Research article

Smad1 STABILIZATION AND DELOCALIZATION IN RESPONSE TO THE BLOCKADE OF BMP ACTIVITY

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Abstract: Signaling at the plasma membrane receptors is generally terminated by some form of feedback regulation, such as endocytosis and/or degradation of the receptors. BMP-Smad1 signaling can also be attenuated by BMP-induced expression of the inhibitory Smads, which are negative regulators of Smad1 transactivation activity and/or BMP antagonists. Here, we report on a novel Smad1 regulation mechanism that occurs in response to the blockade of BMP activity. Lowering the serum levels or antagonizing BMPs with noggin led to upregulation of Smad1 at the protein level in several cell lines, but not to upregulation of Smad5, Smad8 or Smad2/3. The Smad1 upregulation occurs at the level of protein stabilization. Upregulated Smad1 was relocalized to the perinuclear region. These alterations seem to affect the dynamics and amplitude of BMP2-induced Smad1 reactivation. Our findings indicate that depleting or antagonizing BMPs leads to Smad1 stabilization and relocalization, thus revealing an unexpected regulatory mechanism for BMP-Smad1 signaling.

Key words: Smad1, BMPs, Feedback regulation, Degradation, Noggin, BMPRIA, PPM1A, Serum starvation, Intestinal villi, Smad2/3

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INTRODUCTION

Bone morphogenetic proteins (BMPs) are a subfamily of the TGFβ superfamily [1, 2]. Like the TGFβs, BMPs activate the canonical Smad pathway (Smad1/5/8 for BMPs and Smad2/3 for TGFβ) and the non-canonical MAPK pathways [3, 4]. BMPs bind to the plasma membrane-localized BMP receptor II (BMPRII), leading to the formation of the BMPRII-BMPRI complex. BMPRII, which is constantly active as a Ser/Thr kinase, phosphorylates and activates BMPRI, which in turn phosphorylates Smad1/5/8 at the C-terminal SXS motif. Phosphorylated Smad1/5/8 form a complex with Smad4, which is then translocated into the nucleus, where it binds to the BMP-responsive element and regulates the transcription of the target genes [1, 4]. BMP-induced MAPK activation is believed to be mediated by a protein complex containing Tab1-Tak1 (an MAPKKK), which is recruited to BMPRI via Xiap or Traf6 [1, 5]. The physiological function of BMP-induced MAPK activation remains under-explored.

More than a dozen BMPs have been identified. They play critical roles in many cellular processes and in tissue homeostasis and development [6, 7]. BMPs form a morphogen gradient that controls the dorsal-ventral patterning of invertebrate and vertebrate embryos [8, 9]. Mouse genetic studies confirmed the essential roles for BMP-Smad1/5/8 signaling in early embryonic development and postnatal tissue homeostasis [7]. It was shown that any three alleles of Smad1 and Smad5, but not any two alleles, can support the proper development of mouse embryos [10]. In adult mice, BMPs have been shown to play critical roles in regulating the proliferation and differentiation of tissue stem cells, including neural and mesenchymal stem cells [11-13]. The tight regulation of BMP-Smad1/5/8 signaling is physiologically important: dysregulation of this signaling has been found to result in tumor formation and the development of bone and vascular diseases [6, 7, 14-16]. BMP-Smad1 signaling inhibits tumorigenesis via the prominent tumor-suppressive Atm-p53 pathway. In response to DNA damage, Smad1 is upregulated at the protein level. This is important for optimal p53 induction and tumor suppression [17].

Various mechanisms have evolved to ensure the precise regulation of BMP-Smad1 signaling [1-4]. The strength of BMP signaling is reflected by the duration and the amplitude of Smad1/5/8 SXS phosphorylation [1]. Many negative regulators have been found along the pathway [7, 18]. A number of antagonists reside in the extracellular matrix, where they sequester BMPs and inhibit their function. One of the best-studied examples is noggin [19]. At the receptor level, activation is regulated by dephosphorylation, degradation and endocytosis-mediated lysosome localization [20]. Smad1/5/8 activation is regulated by the inhibitory Smads, Smad6 and Smad7, dephosphorylation by protein phosphatases, such as PPM1A, and increased degradation by Smurf1 and Smurf2 [21-25]. Nuclear proteins, such as Sno and Ski, also inhibit the transactivation activity of Smad proteins via direct binding [14]. Some of the

negative regulators, such as Smad6 and Smad7, are direct target genes of BMP-Smad1/5/8 signaling, thus they form a negative feedback regulation loop.

