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博 士 論 文

Regulation of neuropeptide Y Y1 receptor expression by bone morphogenetic protein 2 in C2C12 myoblasts

（C2C12 筋芽細胞における Neuropeptide Y Y1 受容体の BMP2 による発現制御）

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ABSTRACT

The neuropeptide Y (NPY) system is known as one of the major neural signaling pathways. NPY, produced by peripheral tissues including osteoblasts, is known to bind to the Y1 receptor. Recently, osteoblast-specific Y1 receptor knockout mice were developed and were found to have a high bone mass phenotype, indicating a role for the NPY-Y1 receptor axis as a regulator of bone homeostasis. However, regulation of Y1 receptor expression during osteoblastic differentiation remains unexplored. In the present study, we examined the role of bone morphogenetic protein (BMP) 2 signaling in regulating Y1 receptor expression. In C2C12 cells, expression of Y1 receptor mRNA was induced by BMP2. This induction was also observed after co-transfection with Smad1 and Smad4, the intracellular signaling molecules of the BMP2 signaling pathway. In a transfection assay, Smad1/4 up-regulated transcriptional activity through interaction with the Y1 receptor gene promoter. Following transfection of MC3T3-E1 cells with siRNA for the Y1 receptor, the expression of alkaline phosphatase, osteocalcin, Runx2 and osterix were increased. These results show that BMP2 signaling regulates Y1 receptor gene expression, and raises the possibility that NPY acts in osteoblasts via an autocrine mechanism.

Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide characterized as a neurotransmitter in the brain and the autonomic nervous system [1]. In the central nervous system, NPY plays a critical role as a classic neuronal regulator of appetite and energy homeostasis [2]. In the peripheral nervous system, NPY is a constituent of the sympathetic nervous system, co-stored and co-released with noradrenaline during nerve stimulation [2]. Additionally, several sources of NPY have been identified in various cell types including osteoblasts and adipocytes [3,4,5]. Recently, NPY has been shown to regulate bone homeostasis, being involved in processes such as the regulation of bone mass, where it acts both centrally and peripherally [6].

NPY receptors are a family of G-protein coupled receptors, of which five subtypes have been identified: Y1, Y2, Y4, Y5 and y6 [7]. Of these five known receptors for NPY, the Y1 and Y2 receptors have been shown to influence bone mass in mice [8]. Germ-line deletion of Y1 or Y2 receptor produces similar anabolic responses in bone, resulting in an increase in bone mass owing to activation of osteoblasts and an increased rate of bone formation [4,9]. However, the mechanism underlying the action of the Y1 receptor on bone differs from that of the Y2 receptor: both germline and conditional hypothalamic Y2 receptor knockout mice share the same high bone mass phenotype [9,10] demonstrating that central hypothalamic Y2 receptors are crucial for this process, whereas bone tissue is unaltered by conditional deletion of hypothalamic Y1 receptors [4] indicating a nonhypothalamic control of bone mass. Y1 receptor expression has been demonstrated in osteoblastic cells lining endocortical and trabecular bone surfaces [11] and in primary calvarial cultures [12]. A recent study from osteoblast-specific Y1 receptor knockout mice revealed a high bone mass phenotype similar to the bone phenotype of germline Y1 receptor knockout mice, suggesting that the Y1 receptor mediates effects on bone via direct actions on osteoblasts and that

peripheral Y1 receptors play a role in the regulation of bone formation [13].

Bone morphogenetic proteins (BMPs) regulate the proliferation, differentiation and apoptosis of various cell types [14]. BMP2 is reported to trigger osteoblastic differentiation and to up-regulate the expression of several genes that encode osteoblastic phenotype-related proteins *in vitro* [14,15]. In the intracellular BMP2 signaling pathway, Smad1 is phosphorylated directly by type I receptors and then forms complexes with Smad4 and is transported into the nucleus [16,17]. There, the complex interacts with the regulatory elements of the target genes and regulates their gene expression. To date, little is known of the regulation of NPY receptor expression in response to BMP2 in osteoblasts.

Several studies suggest that NPY is indeed capable of directly modulating osteoblast activity. *In vitro* studies have shown that NPY can inhibit the cyclic adenosine monophosphate (cAMP) response to parathyroid hormone and norepinephrine in osteoblastic cell lines [6]. In addition, treatment of osteoblasts with NPY has been shown to reduce markers of osteoblast differentiation [6]. However, while both NPY and Y1 receptor are known to be expressed in osteoblasts, it is unclear whether peripheral NPY acts in a paracrine/autocrine fashion in osteoblasts.

