Prostaglandin E_2 Enhances B-Type Natriuretic Peptide Receptor Expression in Calvarial Osteoblasts through EP1 Subtype of Prostaglandin E_2 Receptor

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The B-type natriuretic peptide receptor (NPR-B) is a specific receptor for the C-type natriuretic peptide (CNP) and the binding of the peptide to NPR-B stimulates the bone formation by osteoblasts. However, the mechanism behind the regulation of NPR-B expression in osteoblasts remains unknown. In this study, we examined the role of prostaglandin E_2 (PGE_2) through the PGE_2 receptor subtypes, EP1, EP2, EP3 and EP4, in the regulation of NPR-B expression using calvarial osteoblasts from rats of various ages. Reverse Transcription-PCR (RT-PCR) and Western blotting analyses revealed that PGE_2 or 17-phenyl-ω-trinor PGE_2, an EP1 agonist, increased the expression of NPR-B of calvarial osteoblasts from 25-week-old rats in a time- and dose-dependent manner. The PGE_2- and EP1 agonist-induced increase in NPR-B expression was blocked by treating with SC19220, an EP1 antagonist. By contrast, agonists for EP2, EP3, and EP4 failed to affect the NPR-B expression. The basal mRNA level of NPR-B and EP1 continuously decreased with the age of cell donors between 10 to 60 weeks and remained constant over 60 weeks. The degree of EP1 agonist-induced increase in NPR-B mRNA level gradually decreased with age of cell donors between 10 to 60 weeks, and no significant effect of EP1 agonist on the NPR-B mRNA level was observed over 60 weeks. From these results, we concluded that PGE_2 acts as a regulator of NPR-B expression through the EP1 receptor in osteoblasts and age-related decrease in EP1 expression causes a decrease in NPR-B expression.

Key words —— natriuretic peptide receptor, prostaglandin E_2, osteoblast, aging

INTRODUCTION

The natriuretic peptide family consists of atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide (CNP).[1,2] CNP stimulates the differentiation of osteoblastic lineage cells through its specific receptor, B-type natriuretic peptide receptor (NPR-B).[3–5] The targeted disruption of the genes coding for CNP[6] and NPR-B[7] causes dwarfism due to an impaired longitudinal growth of long bone, while the transgenic overexpression of CNP causes skeletal overgrowth,[8] suggesting that the CNP/NPR-B signaling pathway has an essential role in the regulation of bone metabolism. Recently, we have shown that the activation of CNP/NPR-B signaling causes the enhancement of bone formation in calvarial osteoblasts from 25-week-old rats, and that the CNP-induced potentiating action is not observed in cells from 120-week-old rats due to a decrease in NPR-B expression.[9] However, the mechanism behind the decrease in receptor expression remains unknown.

Prostaglandins are strong modulators of bone metabolism, and their administration to rodents increases bone mass, resulting in enhanced mechanical strength of the skeleton.[10] Among prostaglandins, prostaglandin E_2 (PGE_2) has proven to be a bone anabolic agent.[11–14] In primary cultured osteoblasts from cyclooxygenase-2 (COX-2) knockout and wild-type mice, the absence of PGE_2 synthesis suppressed the formation of mineralized bone nodules, a maker of bone formation, suggesting that PGE_2 plays an important role in bone formation by osteoblasts.[15] PGE_2 exerts its effect through interaction with specific receptor subtypes, termed EP1, EP2, EP3 and EP4.[16] We have previously shown that the bone formation in primary cul-

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tured osteoblasts from rat calvariae decreases with age of cell donors due to a reduction in EP1 expression.[17, 18] In this study, we examined the effects of PGE_2 on the NPR-B expression using calvarial osteoblasts from rats of various ages, and found that PGE_2 acts as a regulator of NPR-B expression through the EP1 receptor and the action of PGE_2 is inactive in aged rat cells due to a decrease in the expression of EP1.

MATERIALS AND METHODS

Materials —— SC-19220, PGE_2, 17-phenyl-ω-trinor PGE_2, butaprost, sulprostone, 11-deoxy-PGE_1, and the antibodies for EP1, EP2, EP3 and EP4 were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Forskolin and 3-(isobutyl)-l-methylxantine (IBMX) were purchased from BIOMOL International (Plymouth Meeting, PA, U.S.A.). The antibodies for β-actin and NPR-B were purchased from Sigma (St. Louis, MO, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), respectively. The agonists and inhibitors were dissolved in dimethylsulfoxide, and these solutions were kept at -20°C before use.