In this study, we found that serum depletion or noggin treatment resulted in upregulation of Smad1 and relocalization of the Smad1 protein at the perinuclear region. The upregulation of Smad1 under these conditions occurs at the level of protein stability. More importantly, the Smad1 upregulation and relocalization showed an effect on Smad1 reactivation in response to BMPs.

MATERIALS AND METHODS

MEF isolation protocol

Primary mouse embryonic fibroblasts (MEFs) were isolated from 13.5-day old embryos. Female mice that had been pregnant for 13.5 days were killed by cervical dislocation. The uterine horns were removed, rinsed in 70% (v/v) ethanol, and placed into a petri dish containing sterile PBS. The embryos were taken out and the head and liver were removed. The embryos were minced with scissors and then digested in 0.5 ml of trypsin-EDTA in a 6-well plate. After 20 min to 1 h digestion at 37ºC, 2 ml of DMEM medium was added and the samples were passed through 18G syringes to break the tissue paste into a single-cell suspension, which was then transferred to 6-cm plates for further culture.

Cell culture

MEFs were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). For Western blot and RNA isolation, MEFs were plated onto 6-well plates and grown overnight. They were then switched to DMEM containing different concentrations of FBS and further cultured for the time designated. The cells were then washed with PBS and kept at -80ºC. COS7, an African green monkey fibroblast or fibroblast-like cell line, and 293T, a human embryonic kidney cell line, were cultured in DMEM containing 10% FBS. Wild-type and *Bmpr1a*-/- mouse embryonic stem cells (provided by Professor Yuji Mishina) were cultured in ES cell-specific medium and serum in the presence of LIF.

Cell proliferation assays

To test the proliferation capacity of primary MEFs, cells were cultured in 96-well plates in a final volume of 100 μl/well and then either treated with noggin for 48 h or infected with retroviruses expressing SXS-deleted Smad1 (or the empty retrovirus as a control) for 48 h. Then, 10 μl/well of the cell proliferation reagent WST-1 was added to each well and the cells were further incubated for 4 h in a humidified atmosphere. The cell culture plates were then shaken thoroughly for 1 min on a shaker and measured for absorbance at 450 nm using an ELISA reader.

Western blot analysis

Cells were lysed in TNEN buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 and 0.1% Triton X-100) supplemented with 1 mM NaF, 100 mM Na_2VO_3, 1 mM PMSF, and 1 μg/ml of aprotonin, leupeptin and pepstatin. The

protein concentration was determined using a Bio-Rad kit. Proteins were resolved using SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck Millipore, BSN, USA). The antibodies against the following proteins were used for Western blot: actin (sc-81178, 1:1000, Santa Cruz Biotechnology, CA, USA), p-Smad1 (9511, 1:1000, Cell signaling, Danvers, MA, USA), and Smad1 (9743, 1:1000, Cell signaling, Danvers, MA, USA).

RNA extraction and real-time PCR
Total RNA was extracted from cells that were grown on 60-mm dishes using Trizol reagent (Life Technologies, CA, USA). Total RNA was subjected to DNase treatment and quantified. Then, 5 μg of total mRNA was reverse transcribed into complementary DNA (cDNA) using AMV Reverse Transcriptase (Roche, NJ, USA) and used for real-time PCR analysis (Roche LightCycler 480, NJ, USA). The primers were designed following the manufacturer's instructions.

