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## Thrombospondin-1 (TSP-1), a new bone morphogenetic protein-2 and -4 (BMP-2/4) antagonist identified in pituitary cells

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**Running title:** *TSP-1, a BMP-2/4 antagonist at the pituitary level*

**Key terms:** Bone Morphogenetic Proteins, Thrombospondin-1, BMP antagonists, pituitary, C3H10T1/2 cells, Surface Plasmon Resonance, structural model

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### ABSTRACT

Bone morphogenetic proteins (BMPs) regulate diverse cellular responses during embryogenesis and in adulthood including cell differentiation, proliferation and death in various tissues. In the adult pituitary, BMPs participate in the control of hormone secretion and cell proliferation suggesting a potential endocrine/paracrine role for BMPs, but some of the mechanisms are unclear. Here, using a bioactivity test based on embryonic cells (C3H10T1/2) transfected with a BMP-responsive element, we sought to determine whether pituitary cells secrete BMPs or BMP antagonists. Interestingly, we found that pituitary-conditioned medium contains a factor that inhibits action of BMP-2 and -4. Combining surface plasmon resonance (SPR) and high-resolution mass spectrometry helped pinpoint this factor as thrombospondin-1 (TSP-1). SPR and co-immunoprecipitation confirmed that recombinant human TSP-1 can bind BMP-2 and -4 and antagonize their effects on C3H10T1/2

cells. Moreover, TSP-1 inhibited the action of serum BMPs. We also report that the von Willebrand type C (VWC) domain of TSP-1 is likely responsible for this BMP-2/4 binding activity, an assertion based on sequence similarity that TSP-1 shares with the VWC domain of Crossveinless 2 (CV-2), a BMP antagonist and member of the chordin family. In summary, we identified for the first time TSP-1 as a BMP-2/-4 antagonist and presented structural basis for the physical interaction between TSP-1 and BMP-4. We propose that TSP-1 could regulate bioavailability of BMPs, either produced locally or reaching the pituitary via the blood circulation. In conclusion, our findings provide new insights into the involvement of TSP-1 in the BMP-2/-4 mechanisms of action.

### Introduction

Bone morphogenetic proteins (BMPs), members of the transforming growth factor  $\beta$

(TGF $\beta$ ) superfamily, were originally identified by their ability to induce endochondral bone regulate diverse cellular responses during embryogenesis and in adulthood including cell differentiation, proliferation and death in various tissues. The BMP system appears as a critical component of the local regulation in several endocrine tissues including the ovary, pituitary, hypothalamus, adrenal (3, 4). At the pituitary level, BMPs not only govern organogenesis but also participate to the control of hormone secretion and/or cell proliferation in different differentiated cell types like lactotropes (5, 6), corticotropes (7,8) and gonadotropes (9, 10). In these latter cells, BMPs as well as activins, other members of the TGF $\beta$  superfamily, participate to the regulation of follicle-stimulating hormone (FSH) synthesis and release in addition to the gonadotropin-releasing hormone (GnRH) and the gonadal steroids. Whereas activins are potent stimulators of FSH secretion (11, 12, 13), BMPs modulate FSH secretion with a species dependent effect. In rat pituitary cells and murine L $\beta$ T2 gonadotrope cell lines, recombinant human (rh) BMP-6 and BMP-7 at high concentrations or rhBMP-2 and rhBMP15 at lower concentrations stimulate basal FSH secretion and FSH $\beta$  promoter activity (9, 14, 15). Moreover, rhBMP-4 increased the release of FSH in response to activin and activin plus GnRH (16). In contrast, in ovine pituitary cells, rhBMP-4 inhibits FSH secretion and antagonizes the effects of activin (10, 17). Concerning lactotropes and corticotropes, previous studies reported that BMP-4 induces prolactin secretion in lactotropes (6, 18) meanwhile inhibits ACTH secretion in corticotropes (7, 8). Moreover, recent studies have also revealed a role of BMP-4 in the pituitary pathogenesis. It promotes pituitary prolactinoma while it inhibits corticotrope pathogenesis in Cushing's disease (5, 8).

BMP signaling occurs through heteromeric receptor complexes composed of type 1 and type 2 transmembrane serine/threonine kinase receptors. Three type 1 receptors are known to bind BMPs, which include the activin-receptor-like kinase (ALK)2, ALK3 (also known as BMPR-IA) and ALK6 (BMPR-1B) (19, 20). Similarly, three type 2 receptors possess binding affinity for BMPs, including BMPR-II, activin type IIA receptor (ActR-IIA) and ActR-IIB. The ligand binding induces the trans-phosphorylation of the type 1 receptor by the type 2 receptor. Consequently,

formation (1, 2). They are now known to the activated BMPR1 phosphorylates intracellular receptor-activated SMADs (R-SMAD), SMAD1, SMAD5 and SMAD8, which then interact with SMAD4 (Co-SMAD). The R-SMAD-Co-SMAD complex translocates to the nucleus and acts as transcription factors, either activating or repressing gene expression (21). BMP signaling is modulated by several extra- and intra-cellular modulators acting at multiple levels. A large number of extracellular antagonists, such as noggin, the chordin family, gremlin, twisted gastrulation protein and the Dan family (22, 23) bind BMPs and block their interaction with the receptors, thus inhibiting BMP signaling (24). Moreover, non signaling membrane pseudoreceptors or intracellular inhibitory SMADs are also able to block the BMP signaling (25).

Several BMP mRNAs are present in pituitary. For example, BMP-2, BMP-4, BMP-7 and GDF9 mRNAs were detected in ewe pituitary (10, 26). Furthermore, ALK3, ALK6 and BMPR-II are found on different cell types including gonadotropes and corticotropes (10). These data suggest that pituitary BMPs can exert paracrine/autocrine actions on hormone synthesis and release. Alternatively, BMPs can act as endocrine factors supplied by the blood. Indeed, BMP-4, BMP-6 and BMP-9 were found in bovine serum (27) suggesting a potential endocrine role for BMPs at pituitary level.

In this context, the first aim of the present study was to investigate whether pituitary cells produced BMPs and/or BMP inhibitors as well as whether the serum conveys BMPs. In the absence of available antibodies and protein assays for ovine BMPs, a sensitive *in vitro* bioassay based on mouse C3H-B12 cells was used. These cells are mesenchymal embryonic C3H10T1/2 cells stably transfected with an expression construct (BRE-Luc) containing a BMP-responsive element fused to firefly luciferase reporter gene (28). This assay presents the advantage to monitor the bioactivity of the protein and is not isoform specific. It can also allow to detect factors which inhibit BMP action.

Interestingly, we found that pituitary cell conditioned media exhibited an inhibitory activity for BMP induced luciferase activity. We then conducted the identification of the putative inhibitory factor combining surface plasmon resonance and high resolution tandem mass

spectrometry. Lastly, based on sequence and structure analysis, we provide insights into the molecular basis of interaction between BMP-4 and this inhibitor.

## Results

### *Conditioned media (CM) from pituitary cells do not exhibit BMP activity*

Firstly, the BMP effect on the BRE-Luc construct was determined by treating C3H-B12 cells with increasing concentrations of BMP-2, BMP-4, BMP-6 or BMP-7 (0 to 50 ng/mL) overnight and monitoring changes in the luciferase activity. BMPs stimulated luciferase activity in a dose-dependent manner (Fig.1A). BMP-4 was the most potent inducer of luciferase activity with an ED<sub>50</sub> of 1.2 ng/mL and detection threshold of 0.25 ng/mL whereas BMP-7 was the least potent inducer with a detection threshold of 25 ng/mL.

To determine whether CM from ovine pituitary cells exhibited BMP activity, C3H-B12 cells were exposed to CM from cultured pituitary cells which were treated or not with 10<sup>-8</sup> M GnRH for 6 hours (CM GnRH 6 hours), or with either 10<sup>-9</sup> M activin for 48 hours (CM Activin 48h). Luciferase activity from C3H-B12 was not modified compared to C3H-B12 cells exposed to DMEM (DMEM-0.1% BSA *i.e.* non conditioned media, Fig. 1B). This result suggests that conditioned media exhibit no or low amount of bioactive BMP whatever the treatment and the incubation period of the pituitary cells.

### *Conditioned media from pituitary cells exhibit an inhibitory activity on BMP action.*

To evaluate the potential presence of BMP inhibitors in CM, we supplemented pituitary cell media conditioned for 48 hours with increasing doses of BMP-4 and incubated them with C3H-B12 cells. A dose dependent increase in the luciferase activity was observed (Fig. 1C, grey spots). However, this increase was impaired relative to that obtained with DMEM-0.1% BSA supplemented with similar doses of BMP-4 (Fig. 1C, black spots). A dose of 50 ng/mL of BMP-4 in CM was necessary to recover full activity of BMP-4 on C3H-B12 cells compared to 5 ng/mL in DMEM-0.1% BSA (Fig. 1C). These results suggest that an inhibitory activity of BMP action is present in CM. Moreover, the inhibitory activity of BMP action was more elevated in the medium conditioned for 48 hours (CM Basal 48h) than for 6 hours (CM Basal 6h) (72% of

inhibition and 17% respectively vs DMEM + BMP-4) (Fig. 1D). As comparison, we analysed CM Basal 48h from adrenocortical cells cultured in the same conditions than the pituitary cells. The C3H-B12 cell luciferase activity induced by BMP-4 (10ng/mL) supplemented in adrenal cell CM was similar to that of BMP-4 diluted in DMEM-0.1% BSA indicating that the CM from adrenal cells did not exhibit a detectable inhibitory activity of BMP action (Fig. 1D, insert).

### *Treatment of pituitary cells with GnRH for 6 hours increases the CM inhibitory activity on BMP action.*

To determine whether FSH regulatory factors, such as GnRH and activin, were capable to modulate the production of the inhibitory activity, CM from pituitary cells treated with these factors, in dose and time conditions known to affect FSH secretion, were supplemented with 10 ng/mL of rhBMP-4 and incubated with C3H-B12 cells overnight.

In presence of BMP-4, CM from pituitary cells treated with GnRH for 6 hours (CM GnRH 6h) impaired the increase in luciferase activity of C3H-B12 cells vs DMEM + BMP-4 (77% inhibition) ( $p < 0.01$ ) more than did CM from non treated cells (CM Basal 6h) (17% inhibition) ( $p > 0.05$ ) (Fig. 1D). This suggests that GnRH increased the CM inhibitory activity on BMP action.

When the CM from pituitary cells treated with activin for 48 hours (CM Activin 48h) were added with BMP-4, they tend to impair the increase in luciferase activity vs DMEM + BMP-4 more than did CM from non treated cells (CM Basal 48h) (83% of inhibition versus 72%) although the difference was not statistically different (Fig. 1D).

The treatment of C3H-B12 cell with pituitary CM, BMP-4, GnRH or activin did not affect cell proliferation compared to DMEM-0.1% BSA (Fig. 1E).