In this study, we show that BMP2 signaling regulates Y1 receptor expression via the Y1 receptor gene promoter at the transcriptional level. In addition, our results of siRNA-mediated Y1 receptor knockdown demonstrate the possibility that NPY acts by an autocrine mechanism in osteoblasts.

Materials and Methods

Cell cultures

The myoblast cell line C2C12 and the osteoblast cell line MC3T3-E1 were obtained and cultured as described previously [18]. For osteoblastic differentiation, MC3T3-E1 cells were cultured for 3 weeks in α-MEM containing both 5 mM β-glycerophosphate and 100 μg/mL of ascorbic acid, with medium changes every 3 days.

Expression plasmids and transfection

Smad expression plasmids encoding the wild-type Smad4 and constitutively active Smad1 (DVD) were provided by Dr T. Katagiri [19,20]. The plasmid DNA was transfected into cells using Lipofectamine 2000 (Invitrogen) as described previously [18].

Transfection of small interfering RNA (siRNA)

MC3T3-E1 cells were transfected with Silencer® select pre-designed siRNA for the Y1 receptor (Ambion, ID number s70765) or Silencer® negative control siRNA #1 (Ambion) at a concentration of 10 nM using Lipofectamine RNAiMAX (Invitrogen) as described previously [21,22]. Cells were harvested 48 h after transfection.

Reverse transcription-polymerase chain reaction (RT-PCR)

PCR was used to analyze transcript levels of the Y1 receptor. Total RNA was extracted from the cells using Isogen (Nippongene, Toyama, Japan) as described previously [18]. RT-PCR was performed as previously described [18]. The primer sequences for each gene were as follows: Y1 receptor, 5'-CTCGCTGGTTCTCATCGCTGTGGAACGG-3' (forward), 5'-GCGAATGTATATCTTGAAGTAG-3' (reverse), generating a 325 bp fragment; Y2 receptor, 5'- GAGAGCAAGATCTCCAAGC-3' (forward),

5'-TGTACTCCTTCAGGTCCAG-3' (reverse), generating a 442 bp fragment; glyceraldehyde -3-phosphate dehydrogenase (GAPDH), 5'-GTGAAGGTCGGTGTCAACG-3' (forward), 5'-GGTGAAGACGCCAGTAGACTC-3' (reverse), generating a 300 bp fragment. To account for any difference in the amount of RNA, *GAPDH* was chosen as our endogenous control. The amplification products were separated by electrophoresis on 2% agarose gels.

Quantification of gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) was performed using assay-on-demand TaqMan probes (Applied Biosystems) and the StepOne® real time PCR system [21,22]. The relative level of gene expression was quantified using the comparative C_T method with *GAPDH* as the endogenous control.

Reagents

Recombinant human BMP2 was kindly supplied by Astellas Pharma Inc. (Tokyo, Japan).

Reporter constructs and assay for luciferase activity

Luciferase reporter plasmids for the murine *Y1 receptor* promoter were generated as follows. The 871-bp *Y1 receptor* promoter fragment (-731 to +140) was isolated from mouse genomic DNA by PCR and subcloned into the pGL4.12 vector (Promega) to generate the luciferase reporter plasmid (pY1R731-luc). The deletion reporter constructs: pY1R371-luc (-371 to +140 *Y1R* promoter fragment), pY1R311-luc (-311 to +49) and pY1R271-luc (-271 to +49) were made by PCR amplification. The nucleotide sequences of each promoter region were verified by sequencing. The reporter assay

was performed as described previously [18].

Chromatin immunoprecipitation (ChIP) Assay

A ChIP assay was performed as described previously [21] using ChIP-IT® Express Chromatin Immunoprecipitation Kits (Active Motif). Chromatin solutions were incubated with the following antibody solutions: anti-Smad1 (A-4, Santa Cruz) or control IgG (Sigma-Aldrich). Fractions of the purified ChIP DNA or inputs were used for PCR analysis. The reaction was performed with Taq DNA polymerase (Qiagen) for 30 cycles of 30 s at 94°C, 30 s at 58°C, and 60 s at 72°C. PCR primer pairs were generated to detect DNA segments located at -371/-242 (primers P1 and P2) in the mouse *Y1 receptor* promoter. PCRs using primers P3 and P4 of the mouse *Y1 receptor* intron region (+181/+361) served as negative control. The PCR primer sequences used in this study were P1, 5'-CTCCCCTCCATTCTTGACTT-3'; P2, 5'-GCAAGGCGCATTTACAAGCC-3'; P3, 5'-GGTGTCTGATCCAGTCCTGG-3'; P4, 5'-AGTGCAAGACGCCAGGATGC-3'. The PCR products were separated on 3% agarose gels and visualized with ultraviolet light. All ChIP assays were repeated at least two times.