Immunofluorescence staining and immunohistochemical staining
MEFs were cultured on coverslips in 6-well plates. After serum starvation or noggin treatment, the cells were fixed with freshly made 4% paraformaldehyde and blocked with 5% BSA in PBS with 0.5% Triton X-100 for 30 min. The cells were incubated with primary antibody overnight at 4ºC and processed according to a standard protocol for immunocytochemical staining. The anti-Flag antibody was purchased from Sigma-Aldrich. To detect Smad1 in the mouse intestine, intestines of adult mice were fixed in 4% paraformaldehyde in PBS overnight and processed for paraffin embedding. Sections of 4 μm were cut using a rotary microtome. Paraffin-embedded sections were deparaffinized and rehydrated. After blocking the endogenous peroxidase activity with methanol containing 0.3% hydrogen peroxide for 30 min, the sections were incubated with primary antibody overnight at 4ºC, and then washed three times in PBS for 10 min and incubated with secondary antibodies at 1:100 in PBS for 1 h at 37ºC. After washing three times in PBS for 10 min, the slides were incubated with 3,3-diaminobenzidin (DAB) at room temperature without light for 10 min, followed by hematoxylin staining for 1 min. Slides were then dehydrated and mounted with neutral gums.

Statistical analysis
Each experiment was repeated three or more times. Statistical analysis was performed using an unpaired t test (STATISTICA software; StatSoft, Inc.). Significant association was defined when the P value was less than 0.05.

RESULTS

Lowering serum levels led to upregulation of Smad1 in several cell types
During our study of BMP-Smad1 activation in mouse embryonic fibroblasts, we sometimes needed to starve the cells of serum before adding BMPs. Surprisingly, we found that the Smad1 protein level increased in the absence of

serum, while the protein levels of Smad5, Smad8 and Smad2/3 did not change (Fig. 1A). Smad1 upregulation was also observed in other cell lines tested, including COS7 and 293T (Fig. 1B and C), suggesting that this is a rather common cellular response. To confirm this finding, we switched primary MEF cultures from 10% serum to different concentrations of serum for 4 or 8 h and determined the protein levels of Smad1 with western blot analysis. It is obvious
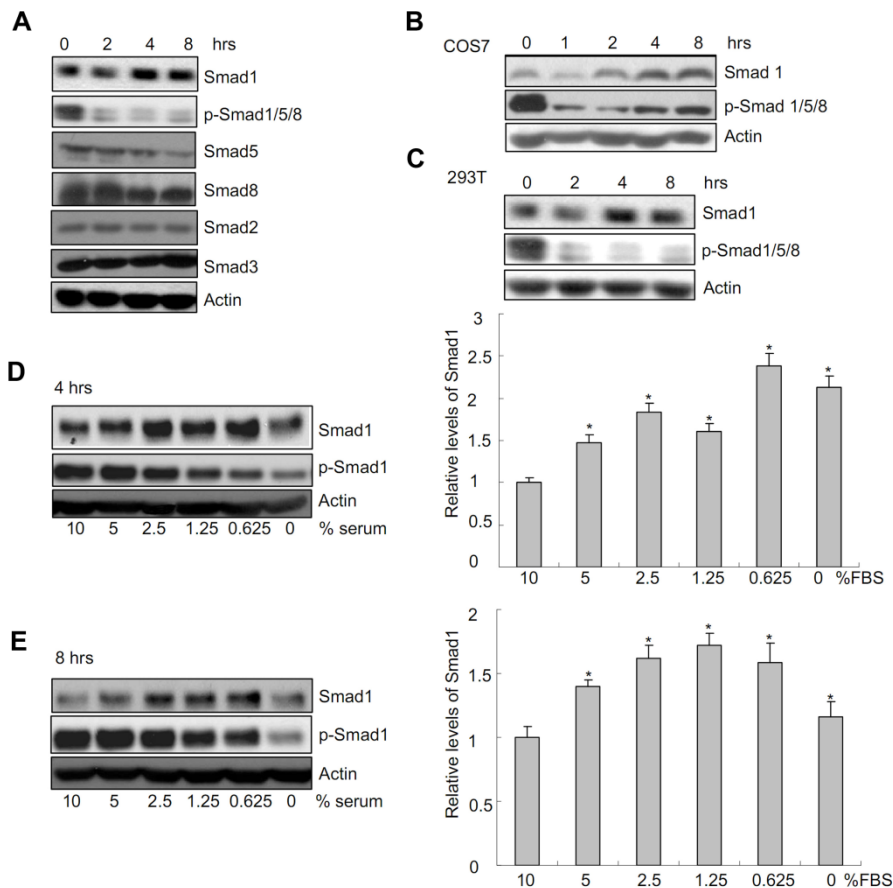
Fig. 1. Serum starvation led to Smad1 upregulation in a dose-dependent manner. A – Serum starvation led to upregulation of Smad1 but not Smad5, Smad8 or Smad2/3 in MEFs. Log phase primary MEFs were cultured in a medium without serum for different periods. The cells were collected and the protein levels of Smad1 were determined with western blot analysis. B – Serum starvation led to Smad1 upregulation in COS7 cells. C – Serum starvation led to Smad1 upregulation in 293T cells. D – Lowering the levels of serum led to an increase in Smad1 at the protein level after 4 h in MEFs. MEFs cultured in 10% serum were switched to a medium containing different concentrations of serum for 4 h. The levels of Smad1 and phosphor-Smad1 were determined with western blot analysis. Right panel: quantification data. E – Lowering the levels of serum led to an increase in Smad1 at the protein level after 8 h in MEFs. Right panel: quantification data.