Moreover, we tested the ability of the pituitary CM to decrease the effect of another BMP, BMP-2. Fig. 1F shows that luciferase activity was impaired when BMP-2 was added to CM conditioned for 48h compared to the addition in DMEM, similarly to the effect observed with BMP-4.

### *Conditioned media from pituitary cells exhibit BMP-4 binding protein*

To explore the hypothesis that the CM factor(s) responsible for the inhibition of BMP action can be BMP-4 binding protein(s), interaction between conditioned media and BMP-4 was analysed using surface plasmon resonance (Biacore). The injection of CM (1/10 diluted) resulted in a binding to high density immobilized rhBMP-4 whereas the injection of DMEM-0.1% BSA led to a low non-specific binding signal (Fig. 2). Moreover, the interaction signal was more elevated with media conditioned for 48 hours compared to media conditioned for 6 hours. To concentrate the binding factor and eliminate small molecules, the CM volumes were 10 fold reduced using high molecular mass PEG dialysis. The concentrated media exhibited an increased interaction signal compared to crude CM (Fig.2). Collectively, these results demonstrate that an interaction occurs between pituitary CM and BMP-4. Note that the differences in interaction signal observed between media conditioned for 6 hours and 48 hours are consistent with the changes observed in the biological effect of the corresponding CM on CH3-B12 cells (Fig. 1D).

*BMP-4 binding protein identified as thrombospondin-1 by tandem mass spectrometry*  
The CM fraction bound to BMP-4 on CM5 sensorchip was eluted and analysed by on-line nanoflow liquid chromatography tandem mass spectrometry after tryptic digestion. The only three detectable peptides allowed the identification of the predicted thrombospondin-1 isoform 1 (TSP-1) (Table 1), a 450 kDa secreted homotrimeric protein that regulates a wide range of functions (35). These peptides were not detected when elution was performed after injection of DMEM-0,1% BSA on CM5 sensorchip instead of conditioned media. These results demonstrated that BMP-4 chip acts as a specific and efficient affinity separation method.

*Recombinant thrombospondin-1 interacts with BMP-4 as well as BMP-2 and antagonizes its action*

To confirm the interaction between TSP-1 and BMP-4, surface plasmon resonance analysis was conducted. The injection of rhTSP-1 on low density BMP-4 immobilized CM5 sensorchip led to a dose-dependent binding (Fig. 3A). To determine affinity data, we used the method of steady-state analysis. The equilibrium dissociation constant ( $K_D$ ) for the interaction was

calculated at  $10^{-8}$  M in two independent experiments.

BMP-4 binding to TSP-1 was further assayed using TSP-1 immobilized CM5 sensorchip. As expected, the injection of BMP-4 resulted in a dose-dependent binding signal (Fig. 3B). BMP-2 binding to TSP-1 was also studied on TSP-1 immobilized CM5 sensorchip. A dose-dependent binding signal was observed (Fig. 3C) with a kinetics slightly different from that of BMP-4. The dissociation constants ( $K_D$ ) were:  $5.95 \times 10^{-7}$  M for BMP-4 and  $1.26 \times 10^{-7}$  for BMP-2. Evaluation of the kinetic parameters showed faster association and dissociation rates for BMP-2 (Table 2).

In contrast, no interaction between TSP-1 and activin, chosen as control, was observed (Fig. 3D).

To add more experimental data showing the interaction between BMP-4 and TSP-1, co-immunoprecipitation experiments were performed. When BMP-4 and TSP-1 are incubated together, precipitation with anti-BMP-4 antibody co-precipitates TSP-1 as detected by western-blot (Fig. 3E, lane 4). In contrast, when the anti-BMP-4 antibody was omitted or replaced by a non specific IgG, TSP-1 was not detected (Fig. 3E, lanes 2 and 3, respectively). These observations confirm the interaction between BMP-4 and TSP-1.

Furthermore, to assess the biological relevance of the BMP-4 and TSP-1 interaction, we studied the ability of TSP-1 to block BMP-4 or BMP-2 action. As shown in Fig. 4A and 4B, incubation of TSP-1 with BMP-4 or BMP-2 leads to antagonize BMP action as demonstrated in CH3-B12 cell bioassay where rhTSP-1 inhibited the activity of BMP-4 or BMP-2 (2.5 ng/mL, *i.e.*  $10^{-10}$  M) on luciferase transcription in a dose dependent manner. Dose response curves were fitted using the Hill equation (Fig. 4E and 4F). Half-maximal inhibitory concentration ( $IC_{50}$ ) values were estimated at  $11.4 \pm 6.3$  and  $8.5 \pm 3.9$  nM, respectively, with Hill slope values of  $0.36 \pm 0.1$  and  $0.61 \pm 0.13$ . These values are in agreement with a negative cooperative interaction. TSP-1 alone did not affect luciferase transcription (Fig. 4A). As comparison, noggin, a well known BMP-2/4 binding protein, inhibited the activity of BMP-4 as well as BMP-2 (2.5 ng/mL, *i.e.*  $10^{-10}$  M) (Fig. 4C and 4D). When the data were fitted with the Hill equation, estimated  $IC_{50}$  were  $0.023 \pm 0.001$  and  $0.021 \pm 0.003$  nM, respectively, with Hill slope values of



7.4 ± 0.6 and 6.9 ± 0.8 (Fig. 4E and 4F). These values suggest a positive cooperative interaction.

#### *Thrombospondin-1 mRNA and protein are expressed in pituitary cells*

To confirm our findings from mass spectrometry identifying TSP-1 as a factor present in pituitary cell CM, the expression of its mRNA was analysed by RT-PCR in cells collected at the time of CM recovery (48 hours). Fig. 5A shows the presence of a single PCR product at the expected size for TSP-1 mRNA, *i.e.* 98 bp, whereas no product was detected when reverse transcriptase was omitted.

Western-blot analysis confirmed the presence of the protein in CM with an apparent *Mr* of 150 kDa or 450 kDa in reducing or non-reducing conditions respectively, corresponding to TSP-1 (Fig. 5B). As expected, the level of TSP-1 was increased in CM 48 hours compared to CM 6 hours. This result is consistent with the increase of the inhibition of BMP-4 action on luciferase activity from B12 cells observed with media from pituitary cells conditioned for 48 hours compared to 6 hours (Fig 1D). TSP-1 level also tended to increase in presence of CM from pituitary cells treated with GnRH for 6 hours compared to CM basal 6 hours although the difference was not significant ( $p < 0.1$ , Fig. 5B).

#### *High molecular mass factor enriched pituitary CM interact with BMP-4 and antagonize its action*

In order to validate that a high molecular mass factor like ovine TSP-1 present in pituitary CM is able to bind BMP-4 and inhibit its action, conditioned media were fractionated using 100 kDa cut-off membranes. The presence of TSP-1 in the retentate (CM >100 kDa) but not in the filtrate (CM <100 kDa) was confirmed by western-blot (Fig. 5C). Fig.5D shows that retentate (CM >100 kDa), but not filtrate (CM <100 kDa), bound to BMP-4 immobilized sensorchip. Consistent with this result is the inhibition of BMP-4 (10 ng/mL) action observed with the retentate, but not with the filtrate on luciferase transcription in CH3-B12 cells (Fig. 5E).

#### *Blood serum exhibit BMP activity*

The above data showed that pituitary cells produce TSP-1 and that TSP-1 is able to bind BMPs. We made the hypothesis that pituitary TSP-1 could bind BMPs reaching the pituitary by the blood way. We first asked whether ovine

serum contains BMPs. To address this question, C3H-B12 cells were exposed to dilutions of serum from adult ewes. Figure 6A shows that serum induced a dose dependent increase of luciferase activity in C3H-B12 cells suggesting that BMP-like activity was present. Serum did not affect cell proliferation or morphology in our assay conditions when diluted ½ or more (data not shown).

To test whether the increase in C3H-B12 luciferase activity was specific of a BMP action, ovine sera were supplemented with increasing doses of noggin (0 to 4.10<sup>-10</sup>M), a BMP-2 /4 binding protein. The serum-induced luciferase activity was decreased in a dose-dependent manner by noggin demonstrating the BMP specificity (Fig. 6B). When serum was supplemented with dorsomorphin (2.5.10<sup>-6</sup>M), a small-molecule inhibitor of BMP type I receptors, which selectively blocks ALK2, ALK3 and ALK6 activity (36), serum induced luciferase activity in C3H-B12 cells was not detected (Fig. 6B). In contrast, follistatin, known as a preferential activin binding protein, did not affect serum induced luciferase activity at the dose of 10<sup>-8</sup> M as illustrated in figure 6B.

To determine whether serum is able to induce BMP signaling in a short delay, SMAD1 pathway in C3H-B12 cells was analysed. Western-blot was performed following cell treatment with serum (1/2 diluted) for 60 minutes. As shown in Figure 6C, treatment with BMP-4 or with serum induced SMAD-1 phosphorylation. When serum was supplemented with dorsomorphin (2.5 µM), phospho SMAD-1 immunoreactivity in C3H-B12 cells was not detected. These results suggest that serum rapidly recruits BMP signaling to stimulate luciferase activity in C3H-B12 cells.

#### *Conditioned media from pituitary cells as well as rhTSP-1 inhibit BMP activity of the serum.*

Treatment of C3H-B12 cells with both CM and serum (1/1) showed that pituitary CM inhibited the BMP effect of serum on luciferase activity from C3H-B12 cells (Fig. 6C). This BMP counteraction was mimicked when rhTSP-1 (10<sup>-7</sup>M) was added to serum ½ diluted (Fig. 6D). Note that TSP-1 alone had no effect on luciferase activity (Fig. 6D).

#### *TSP-1 shares sequence similarity with proteins of the chordin family*

As defined by searching domain databases (HH-PRED Proba 98.85 with Pfam0093), ovine TSP-1 contains a von Willebrand type C domain (VWC), between amino acids 319 and 373. VWC domains have originally been identified in the von Willebrand factor, a large multimeric plasma protein involved in homeostasis, and are present in a wide variety of extracellular proteins. Among these are members of the Chordin modulator family, including Chordin and Crossveinless 2 (CV-2) also known as BMP binding endothelial regulator (BMPER), which binds BMP-2 with nanomolar affinities and compete for binding BMP type I and type II receptors (37). As observed in the whole VWC family, sequence identities are low: only the 10 cysteine residues and spacing are conserved, together with a very limited number of other positions (Fig. 7A). However, these conserved cysteine residues form the cysteine knot that constitutes a signature of these BMP antagonists (23, 38). Fig. 7B represents the domain architecture of TSP-1 including the VWC domain as described in (48).