Statistical analysis

All experiments were repeated three to four times and representative results are shown. The data are reported as the mean ± standard deviation, and were analyzed by Student's t-test, where values of $P < 0.05$ were considered significant.

Results

BMP2 or Smad1/4 induces Y1 receptor expression in C2C12 cells

To evaluate a potential role for BMP2 on NPY receptor expression, we used the C2C12 cell line. C2C12 cells are a well-characterized model system, which have been reported to differentiate not only into myotubes, but also osteoblasts, depending on the specific culture conditions, when incubated in the presence of BMP2 [15]. In C2C12 cells, neither Y1 nor Y2 receptor expression was detected using RT-PCR analyses. The addition of BMP2 induced Y1 receptor mRNA expression in these cells (Fig. 1A). In contrast, Y2R expression was not affected by BMP2 (Fig. 1A). The expression level of the Y1 receptor was also determined by qRT-PCR. The level of Y1 receptor mRNA increased by more than 5-fold in response to 50 ng/mL of BMP2 compared to untreated cells and further increased with increasing doses of BMP2, up to an increase of more than 9-fold at 250 ng/mL (Fig. 1B). Both alkaline phosphatase (ALP) and osteocalcin expression, which are well known to respond to BMP2 in these cells, also increased (Fig. 1C). When we analyzed the time course of Y1 receptor mRNA expression induced by BMP2, we found that the level of Y1 receptor mRNA began to increase after 12 h and a time-dependent increase in Y1 receptor mRNA levels was observed up to 24 h (Fig. 1D). These findings indicate that BMP2 can induce Y1 receptor gene expression in C2C12 cells.

Several previous reports have demonstrated that BMP2 signaling results in the direct transcriptional activation of BMP-responsive promoters by transcription factors that include Smad1 and Smad4 [19,23,24]. Therefore, to explore the effect of Smad1/4 on Y1 receptor expression, we transfected expression plasmids for these proteins into C2C12 cells, instead of adding BMP2. The induction of Y1 receptor expression was observed after transfection of the constitutively active form of Smad1 (Smad1(DVD)) but not Smad4 (Fig. 2). In addition, the Y1 receptor expression level was increased

significantly by co-transfection of Smad1(DVD) and Smad4 (Fig. 2). In contrast, Y2 receptor expression was not affected by Smad1(DVD) and/or Smad4 (data not shown). Our results suggest that regulation of Y1 receptor expression by BMP2 depends on the Smad1/4 signaling pathway.

Regulation of Y1 receptor promoter activity by BMP2 signaling

To investigate the mechanism by which BMP2 signaling activates *Y1 receptor* transcription, we cloned an approximately 0.8 kilobase-pair mouse genomic DNA fragment corresponding to the 5'-flanking promoter region of the mouse *Y1 receptor* gene [25]. The Y1 receptor promoter region was ligated into a luciferase reporter expression vector (pY1R-731luc) to examine its responsiveness to BMP2 signaling. Transient transfection of pY1R731-luc into C2C12 cells together with co-transfection of both Smad1(DVD) and Smad4 cDNA resulted in a significant upregulation of luciferase activity (Fig. 3A). The luciferase activity of the construct in which the 5'-end was deleted up to nucleotide position -371 (pY1R317-luc) or -311 (pY1R311-luc) was also increased by Smad1/4 (Fig. 3A). However, luciferase activity was not altered in the construct in which the 5'-end was deleted up to nucleotide position -271 (pY1R271-luc), used as a reporter for Smad1/4 (Fig. 3A).

To assess whether BMP2-induced Smad1/4 also bound *in vivo* to its target site in the Y1 receptor gene promoter, we performed a ChIP assay. BMP2 induced interaction of Smad1 with the chromatin fragment of the proximal Y1 receptor promoter that contained the putative Smad binding motif (primers P1/P2). In contrast, Smad1 antibody failed to immunoprecipitate chromatin fragments of the Y1 receptor gene first intron that contains no Smad-binding motif (primers P3/P4) (Fig.3B).

Regulation of Y1 receptor expression during osteoblastic differentiation

To investigate whether regulation of Y1 receptor expression depends upon differentiation stage during osteoblastic differentiation, MC3T3-E1 cells were cultured in α-MEM containing ascorbic acid and β-glycerophosphate. These cells have been shown to exhibit characteristics typical of osteoblasts [26]. As shown in Fig. 4A, Y1 receptor expression in differentiated MC3T3-E1 cells (at 2 and 3 weeks) was higher than in undifferentiated MC3T3-E1 cells (at 1 day) (Fig. 4A).