Animals —— Female Wistar rats (4 weeks old) were purchased from CLEA (Tokyo, Japan), and maintained in the animal facility of Toho University under specific pathogen-free conditions. All animal experiments were performed in accordance with the Care and Use of Laboratory Animals Committee of the Faculty of Pharmaceutical Sciences, Toho University.

Cell Culture —— The cells enriched for osteoblast phenotype were enzymatically isolated from calvariae of 25- and 120-week-old female Wistar rats as described previously.[9] In brief, frontal and parietal bones from rats were stripped of soft tissue and periosteum, minced with scissors, and digested with a mixture of 0.2% collagenase (Wako, Tokyo, Japan) and 0.25% trypsin at 37°C for 20 min. The released cells were collected and immediately suspended in F-12 medium (Invitrogen, Rockville, MD, U.S.A.) supplemented with 10% fetal bovine serum (FBS, Invitrogen). This procedure was repeated 5 times every 20 min. The released cells from the last four fractions were grown in F-12 medium supplemented with 10% FBS at 37°C. After reaching sub-confluence, the cells were collected by trypsin treatment, plated in 35-mm dishes at a density of 5×10^3 cells/cm^2, and grown in the same medium for 6 days. To determine the effects of an agonist for a PGE_2 receptor on the expression level of NPR-B in osteoblasts, the cells were treated with various concentrations of test agent in serum-free medium for 24 hr on day 5.

Real-Time Reverse Transcription-PCR (RT-PCR) Analysis —— On day 5 or 6, the cells in a 35-mm dish were homogenized using TRIzol reagent (Invitrogen). Total RNA was extracted in accordance with the manufacturer's protocol and subjected to real-time RT-PCR analyses using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, U.S.A.) in accordance with the method described previously.[9] The sequences of the primer sets for NPR-B, EP1 and β-actin mRNAs and the product sizes determined by PCR are shown in Table 1. The quantities of receptor mRNAs in each sample were normalized using the C_T (threshold cycle) value obtained for β-actin mRNA amplification.

Western Blotting Analysis —— On day 6, the cells in a 35-mm dish were washed with cold phosphate buffered saline and whole cell lysate was prepared by adding an M-PER mammalian protein extraction reagent (PIERCE Biotechnology, Rockford, IL, U.S.A.) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, U.S.A.) to the cells. Western blotting analyses were performed in accordance with the method described

Table 1. Sequences of Primers Used in Real-Time PCR Analysis

Gene	GenBank™ accession number	Sequence of primers[a)]	Product size (bp)
NPR-B	NM_053838	F: 5′-TGAGCAAGCCACCCACTTC-3′	111
		R: 5'-CAGCGGGCCGCAGATA-3'	
EP1	NM_013100	F: 5′-AAGGCAGTGACAGGTGAAGTGG-3′	270
		R: 5′-CCCCATCCTTCTTGCTGT-3′	
β-actin	NM_031144	F: 5′-CATGAAGATCAAGATCATTGCTCCT-3′	109
		R: 5′-CTGCTTGCTGATCCACATCTG-3′	

a) F, forward primer; R, reverse primer.