that lowering the serum levels led to an increase in Smad1 levels in a dose-dependent manner (Fig. 1D and E). These results suggest that fetal bovine serum contains bioactive agents that modulate Smad1 expression.

Inhibition of BMPs led to upregulation of Smad1
Since lowering serum concentration also resulted in a dose-dependent decrease in Smad1 SXS phosphorylation in addition to upregulation of Smad1 (Fig. 1D and E), we suspected that BMPs might occur in the serum and affect Smad1 expression. To test this possibility, we treated the cells with noggin, which markedly inhibited Smad1/5/8 SXS phosphorylation (Fig. 2A), confirming that the serum does contain BMPs. More importantly, noggin treatment also led to upregulation of Smad1 (Fig. 2A), supporting our hypothesis that withdrawal of
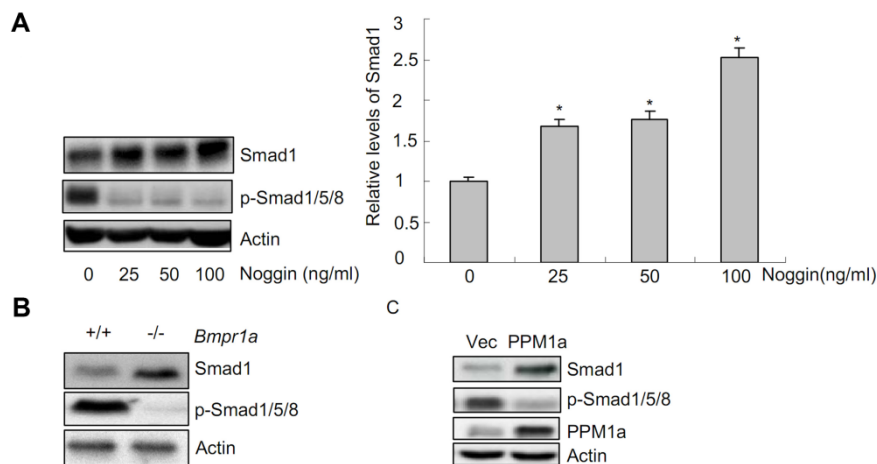

Fig. 2. Deactivation of BMP-Smad1 signaling led to Smad1 upregulation. A – Noggin treatment led to Smad1 upregulation in MEFs. Different concentrations of noggin were added to the normal culture medium for 24 h and the Smad1 protein levels were determined with western blot analysis. Right panel: quantification data. B – *Bmpr1a* deficiency also resulted in an increase in Smad1 in mouse ES cells. Wild-type and *Bmpr1a-/-* ES cells were cultured to log phase and collected. Smad1 protein levels were determined with western blot analysis. C – Ectopic expression of PPM1A also led to Smad1 upregulation in MEFs. PPM1A was expressed in MEFs using a retrovirus vector. Smad1 and PPM1A protein levels were determined with western blot analysis.