Knock-down of the Y1 receptor upregulates ALP, osteocalcin, Runx2 and osterix expression in MC3T3-E1 cells

To evaluate the potential biological relevance of regulation of the Y1 receptor in osteoblasts, we examined the effect of Y1 receptor knockdown using RNA interference. Following transfection of MC3T3-E1 cells with siRNA for the Y1 receptor, the expression levels of Y1 receptor dramatically diminished to undetectable levels, confirming that the siRNA was effective in silencing the endogenous Y1 receptor gene (Fig. 4B). In the same cultures, expression of specific genes which characterize osteoblastic differentiation, such as ALP, osteocalcin, Runx2 or osterix were increased by Y1 receptor inhibition. In contrast, NPY expression was down-regulated (Fig. 4B). These results indicate that osteoblastic gene expression is NPY-Y1 receptor dependent, and that the NPY signaling pathway may be involved in regulating osteoblastic gene expression.

Discussion

NPY binds predominantly to a G-protein coupled receptor, the Y1 receptor, which is widely expressed in the brain and in several types of peripheral tissue [7]. In bone tissue, the Y1 receptor appears to be constitutively expressed by osteoblasts [13]. Our study shows that unstimulated C2C12 cells express undetectable levels of Y1 receptor, but that this expression is induced by BMP2 treatment, suggesting that the induction of Y1 receptor expression may be regulated during osteoblast differentiation. Our present results, showing that Y1 receptor expression is high in differentiated MC3T3-E1 cells may support the idea that the Y1 receptor is predominantly expressed by osteoblastic cells at a particular differentiation stage. Y1 receptor expression might be characteristic of osteoblasts and be regulated during osteoblast differentiation. Therefore, induction of Y1 receptor expression may represent a useful marker of osteoblast differentiation. Although BMP2 induced Y1 receptor expression, BMP2 was not able to induce Y2 receptor expression in C2C12 cells. Consistent with our results, other investigators have also shown that the Y2 receptor could not be detected in bone cells using these methods [11]. Our observation suggests that the Y2 receptor could not mediate effects on bone via direct actions on osteoblasts.

To date there are few reports concerning regulation of Y1 receptor expression by growth factors and signaling molecules. Mannon *et al.* reported that retinoic acid down-regulates Y1 receptor expression in SK-N-MC cells [27]. In this study, we show that BMP2 signaling activates the Y1 receptor gene promoter. Upon activation by BMP2, Smad1 can form a complex with Smad4. The complex then acts as a transcriptional regulator of target genes. A number of target genes such as Id1, Tlx-2, or Mix.2 have been identified that respond to BMP signaling and that have a Smad binding motif [19,23,24]. Several DNA binding motifs for Smads have been identified [28]. PCR-based screening of random sequences identified the GTCT motif as a Smad-binding motif [29].

Our analysis implies the presence of transcriptional machinery that is sensitive to interference by BMP2 signaling and that regulates transcriptional activity through interaction with the Y1 receptor gene promoter between nucleotide position -311 and -271. We performed a search of Y1 receptor gene promoter sequences downstream from the nucleotide sequence from position from -311 to -271 for potential Smad binding motifs. This search identified a GTCT motif located at positions -294 to -284. Consistent with our studies, sequences consisting of the GTCT motif were bound and transactivated by Smad1/4 [30]. Our present results suggest that the Smad-binding motif in the Y1 receptor gene promoter functionally interacts with Smad1/4, and that these complexes then up-regulate expression of the Y1 receptor.

Evidence from several *in vitro* and *in vivo* studies supports the idea that NPY inhibits osteoblast differentiation. For example, it has been shown that NPY reduces ALP and osteocalcin expression and inhibits mineralization in bone marrow stromal cells [31]. Recently, Matic *et al.* developed a mouse model of osteoblast lineage-specific NPY overexpression [32]. In these animals, trabecular and cortical bone volume is reduced. Calvarial osteoblast cultures from these mice also exhibit reduced expression of several osteogenic marker genes such as osteocalcin [32]. In our present study, inhibition of the Y1 receptor using siRNA regulates Runx2, osterix, ALP and osteocalcin mRNA expression. Runx2 and osterix are osteoblast-specific transcription factors essential for the development of osteoblastic cells and bone formation [33]. Inhibition of the Y1 receptor may induce expression of the osteoblast phenotype by increasing Runx2 activity and consequently stimulating the expression of osteocalcin and ALP. Inhibition of the Y1 receptor has been shown to specifically regulate NPY expression, indicating the possibility that not only differentiation-related mRNA but also NPY expression is regulated by the Y1 receptor. It has been reported that NPY downregulates expression of Y1 receptor mRNA in bone marrow stromal cells [31].