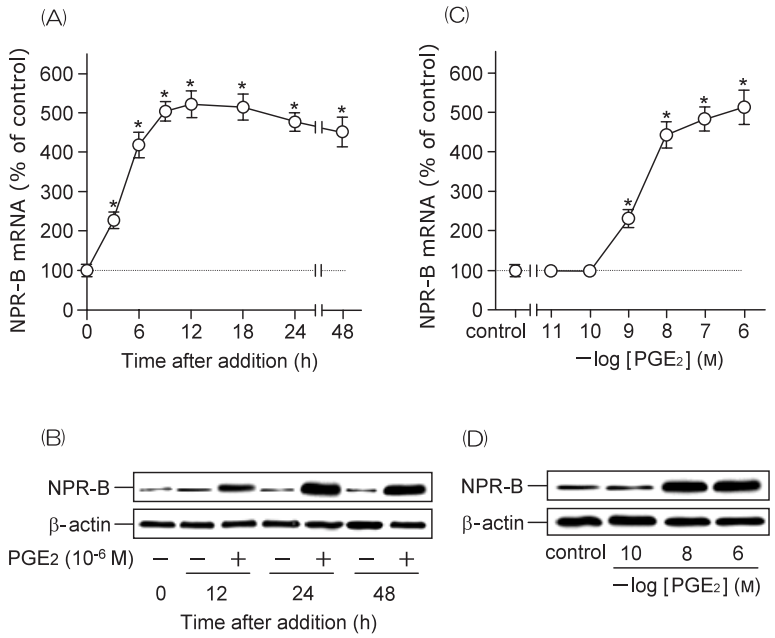

Fig. 1. Effects of PGE_2 on NPR-B Expression in Osteoblasts from 25-Week-Old Rats

(A, B) On day 5, calvarial osteoblasts from 25-week-old rats were treated with vehicle or PGE_2 (1×10^{-6} M) for the indicated periods. (C, D) On day 5, the cells were treated with vehicle or the indicated concentrations of PGE_2 for 24 hr. After treatment, total RNA and protein were extracted from the cells, and subjected to real-time RT-PCR (A, C) and Western blotting (B, D) analyses as described in Materials and Methods. (A, C) The relative expression levels of NPR-B mRNA were normalized to that of β-actin in the same sample. Each point and vertical bar represents the mean ± S.D. of four cultures. $^*p < 0.05$; compared with the level of time-matched control (A) or that of vehicle treatment (C). (B, D) NPR-B and β-actin protein levels. The experiment was repeated twice and the results were essentially the same as those depicted.

previously.[9)]

Statistical Methods —— Data were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. $p < 0.05$ was considered significant. All data are represented as the mean ± S.D. of four cultures.

RESULTS AND DISCUSSION

We first examined the effect of PGE_2 on NPR-B expression in calvarial osteoblasts from 25-week-old rats (Fig. 1). The mRNA level of NPR-B was significantly enhanced within 3 hr by treating cells with PGE_2 (1×10^{-6} M) relative to that obtained using time-matched control, reaching the maximum at 12 hr, and the level was kept until 48 hr (Fig. 1A). PGE_2 also increased the protein level of NPR-B time-dependently (Fig. 1B). The 24-hr treatment with PGE_2 in the concentration range of 10^{-9}–10^{-6} M caused a dose-dependent increase in NPR-B mRNA level (Fig. 1C; $EC_{50} = 2 \times 10^{-9}$ M). The maximal mRNA level of NPR-B obtained at 1×10^{-6} M PGE_2 was 5.1-fold higher than the control level. PGE_2 also increased the protein level of NPR-B dose-dependently (Fig. 1D). These results indicate that PGE_2 induces NPR-B expression in calvarial osteoblasts from 25-week-old rats.

Puggina and Selliti[19)] examined the effect of cyclic adenosine-3′,5′-monophosphate (cAMP) on NPR-B expression in human vascular smooth muscle cells and found that the transcriptional level of NPR-B is significantly enhanced by treating with dibutyryl cAMP, a membrane-permeable cAMP analogue, or forskolin, an adenylate cyclase activator. It is known that the biological activities of PGE_2 are mediated through four well-characterized receptors, EP1–EP4, of which EP2 and EP4 produce cAMP through the activation of adenylate cyclase.[16)] To determine which subtype receptors are involved in the PGE_2-induced increase in NPR-B expression, calvarial osteoblasts from 25-week-old rats were treated with PGE_2 receptor-selective agonists, 17-phenyl-ω-trinor PGE_2 (EP1A) for EP1, butaprost (EP2A) for EP2, sulprostone (EP3A) for EP3, and 11-deoxy-PGE_1 (EP2/EP4A) for EP2 and EP4, and then the expression level of NPR-B was measured by real-time RT-PCR and Western blotting analyses. Of the agonists tested,