#### *Model of the 3D-structure of the TSP-1 VWC domain in complex with BMP-4*

We built a model of the 3D structure of the TSP-1 VWC domain in complex with BMP-4 (ovine sequences), based on the experimental 3D structure of *Danio rerio* Crossveinless 2 (CV-2) in complex with human BMP-2 (HH-Pred Proba 98.67 with pdb 3bk3) (39) (Fig. 7C). The TSP-1 VWC domain (amino acids 317-374) shares the global architecture of the CV-2 VWC domain, made of a tripartite organization with a N-terminal clip segment and two small subdomains, called SD1 and SD2. Based on the similarity we observed with the CV-2 : BMP-2 interaction, we suggest that VWC domain of TSP-1 behaves as a paperclip relative to the BMP-4 3D structure, with on the one hand the N-terminal clip folding into the BMP-4 wrist epitope (blocking interaction with type I receptor) and on another hand the SD1 interacting with the BMP-4 knuckle epitope (blocking interaction with type II receptor) (Fig. 7C, top and bottom left).

BMP-2 and BMP-4 share high sequence identity (62%), whilst there is little sequence similarity between CV-2 and TSP-1 (26% identity), a general trend observed in the whole VWC family. However, some positions are highly conserved, such as two generally occupied by aromatic amino acids (in orange in Fig. 7C

bottom right, Y325 and W331 in TSP-1). Interestingly, the side chain W331 is located inside a groove at the surface of BMP-4.

## Discussion

BMPs regulate a large array of cellular processes in diverse tissues. In the anterior pituitary, they play various roles both for the initiation of organogenesis during the development and for the regulation of a number of pituitary functions in adult. Some of their actions regard the regulation of prolactin, ACTH or FSH synthesis and release (6, 8, 9, 10). Previous results reported that several BMPs, in particular rhBMP-4 and rhBMP-6, are able to modulate *FSH $\beta$*  mRNA expression and FSH release from pituitary cells (10, 17). Together with the presence of some *BMP* mRNAs as well as BMPR-IA (ALK3), BMPR-IB (ALK6) and BMPR-II receptors in the pituitary (10), these data suggest that BMPs can be produced locally and exert paracrine action on hormone synthesis. To explore the presence of BMPs in biological samples, the bioassay based on C3H-B12 cells represents a meaningful approach and exhibits several advantages since it is rapid, sensitive and detects bioactive BMPs (28).

In this context, our results showed that conditioned media from ovine pituitary cells did not exhibit BMP activity whatever the treatment (GnRH, activin) and the incubation period (6 hours or 48 hours) of the pituitary cells suggesting that, at least *in vitro*, pituitary cells do not release BMPs or in very low amounts and that GnRH or activin do not modify their levels. Alternatively, pituitary cells can release BMP isoforms that are not detected in the bioassay. For example, the detection threshold for rhBMP-7 is around 25 ng/mL and the bioassay could fail to detect small concentrations of BMP-7. However, even when concentrated pituitary media were used, we did not observe any increase in luciferase activity (data not shown). One also must consider that BMPs can function in a highly locally manner limiting their diffusion in the medium. Furthermore, our bioassay only detect BMPs biologically active, thus the BMPs present in the conditioned medium can be biologically inactive. Indeed, their activities depend on the ability of cell environment to convert the protein from an inactive to an active form (40). This process relies on pro-protein convertase whose

expression pattern is cell specific. No data concerning pituitary cells are available. Another point we considered is that the biological activity of BMPs can be blocked by binding proteins produced locally.

In agreement with this last point, our results support the assumption that pituitary cells produce BMP antagonist(s). Indeed, when pituitary CM were supplemented with BMP-4 or BMP-2, the increase in the C3H-B12 cells luciferase activity was impaired relative to that obtained with DMEM supplemented with BMP-4 or BMP-2. Additional preliminary data indicate that pituitary CM also reduced BMP-6 and BMP-7 action. This suggests that inhibitor(s) of BMP action are present in CM. Moreover, the inhibitory activity was more elevated when pituitary cell media were conditioned for 48 hours compared to 6 hours indicating that inhibitor(s) of BMP action accumulate during the culture. In contrast, pituitary CM did not affect C3H-B12 cell proliferation or morphology in our assay conditions. Our data also underlined a selective presence of inhibitor(s) in pituitary cells since CM from adrenocortical cells did not inhibit BMP-4 activity. Treatment of pituitary cells with GnRH for 6 hours induced an increase of the inhibitory activity of BMP action compared to untreated pituitary cells (Basal 6h). This effect suggest an action of this inhibitor on gonadotrope function. It can be postulated that GnRH could block, through such a BMP inhibitor, BMP signaling induced by low amounts of local BMPs or more probably by circulating BMPs. Such an action could promote FSH synthesis and release since BMP-4 inhibits FSH secretion in ewe pituitary (10, 17).

The following focus of the study aimed at identifying the pituitary factor(s) responsible for the inhibition of BMP action. In agreement with the hypothesis that the inhibitory action can be attributed to extracellular BMP antagonist(s), *i.e.* binding protein(s) able to prevent BMP signaling, real time SPR analysis revealed an interaction between BMP-4 and pituitary conditioned media highlighting the presence of a BMP binding protein. Notably, the interaction signal intensity was correlated to the inhibitory action of the CM on C3H-B12 cell luciferase activity, with a lower interaction with media conditioned for 6 hours compared to 48 hours. Following step combining SPR and tandem mass spectrometry allowed to identify for the first

time thrombospondin-1 (TSP-1) as BMP-2/-4 binding protein.

TSP-1 is a 450 kDa multifunctional homotrimeric glycoprotein that belongs to a family of extracellular matrix binding proteins. It is produced by different cell types like platelets, endothelial cells, macrophage and is involved in a variety of biological functions including angiogenesis, inflammation, cell proliferation, differentiation, apoptosis, and cell-cell interactions (41, 42). The multifaceted action of TSP-1 depends on its ability to physically interact with different ligands like components of the extracellular matrix, cell receptors, growth factors or cytokines (41, 42). Among the TGF $\beta$  family, the only known example is the interaction between TSP-1 and TGF $\beta$ . Indeed, TSP-1 binding to the latent TGF $\beta$ 1 complex induces activation by stimulating a conformation change (43, 44).

Our present data contribute to expand the field of TSP-1 interactome by demonstrating, for the first time, that TSP-1 physically interacts with BMP-4 and BMP-2. This interaction is revealed when either BMP-4 or TSP-1 are immobilized on sensor chip. Recombinant TSP-1 binds BMP-4 or BMP-2 with relatively high affinity, the  $K_D$  value calculated from SPR experiments being around  $10^{-7}$  -  $10^{-8}$  M. The association and dissociation rates were higher for BMP-2 compared to BMP-4. In contrast, TSP-1 does not bind activin A. The interaction between TSP-1 and BMP-4 was further confirmed by the co-immunoprecipitation assays. Furthermore, we show that the binding between TSP-1 and BMP-4 or BMP-2 antagonizes BMP activity. Indeed, rhTSP-1 as well as high molecular mass enriched pituitary CM containing TSP-1 prevent BMP-4 bioactivity on C3H-B12 cells. Even if the affinities of TSP-1 for BMP-4 or BMP-2 ranging around  $10^{-8}$  M are weaker than the affinities of known BMP binding proteins like noggin ( $10^{-11}$  M) or chordin which range around  $10^{-10}$  M (45) and  $10^{-13}$  M (46), the abundance of TSP-1 in CM as well as its high molecular mass may explain the relevance of the antagonism. Alternatively, TSP-1 may need other factors present in CM for optimal function. In the bioassay,  $10^{-7}$  M rhTSP-1 did not inhibit BMP-4 action to a similar extent than pituitary media conditioned for 48h. This result suggests either that rhTSP-1 has a less potent biological effect than pituitary TSP-1 or that TSP-1 concentrations in the CM are higher than  $10^{-7}$  M. Also, CM may contain



additional inhibitory factors. Interestingly, we did not identify other BMP binding protein(s) than TSP-1 after combining SPR and MS suggesting that, if they are present, they are in low amounts. Moreover, we can notice that our data argue in favour of a negative cooperative interaction between TSP-1 and BMPs in contrast to the positive cooperative binding shown between noggin and BMPs.

The bioinformatics analysis we made here reinforce evidence for binding between TSP-1 and BMP-4, by suggesting that the N-terminal VWC domain of TSP-1 mediates this interaction, as observed for BMP antagonists of the chordin family (39). We indeed showed that the VWC domain of TSP-1 shares significant sequence similarities with the VWC domains of the chordin family, supporting the fact that the TSP-1 VWC domain may also adopt a modular tripartite architecture, with a N-terminal clip segment and two subdomains termed SD1 and SD2. Crystal structure analysis revealed how the CV-2 VWC domain blocks BMP-2 receptor binding, by acting on two distinct epitopes specific for BMP type I and type II receptors, respectively (39, 47). According to the model of the 3D structure we built of the TSP-1 VWC domain in complex with BMP-4, structurally close to BMP-2, we thus suggest that TSP-1 could adopt a similar mechanism to antagonize BMP-4.

This mode of interaction between TSP-1 and BMP-4 is different of the binding between TSP-1 with TGF $\beta$  which involves the WSHWSPW sequence located in the second TSR domain of TSP-1 adjacent to the N-terminal (43, 48, 49). The interaction between this motif and TGF $\beta$  is critical for TSP-1 mediated activation of the TGF $\beta$  latent complex (50).

The study presented here identify a novel role for TSP-1 as a BMP-2/-4 binding protein. Our results underlined that TSP-1 is synthesized by pituitary cells and released in the extracellular compartment. The cell type responsible for TSP-1 synthesis is not known although in the rat, TSP-1 immunoreactivity is partly distributed in pituitary endothelial cells surrounding the vessels (51). One possible role of TSP-1 at the pituitary level is to block the action of BMPs produced locally or to prevent the action of BMPs reaching pituitary by the blood way. Indeed, using the C3H-B12 cells, we detected BMP activity in ovine serum, both at SMAD1 signaling and luciferase activity levels extending the results of Herrera and Inman (27). This BMP

activity was inhibited in a dose-dependent manner by noggin, a BMP-2 /4 antagonist, or by dorsomorphin, an ALK2/3/6 inhibitor but not by follistatin, an activin binding protein. Serum factors are able to recruit BMP signaling in a short delay since they stimulated SMAD-1 phosphorylation after one hour treatment as BMP-4 did. The SMAD-1 phosphorylation was prevented by dorsomorphin. These data demonstrate the BMP specificity of the response observed with sera. Furthermore, the conditioned media from pituitary cell cultures, as well as rhTSP-1, antagonized the effect of ovine serum on the luciferase activity from C3H-B12 cells.

The ability of TSP-1 to antagonize BMP-4 action suggests that TSP-1 might be important in pituitary processes where BMP-4 is involved. Our previous work demonstrated that the treatment of ewe pituitary cells with BMP-4 inhibits FSH secretion (10). Given the presence of BMPs in serum if not in pituitary, we postulate that TSP-1 can modulate BMP action on FSH secretion through their interaction. The demonstration of such a biological relevance will require additional investigation under physiological conditions. In TSP-1 deficient mice, the homozygous animals are viable and fertile but they produced fewer litters than the wild-type animals (52). Whether FSH is altered was not studied. Further exploration of the pituitary function of these animals will help to gain insight into the role of TSP-1.

