BMPER Is Upregulated by Statins and Modulates Endothelial Inflammation by Intercellular Adhesion Molecule–1

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Objective—In addition to lowering cholesterol, statins exert pleiotropic effects on endothelial cells. Bone morphogenetic proteins (BMPs) have recently been implicated in vascular inflammation and disease. We set out to investigate the effect of statins on BMP endothelial cell precursor–derived regulator (BMPER), a novel member of the BMP pathway.

Methods and Results—Mevastatin enhanced BMPER expression in cultured endothelial cells in a time- and concentration-dependent manner as determined by immunocytochemistry, RT-PCR, and Western blotting. Similar effects were observed in vitro and in vivo using simvastatin. Actinomycin D chase analysis and BMPER promoter reporter assays revealed that this is mostly a posttranscriptional event resulting in prolonged BMPER RNA half-life. We confirmed that the RhoA/Rho-associated coiled-coil containing protein kinase Rho kinase (Rock)/actin pathway is involved using the specific pathway activator cytotoxic necrotizing factor of Yersinia pseudotuberculosis, which prevented upregulation of BMPER expression by mevastatin and pathway inhibitors (C3-toxin, RhoAN19 mutant, fasudil, and cytochalasin D) that enhanced BMPER expression. Increasing concentrations of BMPER exert antiinflammatory features in endothelial cells as reflected by intercellular adhesion molecule–1 downregulation. Accordingly, silencing of BMPER enhances intercellular adhesion molecule–1 expression. Furthermore, mevastatin reduced the expression of proinflammatory BMP4, a well-known direct interaction partner of BMPER.

Conclusion—Mevastatin modulates the BMP pathway by enhancing BMPER via the RhoA/Rock/actin pathway, as well as by reducing BMP4 expression. BMP4 downregulation and BMPER upregulation contribute to the antiinflammatory pleiotropic effects of statins. (Arterioscler Thromb Vasc Biol. 2010;30:554-560.)

Key Words: statins ■ ICAM-1 ■ bone morphogenetic proteins ■ vascular biology

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily. BMPs are important regulators in blood vessel formation and vascular disease.1 BMP2 and BMP4 are upregulated in athero-prone regions in blood vessels, induce2,3 a proinflammatory endothelial phenotype, and may contribute to the development of atherosclerotic plaques and vascular calcification.4,5 Infusion of BMP4 in vivo leads to endothelial dysfunction and arterial hypertension.6,7 Important insights have also come from the discovery of mutations of the BMP receptors in patients with familial pulmonary artery hypertension or teleangiectasia.8

BMP endothelial cell precursor–derived regulator (BMPER) is a secreted glycoprotein that binds directly to BMPs and modulates their function in a dose-dependent fashion. In gain-of-function assays, BMPER behaves as a BMP antagonist,9,10 whereas in loss-of-function models, BMPER may also exert pro-BMP functions.11–14 BMPER was originally identified in a screen for differentially expressed proteins in embryonic endothelial precursor cells.9 In mouse and zebrafish, it is expressed at sites and at the time of vasculogenesis, consistent with a regulatory role for BMPER in vascular events. When BMPER is inactivated in zebrafish embryos, intersomitic angiogenesis is severely perturbed.11 Consistent with this vascular phenotype, BMPER may confer proangiogenic activity in endothelial cells in a dose-dependent fashion.15 Taken together, these data indicate that BMPER acts as a context-dependent BMP modulator and is essential for BMP4 function in endothelial cells.15

It has been shown that BMP4 exerts its proinflammatory effects by increased nuclear factor-κB activation and induction of intercellular adhesion molecule–1 (ICAM-1).16,17 ICAM-1 is an adhesion molecule that is expressed on the endothelium and leukocytes and is upregulated in inflammation by proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interferon-γ.18 Increased expression of ICAM-1 was identified in all subtypes of atheroscle-
rotic lesions and is involved in the recruitment of monocytes to the lesion, as suggested by its role in the entry of leukocytes into foci of inflammation. Along the same lines, ICAM-1-enhanced monocyte recruitment is a potential mechanism for the growth of an atherosclerotic plaque. Therefore, it is important to understand the regulation of ICAM-1 on the endothelial surface and to identify regulators of ICAM-1 expression because of their potential in the treatment of vascular inflammation.