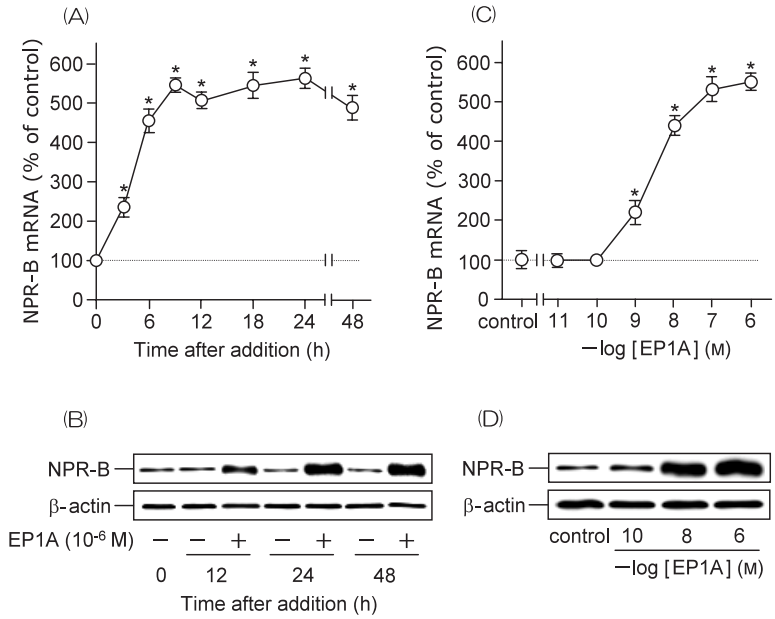

Fig. 2. Effects of Agonist for EP1 on NPR-B Expression in Osteoblasts from 25-Week-Old Rats

On day 5, calvarial osteoblasts from 25-week-old rats were treated with vehicle or 17-phenyl-ω-trinor PGE_2 (EP1A) as an EP1 agonist under the same conditions as described in Fig. 1. (A, C) The relative expression levels of NPR-B mRNA were normalized to that of β-actin in the same sample. Each point and vertical bar represents the mean ± S.D. of four cultures. $^*p < 0.05$; compared with the level of time-matched control (A) or that of vehicle treatment (C). (B, D) NPR-B and β-actin protein levels. The experiment was repeated twice and the results were essentially the same as those depicted.

only the EP1A increased the expression of NPR-B in a time- and dose-dependent manner (Fig. 2; $EC_{50} = 3 \times 10^{-9}$ M). By contrast, EP2A, EP3A, and EP2/EP4A failed to increase the mRNA and protein levels of NPR-B in the concentration range of 10^{-11}–10^{-5} M for 24, 48 and 72 hr (data not shown). In addition, the treating of cells with SC-19220 (1×10^{-5} M) as an antagonist for EP1, effectively inhibited the potentiating effect of PGE_2 or EP1A (each 1×10^{-8} M) on the mRNA and protein levels of NPR-B (Fig. 3). SC-19220 significantly decreased the basal mRNA and protein levels of NPR-B, suggesting that the endogenous PGE_2 is involved in the expression of NPR-B. These results demonstrate the important role of EP1, but not of EP2 or EP4, in the PGE_2-induced increase in NPR-B expression in calvarial osteoblasts from 25-week-old rats.

The effect of PGE_2 on the calvarial osteoblasts from rats at this age is mainly exerted through EP1[17,18] although the expression level of EP2 and EP4 is higher than that of EP1 in these cells.[20] The binding of PGE_2 to the EP2 or EP4 receptor leads to the activation of adenylate cyclase to produce cAMP. On the other hand, the binding of PGE_2 to the EP1 receptor leads to the elevation of intracellular Ca^{2+} level through the activation of phosphatidylinositol-specific phospholipase C. The Ca^{2+} mobilization activates a phosphodiesterase that blocks the signaling through EP2 or EP4 by degrading cAMP.[17,18] Therefore, there is a possibility that the accumulation of cAMP cannot reach a level at which an increase in NPR-B expression is induced. To clarify this point, calvarial osteoblasts from 25-week-old rats were treated with EP2/EP4A or forskolin in the presence of IBMX, a phosphodiesterase inhibitor, and then the mRNA level of NPR-B was measured by RT-PCR (Table 2). Contrary to our expectation, EP2/EP4A and forskolin had no effect on NPR-B mRNA level regardless of the inhibition of phosphodiesterase, suggesting that an accumulation of cAMP does not affect NPR-B expression. The discrepancy of NPR-B expression systems between rat osteoblasts in this study and human vascular smooth muscles[19] may be attributed to the difference in cell types or to the difference in species.