Taken together, these observations suggest a feedback role for the local NPY-Y1 receptor axis that regulates both NPY and Y1 receptor expression in osteoblasts. With this work, we have uncovered a new mechanism by which the NPY-Y1 receptor axis may function as an autocrine mechanism expressed by osteoblasts.

Recently, antagonism of the Y1 receptor, achieved by oral administration of BIBO3304, was shown to enhance osteoblast activity resulting in an increased mineral apposition rate in both cortical and cancellous bone of mice [34]. The authors reported that oral administration of BIBO3304 does not produce significant extra-skeletal side effects with regard to body weight, energy metabolism, glucose homeostasis or food intake, that would contraindicate its use as a bone anabolic agent. Our findings suggest that siRNA might act as a potent Y1 receptor blocker and could thus provide new anabolic agents that could be used therapeutically to prevent or reverse bone loss in such conditions as osteoporosis.

In conclusion, we have shown that BMP2 signaling regulates Y1 receptor gene expression, and that inhibition of the Y1 receptor enhances osteoblastic gene expression. This is the first study linking NPY receptor expression to BMP signaling. As such, these investigations may provide important new information pertaining to the molecular basis of the regulation of osteoblastic differentiation in bone tissue.

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FIGURE LEGENDS

Figure 1

BMP2 induces Y1 receptor expression in C2C12 cells

C2C12 cells were plated at 1×10^5 cells/cm^2 and cultured as described. After 24 h, BMP2 was added at the indicated concentration (A and B) or at 250 ng/mL (C and D), after which the cells were cultured for 24 h (A, B and C) or for the indicated period (D). Total RNA was extracted from the cells and the Y1 receptor (Y1R) and Y2 receptor (Y2R) mRNA level was determined by RT-PCR (A) or qRT-PCR (B, C and D). Data are presented as means ± S.D. *$P < 0.05$.

Figure 2

Smad1/4 induces Y1 receptor expression in C2C12 cells

C2C12 cells were transiently transfected with 100 ng/well of the expression construct for Smad1, Smad4 or an empty expression plasmid (pcDNA3), and then cultured for 48 h. qRT-PCR was performed to quantify the Y1 receptor expression level. Data are means ± S.D. *$P < 0.05$.

Figure 3

BMP2 signaling regulates Y1 receptor promoter activity

(A) C2C12 cells were transiently co-transfected in 24-well plates with a reporter plasmid (pY1R731-luc, pY1R371-luc, pY1R311-luc or pY1R271-luc) and Smad1/4 or control plasmid (pcDNA3) for 48 h, after which luciferase activity was determined. Black bars represent transfection with Smad 1/4; white bars represent control plasmid. Normalized luciferase activity is shown as the ratio of luciferase activity relative to control plasmid with pY1R731-luc, which is set to a value of 1. Data are means ± S.D. *$P < 0.05$. (B) C2C12 cells were cultured as described. After 24 h, the cells were cultured with or

without 250 ng/mL of BMP2 for 24 h. Protein/DNA complexes were extracted from the cells, and then precipitated without antibody (input), or with a Smad1 antibody or nonspecific IgG. PCR amplification was performed using primers for the Y1 receptor gene promoter site (P1 and P2). PCR using Y1 receptor gene intron primers (P3 and P4) was used as the negative control. SBM: Smad binding motif. PCR using input DNA was used as the positive control. The positions used for PCR analyses of ChIP DNAs are shown (upper diagrams).

Figure 4

The Y1 receptor is upregulated during osteoblastic differentiation and its inhibition increases osteoblastic gene expression

(A) MC3T3-E1 cells were cultured in α-MEM containing ascorbic acid and β-glycerophosphate for 2 or 3 weeks or 1 day and total RNA was extracted. The levels of Y1 receptor (Y1R) and Y2 receptor (Y2R) mRNA expression in the cells were determined by RT-PCR. (B) C2C12 cells were transiently transfected with Y1 receptor siRNA or control siRNA (both at 10 nM) and incubated again for 48 h. Total RNA was extracted and qRT-PCR was performed to quantify the expression level of Y1R, Runx2, osterix, alkaline phosphatase (ALP), osteocalcin (OCN) and neuropeptide Y (NPY). $*P < 0.05$.

