MSK2) or Ribosomal S6 Kinase B (RSK-B), acts downstream of p38 and phosphorylates the transcription factor cAMP Responsive Element Protein (CREB) [23]. Phosphorylated CREB has been shown to form a transcriptional complex with Smad1/5/8 and CREB binding protein (CBP) resulting in enhanced BMP-Smad signalling [24]. Thus RPS6KA4 may potentiate BMP signalling by increasing the pool of phosphorylated CREB which can interact with Smad1 and CBP to activate transcription.

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Taken together, we have identified novel kinases regulating BMP-Smad signalling. If these kinases play a role in oncogenic BMP signalling is subject for further study.

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