Beyond the pituitary, the TSP-1 antagonistic role of BMP-2/-4 may be important in other tissues where they exhibit pleiotropic activities (4, 20, 47) and it is tempting to speculate that their antagonism may be important for cell proliferation, differentiation, apoptosis and/or migration. As example, TSP-1, as well as BMP-4 are also known to play crucial role in the pathogenesis of tumors, including the pituitary (5, 53, 54). Whereas TSP-1 has been shown to control tumor growth by inhibiting angiogenesis in various tissues (48, 55, 56), BMP-4 is able to promote angiogenesis (53, 57). All together, these results raise the possibility that molecular interaction between TSP-1 and BMP-4 may be crucial to maintain an appropriate balance between proliferation and differentiation and/or regulate tissue homeostasis.

In conclusion, we show that ewe pituitary cells release a BMP-2/-4 antagonist, identified as TSP-1, and we provide structural evidence for physical interaction between TSP-1 and BMP-2/-4. This suggest that TSP-1 could regulate the

bioavailability of BMPs either produced locally or reaching the pituitary by the blood way. Further study is necessary to elucidate the role of the couple TSP-1/BMP-2/4. Collectively, these data provide new insights into the mechanisms of BMP action.

## Experimental procedures

### *Reagents*

Cell culture media used were DMEM (Dubelcco's modified eagle's medium) and F12 (Nutrient mixture F12 Ham) from Sigma (Saint Louis, MO, USA). Gentamicin, nystatin, L-ascorbic acid, apo-transferrin, FCS (fetal calf serum), BSA (bovine serum albumin), luciferin substrate, 17 $\beta$ -estradiol and dorsomorphin were purchased from Sigma. Collagenase A and DNase 1 were from Roche diagnostics Ltd (Meylan, France). Geneticin sulfate (G418 sulfate) was from InvivoGen (San Diego, CA, USA). Recombinant human Activin A, BMP-2, BMP-4, BMP-6, BMP-7 and follistatin were obtained from R&D systems (Lille, France). Recombinant human TSP-1 was from R&D systems (carrier-free TSP-1) for surface plasmon resonance studies and from EMP Genetech (Ingolstadt, Germany) for biological assays. Human noggin was from ProSci Incorporated (Poway, CA, USA). GnRH (Relefact® LH-RH) was from Aventis (Frankfurt, Germany). Monoclonal mouse TSP-1 antibody (A6.1) was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Rabbit antibodies against phosphorylated SMAD1 were kindly provided by C.H. Helding (Ludwig Institute for Cancer Research, Uppsala, Sweden).

### *Ewe pituitary cell cultures*

Pituitaries from adult Ile-de-France ewes were collected throughout the year and dissociated for cell cultures as previously described (8). Briefly, pituitaries were finely sliced and placed in F12 containing 5% FCS, 0.4 mg/mL collagenase A and 0.025 mg/mL DNase 1 and incubated for 90 minutes in a shaking water bath at 37°C followed by manual dispersion through different needle sizes. Cells were then centrifuged at 100 g for 10 minutes and washed three times. The pellet was resuspended in DMEM supplemented with 5% FCS (culture medium). Cells were seeded in 6-well plates at  $3.10^6$  cells/well in culture medium, and allowed to attach for 2 days in a humidified atmosphere with 5% CO<sub>2</sub> in air at 37°C.

Adrenals were also collected and cortical cells were dissociated according the same protocol as pituitaries.

### *Preparation of conditioned media from pituitary cell cultures*

At day 2 of culture, media were replaced with serum-free DMEM containing 0.1% BSA. One hour later, the medium was changed and replaced by serum-free DMEM-0.1% BSA supplemented or not with 10<sup>-8</sup> M GnRH for 6 hours, or with 10<sup>-9</sup> M Activin A for 48 hours. The choice of concentrations was based on our previous studies determining 10<sup>-8</sup> M and 10<sup>-9</sup> M as optimal doses for GnRH and activin, respectively, to regulate *in vitro* FSH secretion. Conditioned media (CM) were then collected and non adherent cells and debris were removed by centrifugation at 2000 x g for 5 minutes at 4°C before assaying for BMP bioactivity on C3H-B12 cells.

In another set of experiments aimed to concentrate the BMP inhibitory factor and eliminate small molecules, CM were put in a dialysis bag with a cut-off of 3 kDa placed over a bed of polyethylene glycol (PEG, MW 35 kDa) until the volumes were 10 fold reduced.

Moreover, an ovine BMP inhibitory factor enrichment strategy for high molecular mass factors from CM was performed in some assays using filtration on molecular mass cut-off membranes (Vivaspin® 500, Sartorius, Germany). One mL of CM was applied on a 100 kDa cut-off membrane previously rinsed with deionized water and centrifuged at 15000 x g for 10 minutes. Filtrates and retentates (around 20  $\mu$ L) were recovered for subsequent analysis assaying BMP activity.

### *Ewe blood samples*

Blood samples were collected from the jugular vein from six adult ewes throughout the year. They were stored at 4°C for 24 hours and then centrifuged at 4000 x g for 30 minutes. The blood sera were collected, filtered through 0.2  $\mu$ m and treated at 56°C for 1 hour. They were stored at -20°C until the BMP bioassay was performed.

### *C3H-B12 cell cultures*

The mouse C3H-B12 cells were maintained in DMEM containing 10% FCS, 3  $\mu$ g/mL gentamicin, 2  $\mu$ g/mL nystatine and 200  $\mu$ g/mL geneticin sulfate.

### *BMP bioassay*

C3H-B12 cells were plated at  $2.10^5$  cells per well in 48-well plates with DMEM containing 10% FCS, and allowed to attach for 5 hours in a humidified atmosphere with 5% CO<sub>2</sub> in air at 37°C. The cells were rinsed twice with PBS and treated with serum-free DMEM-0.1% BSA containing or not BMPs or with conditioned media from ovine pituitary cells or with ovine serum at 37°C overnight. Cell extracts were then prepared for the luciferase activity assay. The cells were rinsed twice in ice-cold PBS and then lysed with 75 µL of passive lysis buffer (Promega). Samples were centrifuged at 12 000 x g for 1 minute to pellet the cell debris. The supernatants were collected before luciferase assay (Luciferase Assay System, Promega). Luciferase activity was measured on a Luminoskan Ascent (Thermo Labsystems, Issy Les Moulineaux, France). The reported luciferase activities represent the emitted luminescence normalized over respective controls that are obtained in the absence of stimulating factor. All experiments were performed at least three times with 2 or 3 independent wells per condition.

### *Proliferation measurement*

C3H-B12 cells were plated at  $2.10^5$  cells per well in 48-well plates with DMEM containing 10% FCS and allowed to attach for 5 hours in a humidified atmosphere with 5% CO<sub>2</sub> in air at 37°C. The cells were rinsed twice with PBS and conditioned media from ovine pituitary cells or other reagents were added with 0.1 µCi/well of [methyl-<sup>3</sup>H] thymidine and incubated at 37°C overnight. Cells were then washed with PBS, solubilized in 0.5 N NaOH. Thymidine incorporation was assessed using a liquid scintillation analyser (Packard).

### *Surface Plasmon Resonance (SPR) analysis*

SPR measurements were performed on a BIACORE T100 (GE Healthcare) instrument. Carrier free BMP-4 was immobilised on the flow cell(s) of CM5 sensor chips (GE Healthcare), using standard amine coupling, according to the manufacturer's instructions. The analysis was carried out at 25°C with 10 mM Hepes, pH 7.4, 150 mM NaCl, 0.05% Tween-20 (HBS-P) as running buffer. Conditioned media diluted in HBS-P and 0.2 µm filtered were injected for 180 seconds at a flow rate of 30 µL/min. Dissociation was studied for 120 seconds. The surfaces were regenerated for 60 seconds with 3

M guanidin followed by a wash of injection needle and a 300 second running buffer wash. The sensorgrams were subtracted with the non-specific interaction results obtained on an activated-desactivated control flow cell.

The interaction of BMP-4 with purified rhTSP-1 was studied in near conditions on 60 resonance units (RU) low density immobilised BMP-4. TSP-1 was injected at concentrations of 1–67 nM for 150 seconds at a flow rate of 30 µL/min and dissociation was studied for 600 seconds. Regeneration was achieved by injection of 5 mM EDTA for 60 seconds followed by a 120 second running buffer wash. The sensorgrams were corrected by a double subtraction for the non-specific interaction in the control activated/desactivated flow cell and for the buffer contribution in the same flow cell (29). Affinity was calculated using the steady-state 1:1 binding model (GE Healthcare Biacore T100 evaluation software, version 2.0.4). This model calculates the equilibrium dissociation K<sub>D</sub> for a stoichiometric 1:1 model of interaction from a plot of steady-state binding levels (Req) against analyte concentration (C), here TSP-1.  $Req = (CR_{max}/K_D + C) + RI$ , RI is bulk refractive index contribution, which is assumed to be the same for all sample concentrations. R<sub>max</sub> is the analyte (TSP-1) binding capacity of the surface in RU.

The interaction of BMP-4 as well as BMP-2 with TSP-1 was further studied on carrier free TSP-1 immobilized on the flow cells of CM5 sensor chip on a Biacore T200 (GE Healthcare). BMP-4 or BMP-2 were injected at concentrations of 6.25 to 100 nM or 3.12 to 50 nM respectively, for 180 seconds at a flow rate of 10 µL/min and dissociation was studied for 600 seconds. Regeneration was achieved by injection of 5 mM EDTA for BMP-4 or with 0.01% SDS for BMP-2. Binding affinity and kinetic parameters were evaluated using the bivalent fitting model (Biacore T200 software). This model describes the binding of a bivalent analyte, here BMP, to immobilized ligand. In some assays using TSP-1 immobilized sensorchip, activin A were injected at concentration of 100 nM for 180 seconds at a flow rate of 10 µL/min.

### *Identification of the BMP-4 binding protein combining SPR and high resolution Mass Spectrometry*

BMP-4 was immobilised on the 4 flow cells of a CM5 sensor chip at a level around 4000 RU. A

pool of 48 hours CM filtrated on 0.2  $\mu\text{m}$  and  $\frac{1}{4}$  diluted was injected on these flow cells at a 5  $\mu\text{L}/\text{min}$  flow rate for 180 seconds six fold and this cycle was 4 fold repeated. As control, DMEM-BSA was injected in same conditions. The CM fraction bound to BMP-4 was then recovered using 3 M guanidine for 60 seconds. A volume of 80  $\mu\text{L}$  was acidified at 0.1% formic acid and concentrated with solid phase extraction technique using Zip Tip C4 (Millipore Corporation, Billerica, MA). Cysteine reduction and alkylation were performed by addition of 10 mM dithiothreitol in 50 mM ammonium bicarbonate for 30 minutes at 56°C and 55 mM iodoacetamide in 50 mM ammonium bicarbonate for 20 minutes at room temperature in the dark, respectively. Proteins were digested overnight with 6.25 ng/ $\mu\text{L}$  trypsin (Sequencing Grade, Roche, Paris) and acidified with 0.1% formic acid.