BMPs leads to Smad1 upregulation. This conclusion was further supported by the observation that *Bmpr1a-/-* ES cells showed an increase in the protein levels of Smad1 compared to the levels in wild-type ES cells (Fig. 2B). Moreover, overexpression of PPM1A, a phosphatase for Smads SXS dephosphorylation, led to upregulation of Smad1 (Fig. 2C), suggesting that deactivation/dephosphorylation also leads to Smad1 upregulation. These results indicate that BMP-Smad1 deactivation by serum starvation, noggin treatment, *Bmpr1a* deficiency or PPM1A overexpression leads to Smad1 upregulation.

Smad1 was relocalized at the perinuclear region in the absence of BMPs

Smad1 and other R-Smads shuttle between the cytoplasm and the nucleus. SXS-phosphorylated Smad1 is mainly localized in the nucleus. We determined the localization of Smad1 in MEFs when BMPs are depleted or inhibited. Since the available Smad1 antibodies were not sensitive enough to detect endogenous Smad1 in primary MEFs using the standard immunocytochemistry method, we expressed Flag-tagged Smad1 using retroviruses in MEFs. These could be readily detected with anti-Flag antibodies using immunocytochemical assays. It has been reported that Flag-tagged Smad1 behaves like ectopically expressed non-tagged Smad1 in MEFs [12]. Using these cells, we found that serum starvation led to an increase in the intensity of the immunofluorescence signal (Fig. 3A), confirming that Smad1 had been upregulated. Furthermore, serum starvation led to the localization of Smad1 at the perinuclear region, with some Smad1 molecules on the nuclear envelope (Fig. 3A). When the cells were treated with noggin, Smad1 also localized at the perinuclear region (Fig. 3A), which is different from the localization of Smad1 in response to the proteasome inhibitor MG132. Several studies have shown that MG132 treatment leads to Smad1 relocalization to the proteasome and/or centrosome, and further activation of Smad1, which is supposed to be localized in the nucleus [25-27]. The difference in ubiquitination and activation may explain why Smad1 cellular localization in the absence of serum is different from that in response to MG132 treatment. Cell fractionation experiments confirmed that in response to serum starvation, some of the Smad1 molecules localize in the nuclear fraction, likely the nuclear envelope, with the increase in Smad1 mainly occurring in the cytoplasm fraction (Fig. 3B). Further studies are needed to determine the molecular mechanisms behind Smad1 relocalization. There is a report showing that Smad1 can interact with the nuclear envelope-localized protein Man1, which may help to sequester Smad1 at the nuclear envelope [28]. Moreover, the nuclear envelope contains proteins essential for Smad1 nuclear translocation, such as Imp7/8, Sec13 and Nup93, which might participate in sequestering Smad1 at the perinuclear region in response to BMP blockade [29, 30].