Figure 1

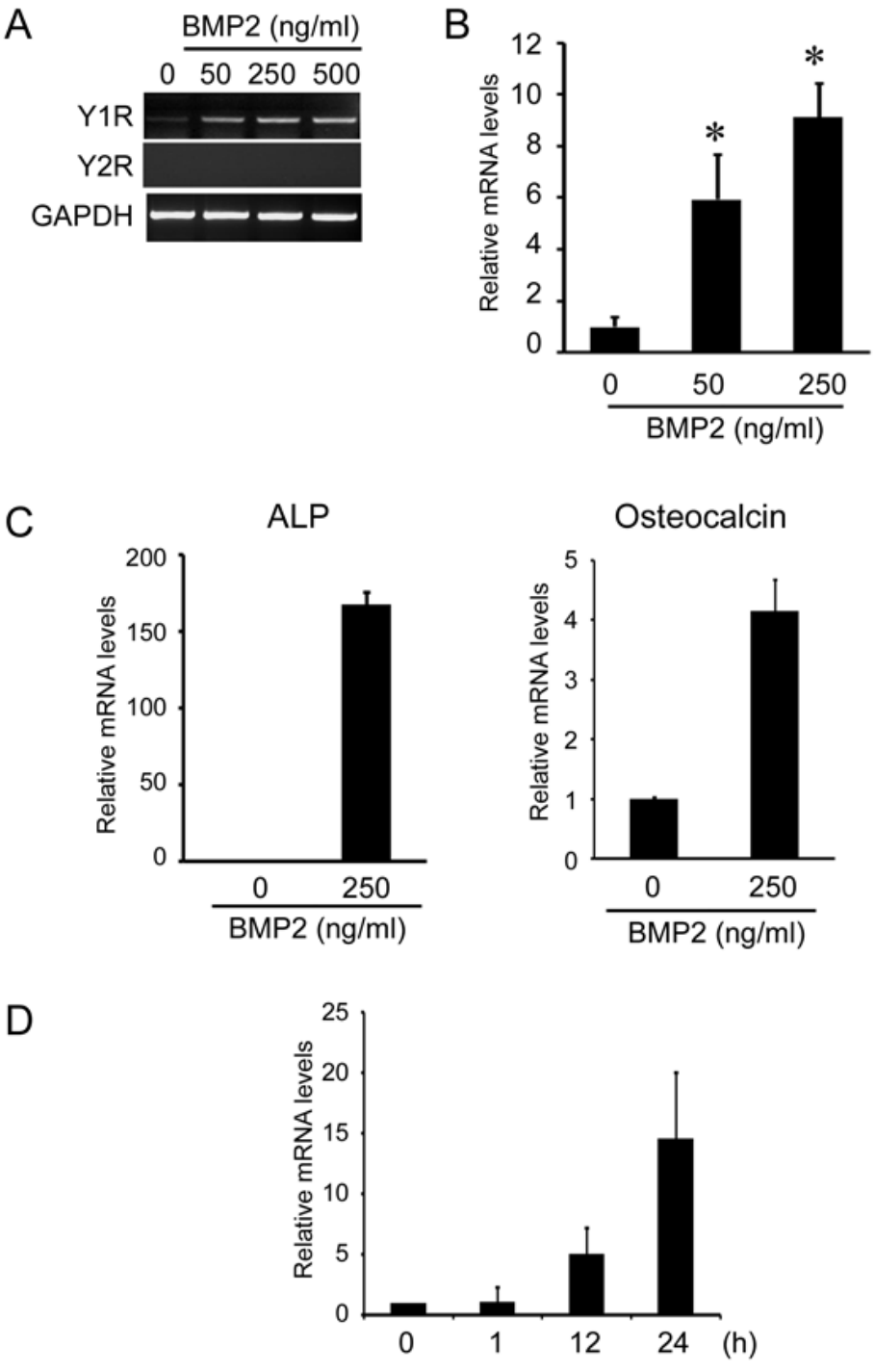

A
BMP2 (ng/ml)
0 50 250 500
Y1R
Y2R
GAPDH
B
Relative mRNA levels
12
10
8
6
4
2
0
*
*
0 50 250
BMP2 (ng/ml)
C
ALP
Relative mRNA levels
200
150
100
50
0
0 250
BMP2 (ng/ml)
Osteocalcin
Relative mRNA levels
5
4
3
2
1
0
0 250
BMP2 (ng/ml)
D
Relative mRNA levels
25
20
15
10
5
0
0 1 12 24 (h)