The in-solution digested peptides were analysed by on-line nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) using a linear ion trap Fourier Transform Mass Spectrometer (FT-MS) LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) coupled to an Ultimate® 3000 RSLC Chromatographer (Dionex, Amsterdam, The Netherlands). Mobile phases consisted of (A) 0.1% formic acid, 97.9% water, 2% acetonitrile (v/v/v) and (B) 0.1% formic acid, 15.9% water, 84% acetonitrile (v/v/v). Sample was desalted for 10 minutes at 5 mL/min with 4% solvent B using an LCPackings trap column (Acclaim PepMap 100 C18, 100 mm i.d. 2 cm long, 3 mm particles) and separated using a LCPackings nano-column (Acclaim PepMap C18, 75 mm i.d. 50  $\mu\text{m}$  long, 3 mm particles). Separation was performed at a flow rate of 300 nL/min by applying gradient of 4–55% B for 60 minutes. The mass spectrometer was operated in positive mode in data-dependent mode with high resolution full scan MS spectra ( $R = 60,000$ ) and low-resolution MS/MS spectra ( $R=7,500$ ). In the scan range of  $m/z$  300-1800, the 20 most intense peptide ions with charge states  $\geq 2$  were sequentially isolated and fragmented by Collision induced Dissociation (CID) mode. Dynamic exclusion was activated during 30 seconds with a repeat count of 1. Polydimethylcyclsiloxane ( $m/z$ , 445.120025) ions were used as lock mass for internal calibration.

All raw data files were converted to Mascot Generic Format (MGF) with Proteome

Discoverer 1.3 software (Thermo Fisher Scientific). All MS/MS data were analysed using MASCOT 2.3 search engine (Matrix Science) against the mammalia section of a locally maintained copy of nrNCBI (1426555 sequences, download 05/14/2013). Enzyme specificity was set to trypsin with two missed cleavages using carbamidomethylcysteine, oxidation of methionine and N-terminal protein acetylation as variable modifications. The mass tolerance was set at 5 ppm for parent and 0.8 Da for fragment ions. Identification mascot results were subjected to Scaffold 3 software (v 3.6, Proteome Software, Portland, USA) for validation using Peptide and Protein Prophet algorithms (30, 31). Peptide identifications were accepted if they could be established at greater than 95.0% probability. Proteins were accepted with at least two distinct peptides.

#### *Co-immunoprecipitation*

Co-immunoprecipitation experiments were performed to further confirm that TSP-1 binds to BMP-4. Recombinant TSP-1 (4  $\mu\text{g}/\text{ml}$ , *i.e.*  $10^{-8}$  M) and BMP-4 (0,2  $\mu\text{g}/\text{ml}$ , *i.e.*  $10^{-8}$  M) were placed alone or in combination in 1 ml binding buffer (PBS with 0.5% BSA) for 1 hour at room temperature with rocking. The pull-down anti-BMP-4 antibody (R&D Systems #MAB757, 500 ng/tube), or non specific IgG (500 ng/tube) were added to each reaction tube and incubated at 4° overnight with rocking. In another control tube, IgG were omitted. The following day, 20  $\mu\text{l}$  of protein A/G-agarose beads (Santa-Cruz Biotechnology) were added to each tube and incubated for 1 hour with rocking. Then, beads were rinsed 5 times in PBS by centrifugation 5 minutes at 1000 x g. Beads were then resuspended in 40  $\mu\text{l}$  SDS sample buffer for western blotting and boiled to remove proteins from beads. Co-precipitated TSP-1 was detected by immunoblotting as described below.

#### *Reverse Transcription and PCR*

Total RNA from pituitary cells were extracted using TRI Reagent (Sigma) according to the manufacturer's instructions. Remaining DNA was removed by RNase-free DNase (Qiagen) treatment. RT-PCR was performed as previously described (26). Complementary DNA was synthesized from 1  $\mu\text{g}$  RNA in a volume of 20  $\mu\text{l}$  containing 150 ng oligodT (Promega), 1mM dNTPs, 20 U of RNasin, 1X RT buffer, and 12 U M-MLV reverse transcriptase (Promega). For the negative control, the reverse transcriptase



was omitted. The RNA denaturation was performed at 70°C for 10 min and the reverse transcription at 37°C for 1h. PCR was run on an iCycler from Bio-Rad, using IQ SYBR Green Supermix (BioRad) (1X) and primers 5'-GACTGGCTTCTGGACTTC-3' (forward) and 5'-GCACTGGCTTATGATGGT-3' (reverse) for ovine TSP-1 amplification (amplicon size = 98 bp). The equivalent of 10 ng of starting RNA was used in each reaction.

#### *Western blotting*

For TSP-1 detection, media from pituitary cells conditioned for 6 or 48 hours (volume equivalent to 10 µg protein) were diluted in reducing or non reducing Laemmli buffer, run in 8% SDS-PAGE and electroblotted onto PVDF membranes. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline containing 0.1% Tween - 1% BSA and incubated with the TSP-1 antibody diluted at 1:1000 for 1 hour at room temperature. After washing, the membranes were incubated with the secondary antibody, a peroxidase-conjugated anti-mouse IgG (Biorad, France) diluted 1:10000 for 1 hour. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, France). Membranes were exposed to Hyperfilm ECL for 1 or 5 minutes.

To investigate activation of SMAD 1 after BMP-4 or serum treatment, C3H-B12 cells were plated at  $2 \times 10^5$  cells per well in 48-well plates with DMEM containing 10% FCS, and allowed to attach for 5 hours in a humidified atmosphere with 5% CO<sub>2</sub> in air at 37°C. The cells were rinsed twice with PBS and treated with serum-free DMEM-0.1% BSA containing either rhBMP4 (10 ng/ml) or with ovine serum at 37°C for 60 minutes. The cells were then washed with ice-cold PBS and lysed in lysis buffer (150 mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.5% Igepal CA-630, 100mM sodium fluoride, 10mM sodium pyrophosphate, 10mM sodium orthovanadate, proteases inhibitor cocktail). The concentrations of the proteins were determined with BC Assay Protein Quantification Kit (Interchim, France). After 4 min at 95°C in the reducing SDS-PAGE buffer containing β-mercaptoethanol, 10 µg proteins were separated in 10% SDS-PAGE gels and electroblotted onto Immobilon-P membranes (Millipore Corporation, USA). Membranes were blocked for 1h at room temperature in Tris-buffered saline-0.1% Tween containing 1% BSA

and incubated with the anti-phospho SMAD1 or the anti-GAPDH protein diluted at 1:20 000 and 1:1000, respectively, overnight at 4°C. After washing, the membranes were incubated with the secondary antibody, a peroxidase-conjugated anti-rabbit IgG (Biorad, France) diluted 1:15 000 for 1 hour. Immunoreactive proteins were detected using enhanced chemiluminescence reagents.

#### *Sequence and structure analysis*

Searches within the domain and structure databases were performed using HH-PRED (32). Modelling was made using Modeller v9.15 (33). Manipulation and visualization of 3D structures were made using Chimera (34).

#### *Data analysis and statistics*

All experiments were performed in duplicate in at least three independent cell cultures. For each figure, the number of cultures is indicated in the legend. All data are presented as the mean ± SEM. Statistical significance was determined by ANOVA with Bonferroni post hoc analysis for multiple group comparison. Differences were considered significant when  $P < 0.05$ . The statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA, USA).

To calculate the IC<sub>50</sub> values and Hill slopes corresponding to the inhibition of BMP-4 or BMP-2 action by TSP-1 or noggin, concentration-response data were fitted using a nonlinear regression analysis to the Hill equation (GraphPad Prism version 6.00).

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#### **Conflict of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

### Author contributions

C. S conducted most of the experiments, analysed the results and wrote the paper with C. T., I. C. performed the bioinformatic analysis and modelling experiments. C. S. and I. B. performed cell cultures, BMP bioassay and Western-Blot. J. F. participated to cell cultures. D. L-A provided the C3H10T1/2 cells for BMP bioassay. X. C. contributed to TSP-1 enrichment

from conditioned media. P. M. participated to the bioinformatic analysis. G. H. and V. L. performed mass spectrometry analysis. S. C., M. P. and C. H. designed and performed SPR experiments. C. T conceived, designed and supervised the study and wrote the paper with C. S. and with comments of other authors. All authors approved the final paper.

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### Footnotes

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The abbreviations used are:

BMP bone morphogenetic protein  
TGF $\beta$  transforming growth factor  $\beta$   
GDF growth and differentiation factor  
GnRH gonadotropin releasing hormone  
ALK activin receptor-like kinase  
BMPR-I BMP receptor type I  
BMPR-II BMP receptor type II  
ActR-II activin receptor type II  
BRE BMP-responsive element  
Luc luciferase reporter  
SPR surface plasmon resonance  
TSP-1 thrombospondin 1

## Figures legends

**Table 1.** Protein and peptides identified by tandem mass spectrometry in the fraction from CM bound to BMP-4 coated CM5 sensorchip.

**Table 2.** Kinetic parameters of the interaction between BMP-4 and TSP-1 analysed by surface plasmon resonance.

**Figure 1.** Effect of BMPs or pituitary conditioned media (CM) on luciferase activity from C3H-B12 cells. (A) Dose-dependent induction of the BRE-Luc construct from C3H-B12 cells by BMPs. C3H-B12 cells were treated overnight with increasing concentrations of either BMP-2 (●), BMP-4 (■), BMP-6 (▲) or BMP-7 (○) prior to assaying for luciferase activity as described under Materials and methods. Note that BMP-6 and BMP-7 curves are superimposed. Results are expressed as arbitrary units. Each point represents the mean  $\pm$  SEM of three experiments. (B, C, D) Effect of pituitary CM on luciferase activity from C3H-B12 cells. C3H-B12 cells were exposed overnight to DMEM or CM prior to assaying for luciferase activity. (B) CM were from ovine pituitary cells treated or not with  $10^{-8}$  M GnRH for 6 hours (CM GnRH 6h) or with  $10^{-9}$  M activin for 48 hours (CM Activin 48h). Values are the mean  $\pm$  SEM from seven experiments. (C) Pituitary media conditioned for 48 hours (CM Basal 48h) were supplemented with increasing concentrations of BMP-4 before exposition to C3H-B12 cells. Values are the mean  $\pm$  SEM from three experiments. (D) Comparison of pituitary media conditioned for 6 hours with  $10^{-8}$  M GnRH (CM GnRH 6h) or without GnRH (CM Basal 6h), or for 48 hours with  $10^{-9}$  M activin (CM Activin 48h) and supplemented with rh-BMP-4 (10 ng/mL) before exposition to C3H-B12 cells. Values are the mean  $\pm$  SEM from seven experiments. Insert: CM were from ovine adrenocortical cell cultures conditioned for 48 hours (CM Basal 48) supplemented with BMP-4 (10 ng/mL) before exposition to C3H-B12 cells. Values are the mean  $\pm$  SEM from three experiments. (E) Effect of pituitary CM on C3H-B12 cell proliferation. C3H-B12 cells were exposed overnight to DMEM supplemented or not with BMP-4 (10 ng/mL), GnRH ( $10^{-8}$  M), Activin ( $10^{-9}$  M) or to pituitary CM (CM Basal 6h or CM Basal 48h) prior to assaying for cell proliferation. (F) Effect of pituitary media conditioned for 48 hours (CM Basal 48h) and supplemented with BMP-2 (10 ng/mL) on luciferase activity from C3H-B12 cells. Values are the mean  $\pm$  SEM from three experiments. Bars with different letters indicate that group means are significantly different at  $p < 0.05$ .