The EP1 pathway does not function in calvarial osteoblasts from aged rats due to the reduction in EP1 expression.[20] We next examined the age-dependent changes in the basal expression levels of EP1 and NPR-B using calvarial osteoblasts from rats of various ages (Fig. 4). The basal mRNA level of these receptors continuously decreased with the

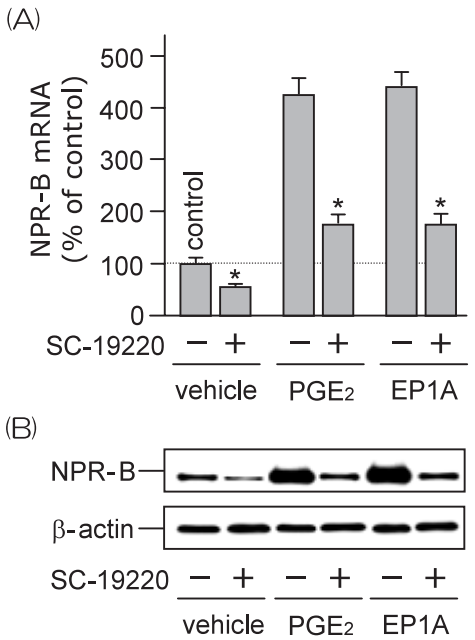

Fig. 3. Effects of EP1 Antagonist on NPR-B Expression in Osteoblasts from 25-Week-Old Rats

On day 5, calvarial osteoblasts from 25-week-old rats were treated with vehicle or SC-19220 (1×10^{-5} M) as an EP1 antagonist for 1 hr, followed by the treatment with PGE_2 (1×10^{-8} M) or 17-phenyl-ω-trinor PGE_2 (EP1A, 1×10^{-8} M) for 24 hr. After treatment, total RNA and protein were extracted from the cells, and subjected to real-time RT-PCR (A) and Western blotting (B) analyses as described in Materials and Methods. (A) The relative expression levels of NPR-B mRNA were normalized to that of β-actin in the same sample. Each point and vertical bar represents the mean ± S.D. of four cultures. $^*p < 0.05$; compared with the level in cells without the SC-19220 treatment in the same stimulation group. (B) NPR-B protein level and β-actin protein levels. The experiment was repeated twice and the results were essentially the same as those depicted.

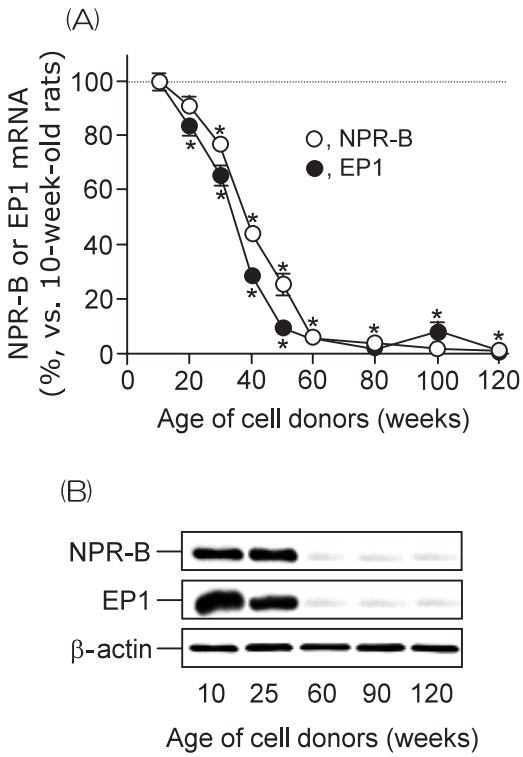

Fig. 4. Age-Dependent Changes in NPR-B and EP1 Expressions

On day 5, total RNA was extracted from calvarial osteoblasts from rats of various ages and subjected to real-time RT-PCR (A) and Western blotting (B) analyses. (A) The relative expression levels of NPR-B or EP1 mRNA were normalized to that of β-actin in the same sample. Each point and vertical bar represents the mean ± S.D. of four cultures. $^*p < 0.05$; compared with the level in cells from 10-week-old rats. (B) NPR-B, EP1 and β-actin protein levels. The experiment was repeated twice and the results were essentially the same as those depicted.