Figure 2

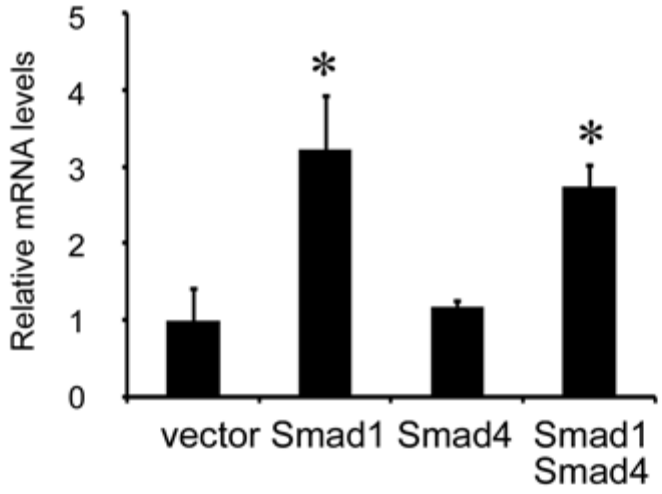

Relative mRNA levels
5
4
3
2
1
0
*
*
vector
Smad1
Smad4
Smad1
Smad4

Figure 3

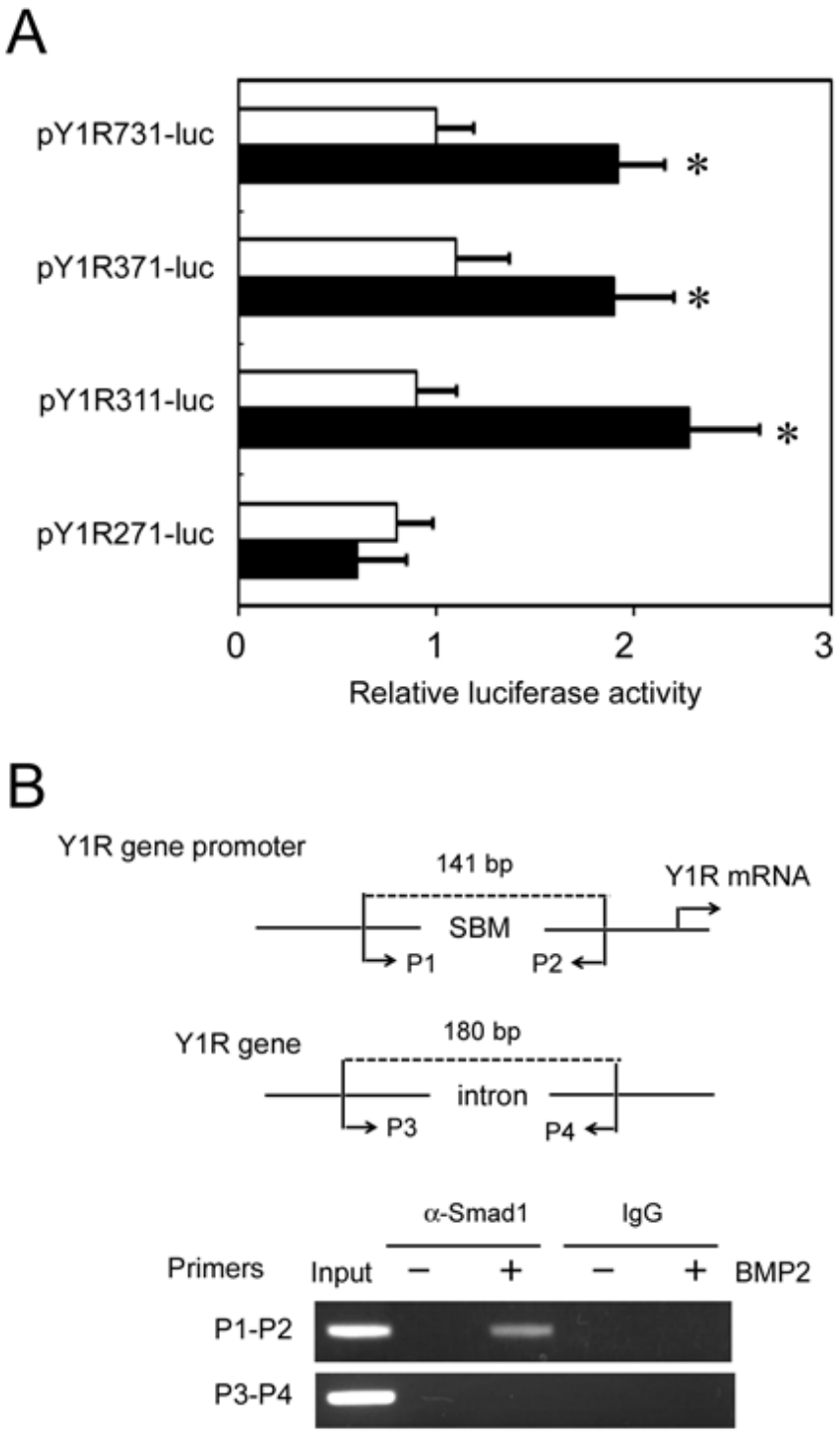
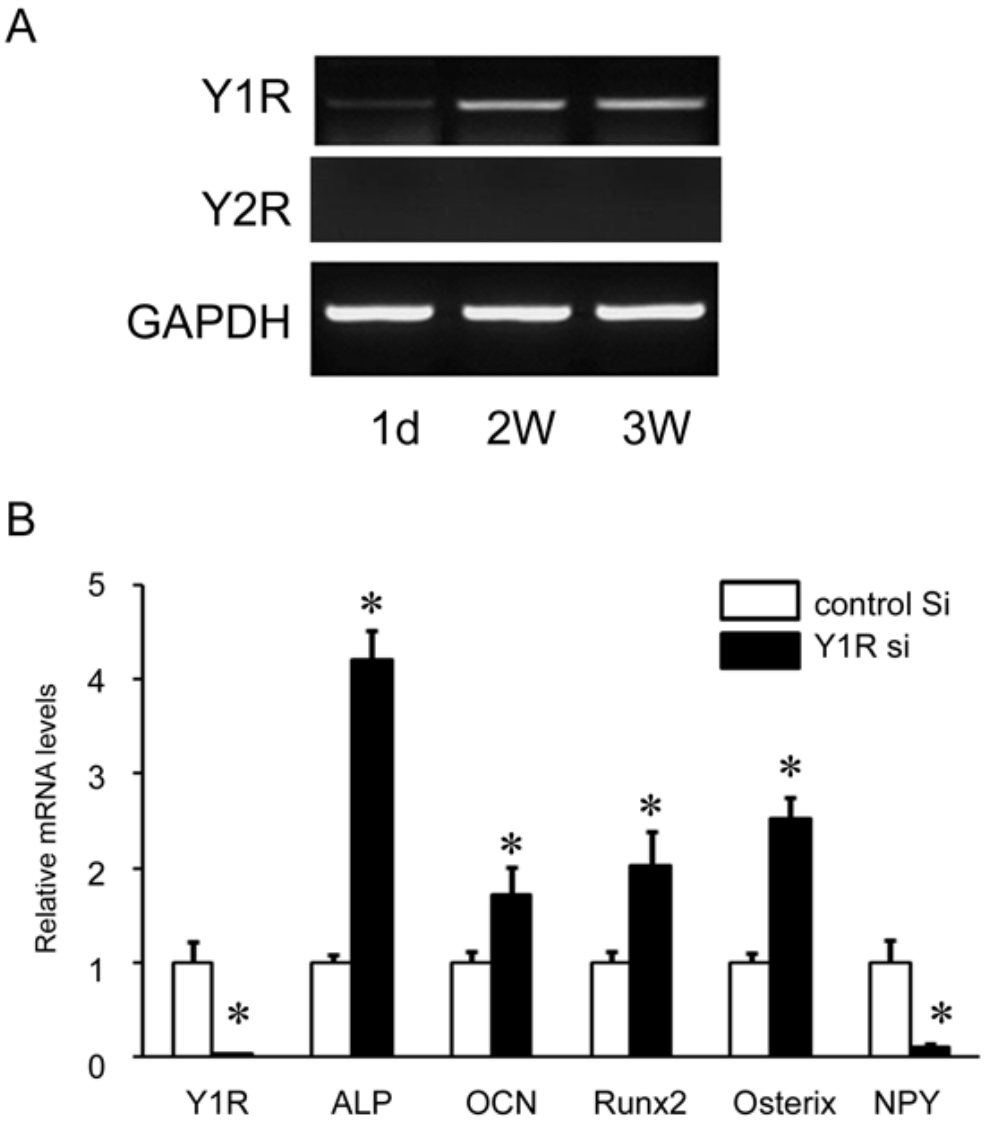

A
pY1R731-luc
pY1R371-luc
pY1R311-luc
pY1R271-luc
*
*
*
0
1
2
3
Relative luciferase activity
B
Y1R gene promoter
141 bp
Y1R mRNA
SBM
P1
P2
Y1R gene
180 bp
intron
P3
P4
α-Smad1
IgG
Primers
Input
−
+
−
+
BMP2
P1-P2
P3-P4

Figure 4


A
Y1R
Y2R
GAPDH
1d
2W
3W
B
Relative mRNA levels
0
1
2
3
4
5
control Si
Y1R si
*
Y1R
ALP
OCN
Runx2
Osterix
NPY