**Figure 2 .** Interaction between pituitary conditioned media and BMP-4 analysed by surface plasmon resonance. BMP-4 was immobilized at intermediate density (4600 RU) on a flow cell of a CM5 sensorchip and DMEM-BSA or 1/10 diluted DMEM-BSA or medium conditioned for 6 hours (CM Basal 6h) or for 48 hours (CM basal 48h) diluted 1/10 were then injected for 180 seconds over the chip at 30  $\mu$ L/min and dissociation studied for 120 seconds (plain lines). The sensorgramms are subtracted with the non-specific interaction values obtained on an activated-desactivated control flow cell.

Spotted lines represent aliquots of media concentrated over PEG as described under Results and 1/50 diluted prior to injection. The figure shows one representative experiment. Similar results were obtained with CM provided by 6 independent pituitary cultures.

**Figure 3.** Interaction between rhTSP-1 and BMP-4. (A) Interaction between rhTSP-1 and BMP-4 analysed by surface plasmon resonance using BMP-4 immobilized sensorchip. To reduce TSP avidity effects, BMP-4 was immobilized at low (60 RU) density on a flow cell of a CM5 sensorchip. Increasing concentrations of rhTSP-1 (from bottom to top: 1, 2, 4, 8, 16, 32, 64 nM) were then injected for 180 seconds over the chip at 30  $\mu$ L.min<sup>-1</sup>. Dissociation was studied for 600 seconds (cut here after 200 seconds to better see the first phase of association). The sensorgramms are double subtracted for the non specific interaction and for the buffer effect. (B) Interaction between rhTSP-1 and BMP-4 analysed by surface plasmon resonance using TSP-1 immobilized sensorchip. TSP-1 was immobilized at 1500 RU on a flow cell of a CM5 sensorchip. Increasing concentrations of BMP-4 from 6.25 to 100 nM were then injected for 180 seconds at 10  $\mu$ L.min<sup>-1</sup>. (C) Interaction between rhTSP-1 and BMP-2 analysed by surface plasmon resonance using TSP-1 immobilized sensorchip. TSP-1 was immobilized at 1500 RU on a flow cell of a CM5 sensorchip. Increasing concentrations of

BMP-2 from 3.12 to 50 nM were then injected for 180 seconds at 10  $\mu\text{L}\cdot\text{min}^{-1}$ . (D) Interaction between rhTSP-1 and rh-activin A analysed by surface plasmon resonance. rhTSP-1 was immobilized on a CM5 sensorchip and 100 nM activin A (a) or BMP-4 (b) were then injected over the chip at 10  $\mu\text{L}/\text{min}$ . (E) Interaction between rhTSP-1 and BMP-4 analysed by co-immunoprecipitation. BMP-4 and TSP-1 were incubated together, immunoprecipitated with anti-BMP-4 antibody and immunoblotted with anti-TSP-1 antibody (*lane 4*). *Lanes 2 and 3* show the result after omission of the antibody or its replacement by non specific IgG, respectively. *Lane 1* shows the detection of rhTSP-1 (100 ng). These experiments were performed three times with similar results.

**Figure 4.** Antagonization of BMP-4 or BMP-2 action by TSP-1 (A, B) or noggin (C, D) on luciferase activity from C3H-B12 cells. C3H-B12 cells were exposed to BMP-4 or BMP-2 (2.5 ng/mL, *i.e.*  $10^{-10}$  M) in presence of increasing concentrations ( $10^{-10}$  to  $10^{-7}$  M) of rhTSP-1 or ( $10^{-12}$  to  $10^{-9}$  M) of noggin overnight prior to assaying for luciferase activity. (E, F) The data points, expressed as percentages of inhibition, were fitted using nonlinear regression to the Hill equation giving IC<sub>50</sub> of  $11.4 \pm 6.3$ ,  $8.5 \pm 3.9$ ,  $0.02 \pm 0.001$ ,  $0.02 \pm 0.003$  nM (A, B, C, D respectively) and Hill slope factors are  $0.36 \pm 0.1$ ,  $0.61 \pm 0.13$ ,  $7.4 \pm 0.6$  and  $6.9 \pm 0.8$  (A, B, C, D respectively). Values are the mean  $\pm$  SEM from at least three independent experiments with duplicate determinations. Bars with different letters indicate that group means are significantly different at  $p < 0.05$ .

**Figure 5.** Expression of pituitary TSP-1 and interaction between TSP-1 enriched CM fractions and BMP-4. (A) TSP-1 mRNA expression in ovine pituitary cells. Cells were cultured for 48 hours before RNA extraction. TSP-1 mRNA were analysed by RT-PCR after 30 cycles (*lane 2*). PCR was performed with omission of reverse transcriptase (*lane 1*) (B) TSP-1 protein expression in CM from pituitary cells. Cells were cultured for 6 hours (CM basal 6h) or 48 hours (CM basal 48h) or 6 hours with GnRH (CM GnRH 6h) before CM recovery and Western-blot analysis in reducing or non reducing conditions. CM from corticoadrenal cells were prepared in the same conditions. As control, rhTSP-1 (50 ng) was loaded. Protein loading was checked by membrane staining with Ponceau S. The histograms represent the mean  $\pm$  SEM of densitometric analysis obtained with CM from 6 independent pituitary cultures. (C) Presence of TSP-1 in high molecular mass fraction from ovine pituitary CM. Media from pituitary cells conditioned for 48 hours were fractionated using 100 kDa cut-off membranes. The presence of TSP-1 in the crude CM, the filtrate (CM <100 kDa) and the retentate (CM >100 kDa) was analysed by Western-blot. Protein loading was checked by membrane staining with Ponceau S. The figure shows a representative experiment. These experiments were performed with CM from 6 independent pituitary cultures. (D) SPR interaction between high molecular mass fraction from pituitary CM and BMP-4. The filtrate (CM <100 kDa) 1/10 diluted and the retentate (CM >100 kDa) 1/50 diluted obtained as described above were injected for 180 seconds at 10  $\mu\text{L}/\text{min}$  on a 60 RU immobilised BMP-4 flow cell of a CM5 sensor chip. The crude CM diluted 1/10 was injected in the same conditions. The sensorgrams are subtracted with the non-specific interaction values obtained on the activated-desactivated control flow cell. The figure shows a representative experiment. Independent experiments were performed with CM from three different pituitary cultures. (E) Antagonization of BMP-4 action by high molecular mass fraction from pituitary CM on luciferase activity from C3H-B12 cells. C3H-B12 cells were exposed to the crude CM 1/2 diluted, the filtrate (CM <100 kDa) 1/2 diluted and the retentate (CM >100 kDa) 1/20 diluted in presence of rhBMP-4 (2.5 ng/mL) overnight prior to assaying for luciferase activity. Values are the mean  $\pm$  SEM from four independent experiments with duplicate determinations. Bars with different letters indicate that group means are significantly different at  $p < 0.05$ .

**Figure 6.** BMP activity in ovine serum. (A) C3H-B12 cells were exposed overnight to different dilutions of ovine serum prior to assaying for luciferase activity. Values are the mean  $\pm$  SEM from one representative experiment with duplicate determinations. These experiments were performed three times with similar results. C3H-B12 cells were exposed overnight to ovine serum 1/2 diluted supplemented with (B) increasing doses of noggin from 0 to  $4 \cdot 10^{-10}$  M,  $2.5 \cdot 10^{-6}$  M dorsomorphin (DM) or  $10^{-8}$  M follistatin or (D) pituitary CM (CM basal 48h) diluted 1/2 or (E)  $10^{-7}$  M rhTSP-1 prior to assaying for luciferase activity. Values are the mean  $\pm$  SEM from four experiments with duplicate

determinations. (C) Activation of SMAD1 in C3H-B12 cells by ovine serum. C3H-B12 cells were exposed for 1 hour to BMP-4 (10 ng/mL) or serum ½ diluted or serum ½ diluted supplemented with dorsomorphin ( $2.5 \cdot 10^{-6}$ M). Total proteins were isolated from the cells and western blotting was performed with Phospho-SMAD1 antibody. Equal loading of the proteins was confirmed by using GAPDH antibody. Bars with different letters indicate that group means are significantly different at  $p < 0.05$ .

**Figure 7.** (A) Multiple sequence alignment of the VWC domain of TSP-1 with other VWC domains from chordin family, including that of CV-2 (Q5D734\_DANRE) whose 3D structure is known (pdb 3BK3). Black boxes indicate highly conserved cysteine residues. Observed secondary structures and disulfide bonds are reported above and below the alignment, respectively. Sequences are designated with their UniProt identifiers. (B) Schematic diagram of domain architecture of TSP-1 (modified from 49) vWC= vWC domain; TSR=thrombospondin type 1 domain repeats; EGF = EGF like domain repeats; Ca binding = calcium binding type 3 repeats (C) **Top.** Model of the 3D structure of the TSP-1 VWC domain (ribbon representation) in complex with BMP-4 (surface representation). The three subdomains (clip, SD1 and SD2) are shown together with the five disulfide bridges (numbering is as depicted in Figure 1). The model was built, based on the 3D structure of Crossveinless 2 (CV-2) in complex with BMP-2 (pdb 3bk3) (37) and on the alignment shown in Figure 1. **Bottom left.** Experimental structure of BMP-2 bound to its high-affinity type I receptor BMPR-IA and its low-affinity type II receptor ActR-IIB (pdb 2h62) (45), in order to show the overlap with the VWC domain binding sites. **Bottom right.** Focus on the W331 amino acid, fitting with a groove at the surface of BMP-4.