Table 2. Effects of EP2/EP4A and Forskolin on mRNA Levels of NPR-B

Pretreatment	Treatment	NPR-B mRNA (% of control)
vehicle	vehicle	100 ± 4.1
vehicle	EP2/EP4A	108 ± 8.2
vehicle	forskolin	105 ± 5.8
IBMX	vehicle	101 ± 9.2
IBMX	EP2/EP4A	116 ± 6.0
IBMX	forskolin	112 ± 7.7

On day 5, after pretreatment with vehicle or IBMX (1×10^{-6} M) for 15 min, calvarial osteoblasts from 25-week-old rats were treated with vehicle, 11-deoxy-PGE_1 (EP2/EP4A, 1×10^{-5} M) or forskolin (1×10^{-5} M) under the same conditions as described in Fig. 1. The relative expression levels of NPR-B mRNA were normalized to that of β-actin in the same sample. Each data represents the mean ± S.D. of four cultures.

age of cell donors between 10 to 60 weeks and then remained constant over 60 weeks (Fig. 4A). The basal mRNA levels of NPR-B and EP1 in calvarial osteoblasts from 10-week-old rats were respectively 70- and 90-fold those in cells from 120-week-old rats. The age-related decrease in the basal protein levels of NPR-B and EP1 was also observed (Fig. 4B). These results can be explained by assuming that age-related decrease in EP1 expression causes a decrease in NPR-B expression. To demonstrate this hypothesis, calvarial osteoblasts from rats of various ages were treated with PGE_2 or EP1A (each 1×10^{-6} M) for 24 hr and then the mRNA level of NPR-B was assayed by real-time RT-PCR (Fig. 5). The degree of PGE_2- or EP1A-induced increase in NPR-B mRNA level gradually decreased with the age of cell donors between 10 to 60 weeks, and EP1 agonists had no effect on the NPR-B mRNA level between 60 to 120 weeks. These results clearly reflect that the activation of EP1, but not EP2 or EP4, leads to the increase in NPR-B expression.

In conclusion, we have shown that PGE_2 in-

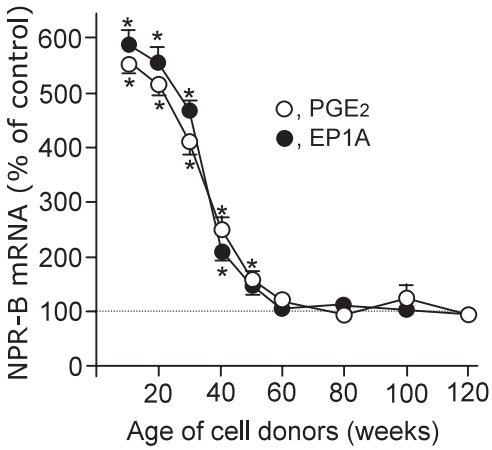

Fig. 5. Age-Dependent Changes in EP1 Agonist-Induced Enhancement of NPR-B mRNA Expression

On day 5, calvarial osteoblasts from rats of various ages were treated with vehicle, PGE_2 (1×10^{-6} M) or 17-phenyl-ω-trinor PGE_2 (EP1A, 1×10^{-6} M) for 24 hr. After treatment, total RNA was extracted from the cells and subjected to real-time RT-PCR analysis. The relative expression levels of NPR-B mRNA were normalized to that of β-actin in the same sample. Each point and vertical bar represents the mean ± S.D. of four cultures. $^*p < 0.05$; compared with the level of vehicle treatment. The basal levels of NPR-B mRNA were shown in Fig. 4. The experiment was repeated twice and the results were essentially the same as those depicted.

creases the NPR-B expression of rat calvarial osteoblasts through the EP1 subtype of PGE_2 receptor and age-related decrease in EP1 expression causes a decrease in NPR-B expression.

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