To verify our cell-based findings, we analyzed Smad1 protein levels in mouse intestine villi. It has been well established that BMPs form a concentration gradient from the intestinal villi to the crypt, with the villi top showing the highest concentration and the crypt showing the lowest [31-34]. We histochemically stained Smad1 on sectioned mouse intestine slides and found that Smad1 is more concentrated at the crypt, which correlates well with low concentrations of BMPs (Fig. 4A and B). Moreover, Smad1 could be seen in the perinuclear region (Fig. 4C and D). These *in vivo* data also support the conclusion that BMP blockade leads to Smad1 upregulation and relocalization to the perinuclear region.
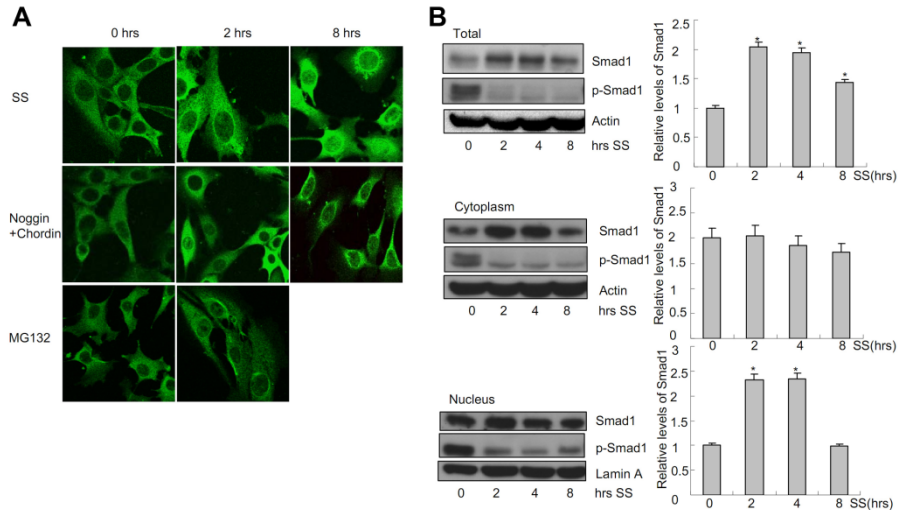
Fig. 3. Elevated Smad1 is localized at the perinuclear region after serum starvation or noggin treatment. A – Immunocytochemical staining results showing that serum starvation or noggin treatment induced redistribution of Smad1 to the perinuclear region, which is different from MG132-induced Smad1 localization. B – Fractionation experiments confirmed the redistribution of Smad1 in the cytoplasm fraction in response to noggin treatment. Right panels: quantification data.
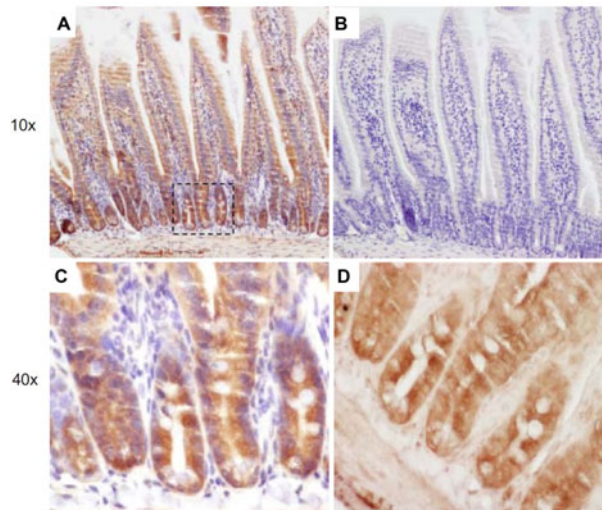

Fig. 4. Histochemical staining of mouse intestinal villi for Smad1. Mouse small intestine samples were paraffin embedded, sectioned and immunostained for Smad1, with hematoxylin staining the nucleus. A – Staining of Smad1 and the nucleus. Brown color was DAB staining of Smad1, purple blue color was hematoxylin staining of the nucleus. B – Staining of the nucleus alone. C and D –The area within the dotted lines in panel A was observed at higher magnification showing the perinuclear localization of Smad1. C – Co-staining of Smad1 and nucleus. D – Smad1 alone.

Smad1 upregulation occurs at the level of protein turnover

To determine what causes upregulation of Smad1 when BMP activity is inhibited, we first analyzed the mRNA levels using real-time PCR. We found that serum starvation or noggin treatment did not affect Smad1 mRNA expression (Fig. 5A and data not shown). Moreover, we found that ectopically expressed Smad1 (under the control of the CMV promoter) was also upregulated by serum starvation in COS7 cells (Fig. 5B). In co-expression experiments, Smurf1, which is an E3 ligase for Smad1 and other molecules, such as MAPKs, lowered the protein levels of Smad1 [35-37]. However, the Smad1 protein level still went up in response to serum starvation (Fig. 5B), suggesting that overexpression of Smurf1 is not sufficient to block Smad1 upregulation. Smurf1 targets Smad1 for degradation by recognizing the proline-rich motif located in the linker region between the MH1 and MH2 domains [36, 38-40], which contain several Ser/Thr residues that can be phosphorylated by kinases, such as MAPK, Atm and GSKs [41]. Linker phosphorylation by MAPKs may affect the stability of Smad1 [41]. Moreover, BMPRI-mediated SXS phosphorylation (activation) of Smad1 may affect linker phosphorylation [17]. This may explain why ectopic Smurf1 expression downregulated Smad1 at the basal level, but not in the absence of serum. We then analyzed Smad1 degradation after inhibiting protein synthesis with cycloheximide. It was clear that serum starvation led to a stabilization of Smad1 (Fig. 5C). These findings suggest that blockade of BMP activity stabilizes Smad1 protein and leads to the accumulation of Smad1. Whether Smad1 perinuclear localization plays a role in Smad1 stabilization warrants further investigation.