Table 1

Identified peptides	Protein description	Accession number (nrNCBI)	Taxonomy	Gene	Theoretical molecular weight (kDa)
TGDEWTVDSCTECR CENTDPGYNCLPCPPR QVTQSYWDTPTR	} Predicted Thrombospondin-1	gi 426232958	Ovis aries	TSP-1	129

Table 2

<b>Interacting proteins</b>	<b>ka (M<sup>-1</sup> s<sup>-1</sup>)</b>	<b>kd (s<sup>-1</sup>)</b>	<b>K<sub>D</sub> (M)</b>	<b>Rmax</b>	<b>Chi<sup>2</sup> (RU<sup>2</sup>)</b>
BMP4-TSP1	6.9x10 <sup>4</sup>	0.037	5.95x10 <sup>-7</sup>	421.76	2.58
BMP2-TSP1	1.23x10 <sup>6</sup>	0.154	1.26x10 <sup>-7</sup>	609.8	4.52

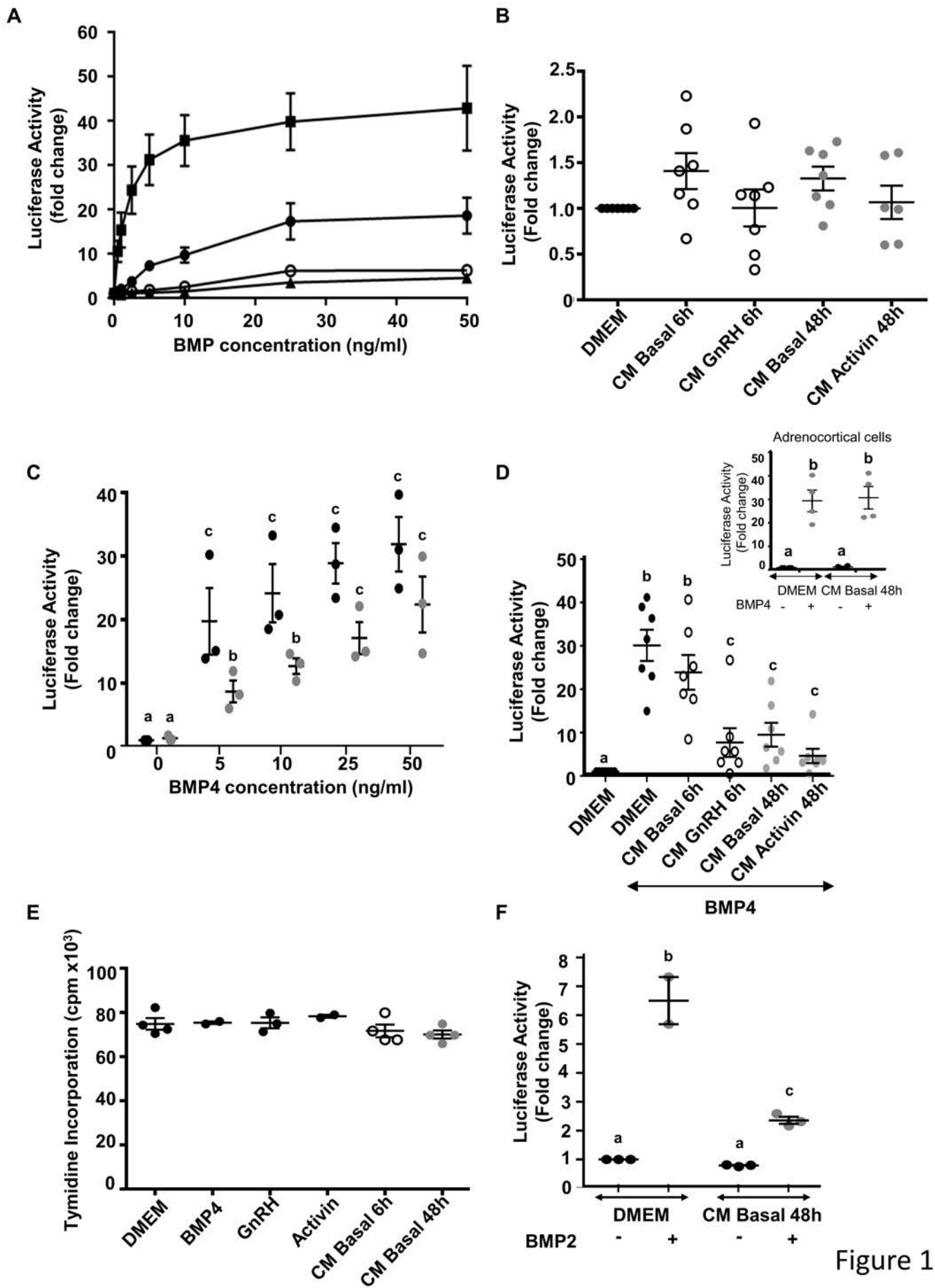


Figure 1

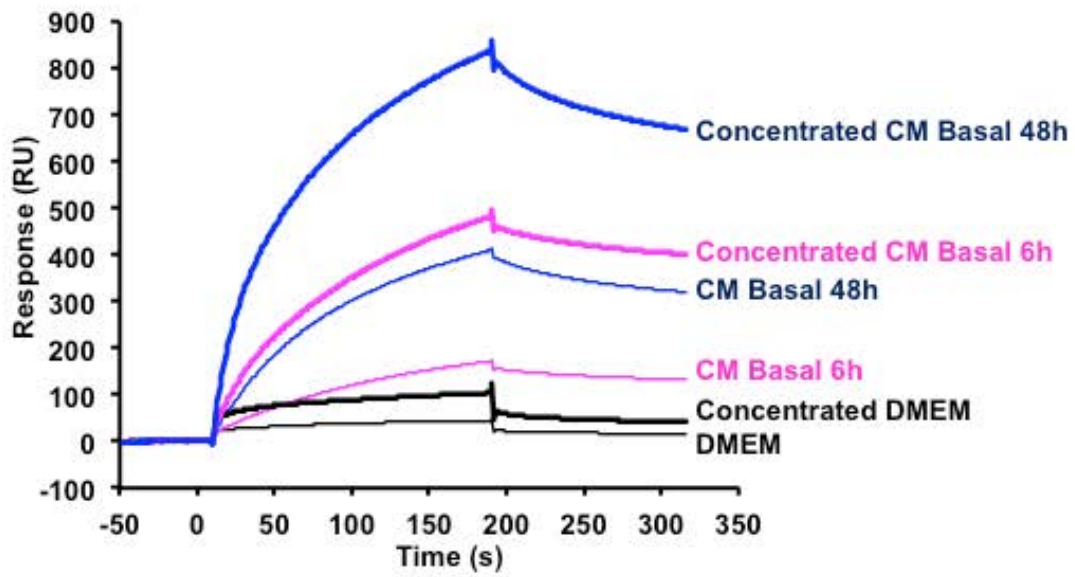