Serum starvation altered the dynamics and amplitude of BMP-Smad1 reactivation

What is the function of Smad1 upregulation in the absence of BMP activity? It has been reported that BMPs can stimulate cell proliferation under certain conditions [13, 42]. Previous studies have also established that SXS phosphorylation is essential for Smad1 function. It is predicted that Smad1 upregulation without SXS phosphorylation should have little effect. In primary MEFs, noggin treatment or ectopic expression of Smad1 with SXS deleted had little effect on cell proliferation (Fig. 5D and E). However, Smad1 upregulation may enable the cells to respond to BMPs more efficiently, thus facilitating the cell response to BMP restimulation. To test this, we compared low-dose BMP2-induced Smad1 activation in MEFs cultured in normal medium and in cells starved of serum for 4 h. Time point analysis revealed that in cells cultured in 10% serum, in response to 25 ng/ml BMP2, the levels of Smad1 phosphorylation increased at 0.5 and 1.0 h to a modest level, which quickly returned to the basal level (Fig. 5F). However, in serum-starved cells, the increase in Smad1 phosphorylation was continuous up to 8 h, although the magnitude of Smad1 SXS phosphorylation was much lower (Fig. 5F). These results indicate that blockade of BMP activity has profound effects on both the magnitude and the dynamics of BMP-2 induced Smad1 reactivation.
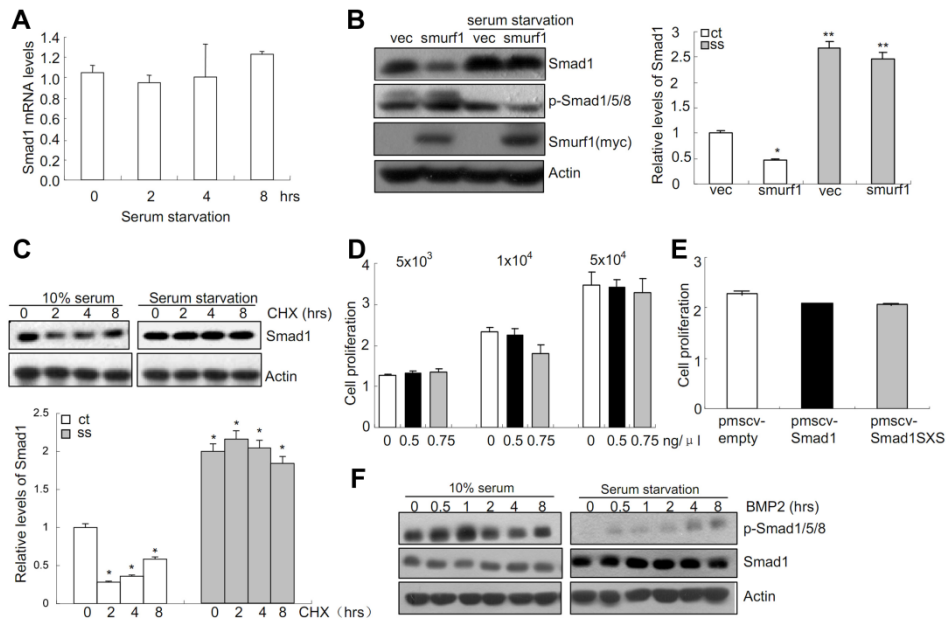
Fig. 5. Smad1 upregulation mainly occurs at the level of protein turnover and affects Smad1 reactivation. A – Serum starvation did not alter the mRNA levels of Smad1 in MEFs, as assayed by real-time PCR. B – Ectopically expressed Smad1 could also be upregulated in response to serum starvation. Smad1 alone or Smad1 plus Smurf1 were transfected into COS7 cells for 48 h. The cells were then serum starved for 8 h. Smad1 protein levels were determined with western blot analysis. Right panel: quantification data. C – Serum starvation led to decreased turnover of Smad1 at the protein level. MEFs cultured in 0 or 10% FBS were treated with cycloheximide for different periods. Smad1 protein levels were determined with western blot analysis. Bottom panel: quantification data. D – Noggin treatment did not affect cell growth. E – Overexpression of SXS-deleted Smad1 showed little effect on cell growth. F – Serum starvation affected the rate and the extent of BMP2-induced Smad1 activation. MEFs cultured in fresh medium containing 0 or 10% of FBS were stimulated with 25 ng/ml of BMP2 for different periods. Activation of Smad1 was determined with western blot analysis.

DISCUSSION

BMPs form a morphogen gradient that decides cell fates during early embryonic development [8, 9, 43]. In addition, BMP-Smad1 signaling controls many other aspects of development and tissue homeostasis and its dysregulation leads to the development of a number of diseases, including cancer and skeletal and vascular diseases. In addition, BMPs are present in the bloodstream and in the microenvironments of many adult stem cells, including neural stem cells, mesenchymal stem cells and skin stem cells [7, 11, 13]. Therefore, it is reasonable to predict that many cells encounter BMPs *in vivo*. As a critical

signaling molecule, Smad1 activation is strictly and precisely controlled, especially by various negative regulators [7, 21-23].

Here, we have uncovered a novel regulatory mechanism in response to the blockade of BMP activity. This not only occurs in *in vitro* cultured cells but also in the cells of the mouse intestine villi, which undergo rapid turnover [33, 44]. The stem cells in the crypts generate new enterocytes, which undergo transient proliferation, then terminal differentiation, and finally move up from the crypt to the villi top, where they die by apoptosis [33]. BMPs and their antagonists, such as noggin, are secreted by the niche cells, including mesenchymal cells and fibroblasts, to form a gradient along the villi, with the top showing the highest BMP activity and the crypt showing the lowest [31, 32]. We found that Smad1 is upregulated and localized at the perinuclear region in the crypts compared to the cells located at the villi top. However, this situation may change when the intestine villi are damaged and repair is needed. Repair calls for increased cell proliferation and differentiation, which may involve Smad1 reactivation [45].

In theory, Smad1 upregulation in response to BMP blockade could help to stock up the signaling molecules and enable the cells to respond to BMP restimulation more efficiently [1, 2]. However, we found that Smad1 activation is slower but lasts longer, in contrast to the rapid Smad1 activation in cells cultured in 10% serum. It is likely that both Smad1 upregulation and relocalization play a role in Smad1 reactivation. In many of the previous studies, cells were serum starved for a period of time before the addition of BMPs, to test the signaling response of Smad1/5/8 [1]. This study shows that the duration and magnitude of Smad1/5/8 activation are quite different in the presence or absence of basal levels of BMPs. The perinuclear and nuclear envelope localization of phosphorylated Smad1 may hinder the access of Smad1 to BMPRI. Previous studies suggest that Smad1, in the presence of BMP-containing serum, constantly shuttles between the nucleus and the cytoplasm [3, 4]. As such, cells respond to further BMP stimulation in a brief but less drastic manner. On the other hand, in cells that do not see BMPs, the response is slow but long lasting. These two different response modes may have distinct effects on cell proliferation and differentiation, and likely on cell fate determination *in vivo*.

In summary, we have identified another way in which BMP-Smad1 signaling is regulated. In the absence of BMP activity, Smad1 is upregulated and relocalized at the perinuclear region. Both events appear to play a role in Smad1 reactivation.

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