Figure 2



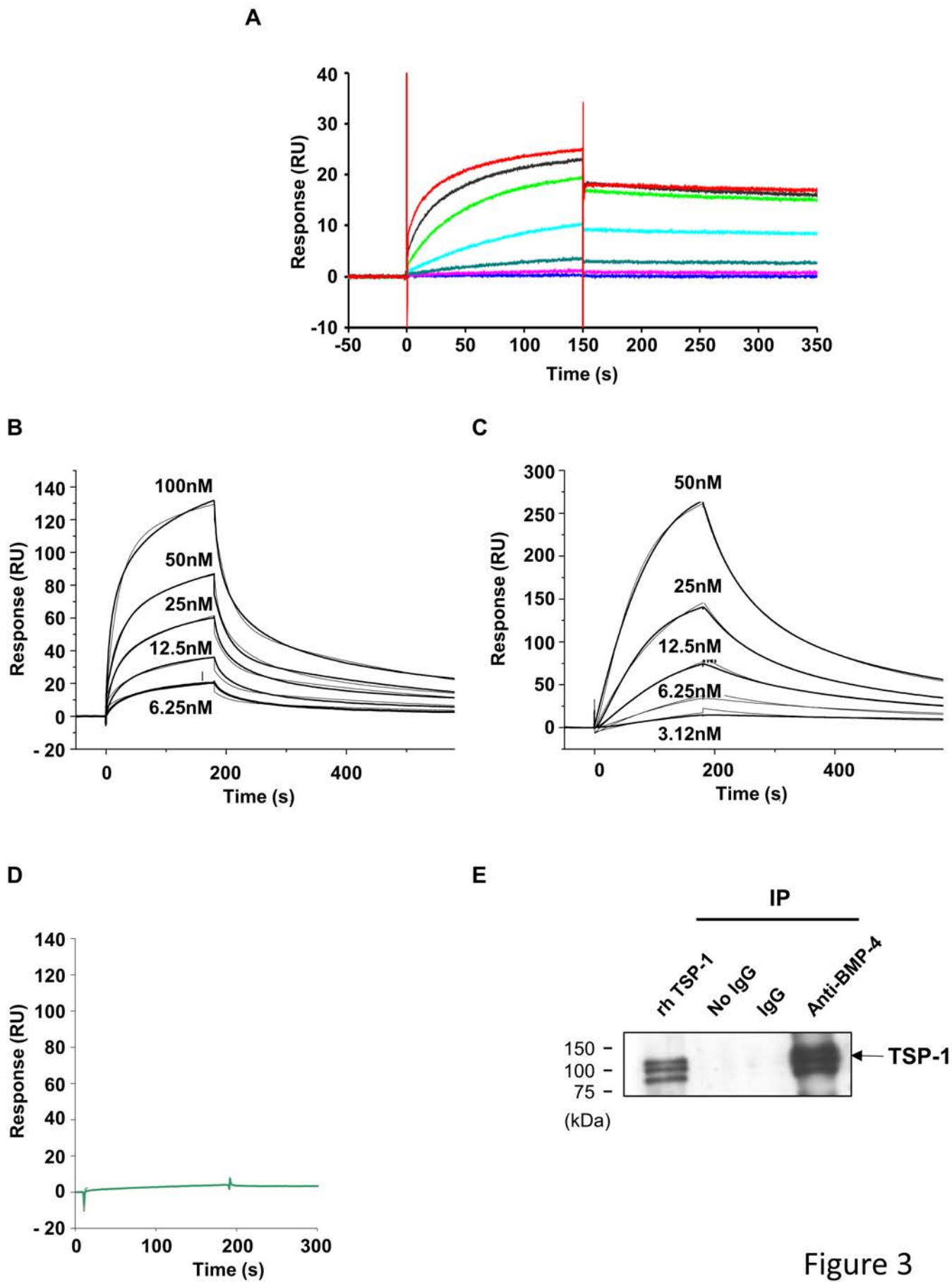


Figure 3

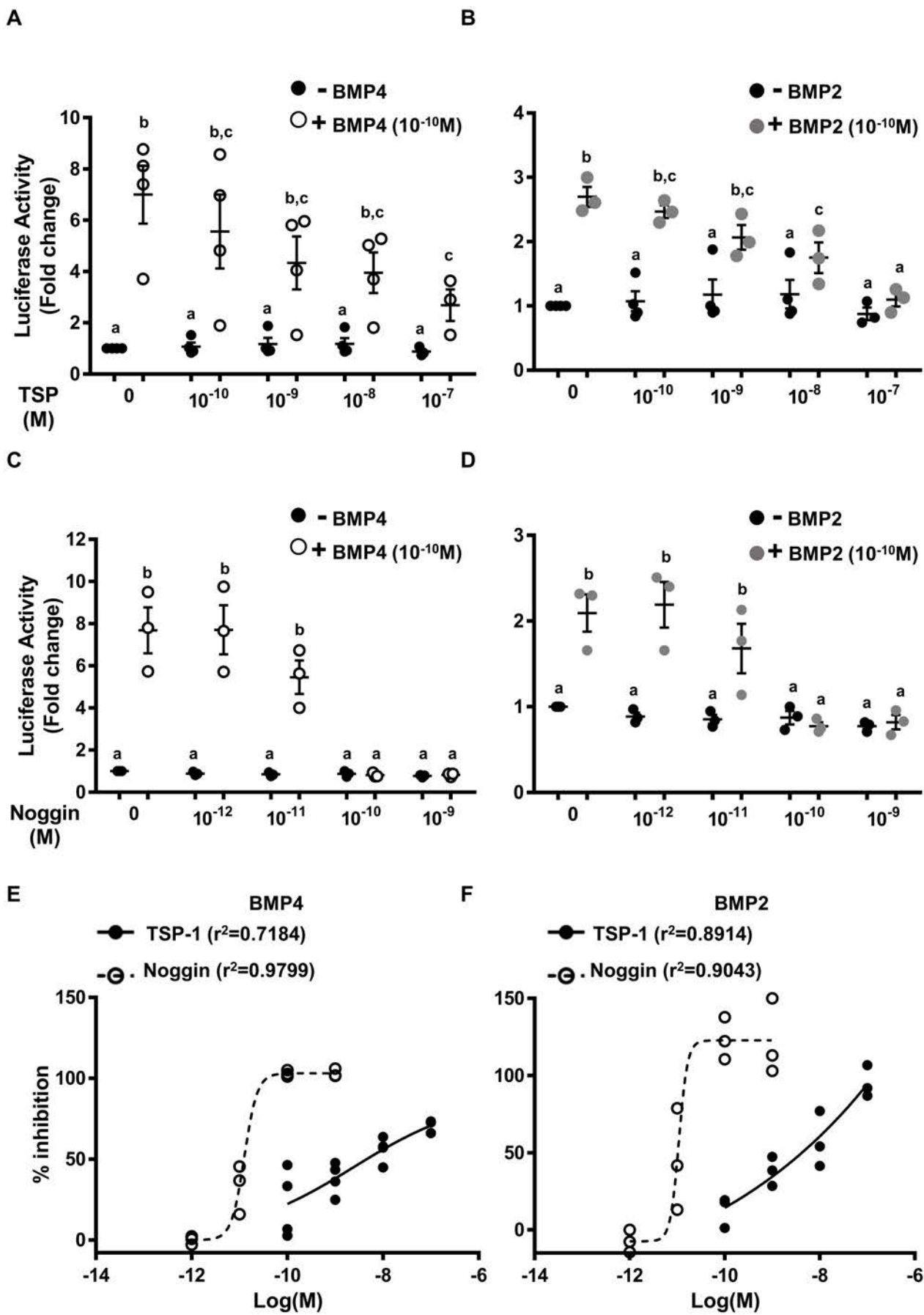


Figure 4

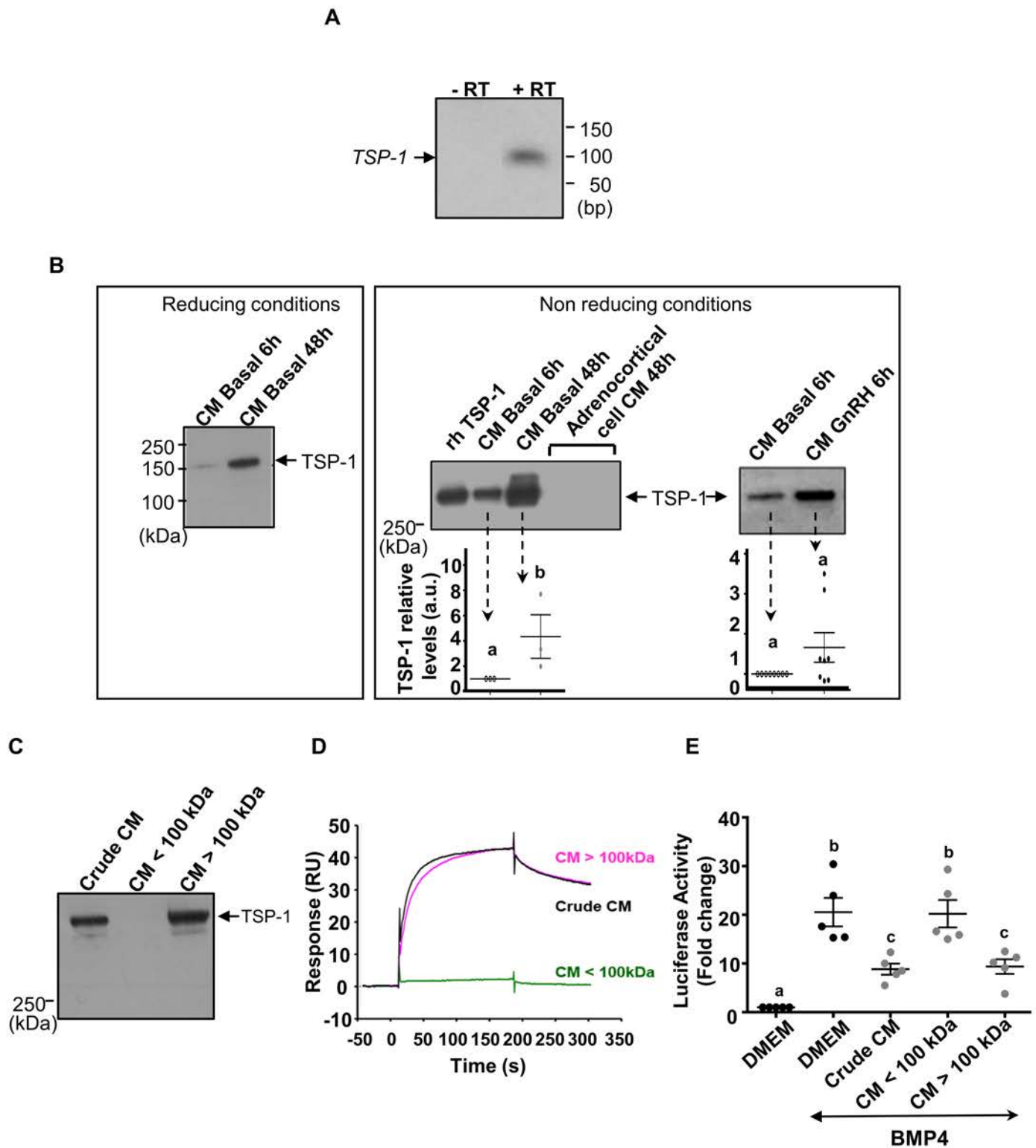


Figure 5

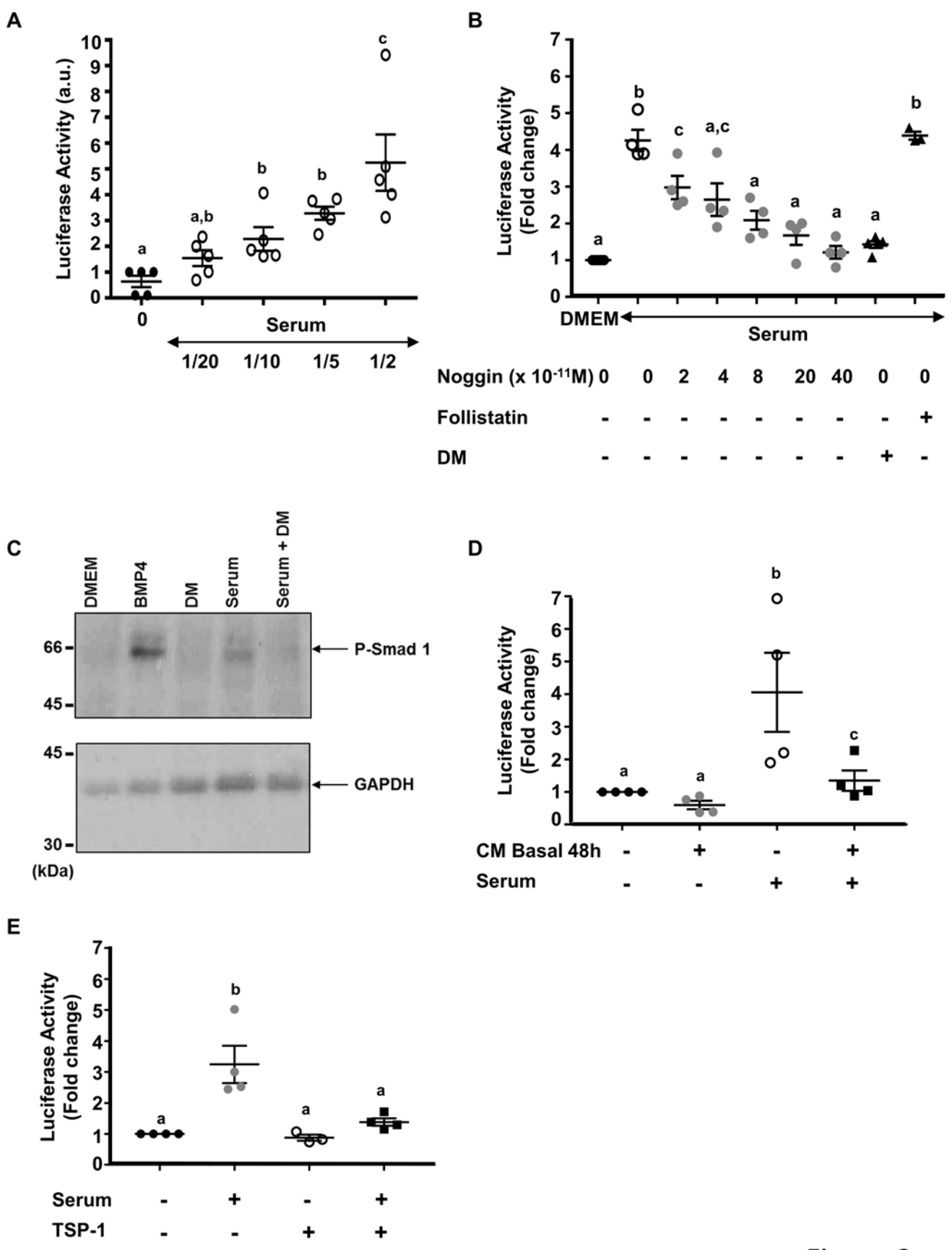


Figure 6





**Thrombospondin-1 (TSP-1), a new bone morphogenetic protein-2 and -4 (BMP-2/4) antagonist identified in pituitary cells**

Céline Sallon, Isabelle Callebaut, Ida Boulay, Joel Fontaine, Delphine Logeart-Avramoglou, Corinne Henriquet, Martine Pugnère, Xavier Cayla, Philippe Monget, Grégoire Harichaux, Valérie Labas, Sylvie Canepa and Catherine Taragnat

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