In addition to their ability to lower plasma cholesterol levels, statins have been shown to decrease ICAM-1 expression in endothelial cells. They demonstrate antiatherogenic properties by improving endothelial function, stabilizing atherosclerotic plaques, and reducing oxidative stress, as well as endothelial inflammation and thrombogenecity. Therefore, statins are used in the primary and secondary prevention of cardiovascular disease. By inhibition of the 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, statins block the conversion of HMG-CoA to mevalonate and cause a depletion of isoprenoids, such as mevalonate, farnesylpyrophosphate (FPP), and geranylgeranylpyrophosphate (GGPP). These isoprenoids serve as important lipid anchors for the posttranscriptional modification of small GTPases, such as Ras, Rho, Rac, and Rap, by isoprenylation. Small GTPases are involved in cell signaling, and perturbed isoprenylation of small GTPases by statins mediates antiinflammatory effects partially by downregulation of proinflammatory BMP2.

In this study, we identified the extracellular BMP modulator BMPER as a new mediator of antiinflammatory effects of statins in endothelial cells.

**Methods**

Reagents, antibodies, cell culture, immunocytochemistry, transfection of promoter constructs, luciferase assays, RT-PCR, quantitative real-time polymerase chain reaction, small interfering RNA (siRNA) transfection, Western blotting, and animal procedures are described in the online Data Supplement, available at http://atvb.ahajournals.org.

**Statistical Analysis and Quantification**

Statistical analyses were performed using GraphPad Prism 4.0. Data are presented as mean±SD, and comparisons were calculated by Student’s t test (2-sided, unpaired). Results were considered statistically significant when P<0.05. Densitometric analysis of Western blots was performed using Quantity One 1-D Analysis Software (version 4.4, Bio-Rad) and levels of significance were calculated by 1-sample t test.

**Results**

**Mevastatin Upregulates BMPER Expression in Endothelial Cells**

To test the hypothesis that statins may exert pleiotropic effects by regulating BMPER, we treated human umbilical vein endothelial cells (HUVECs) with mevastatin. Indeed, as visualized by immunocytochemistry and quantified by Western blotting, mevastatin increased BMPER protein expression in HUVECs (Figure 1A). Treatment with 10 μmol/L mevastatin for 24 hours resulted in a 4-fold upregulation of BMPER protein. Similar results were obtained for BMPER RNA levels in vitro (Figure 1C). In C57/BL6 mice, treatment with activated simvastatin increased BMPER RNA level in HUVECs in vitro (Figure 1E).
suggesting a class effect of statins on BMPER regulation (Figure 1E). These data were confirmed in vivo by treating C57/BL6 mice with subcutaneous injection of simvastatin for 14 days. In simvastatin-treated animals, BMPER RNA levels were upregulated in the lungs compared with control. These data clearly demonstrate that statins increase BMPER expression in vitro and in vivo.

Mevastatin Increases BMPER Expression by Posttranscriptional Modification

To analyze the mechanism by which statins regulate BMPER expression, we pursued 2 separate approaches. First, we tested the effect of mevastatin on 2 BMPER promoter constructs of different sizes that contain luciferase as a reporter of BMPER promoter activity. Krüpple-like factor 15 (KLF15) was used as a positive control for BMPER promoter activation. Values represent the mean ± SD of 3 independent experiments normalized to β-galactosidase. B, After 24 hours of preincubation with or without mevastatin, HUVECs were treated with de novo transcription inhibitor actinomycin D (ActD) for the indicated times. RNA levels were normalized to the RNA level at 0 hours of the same group. Each experiment was performed at least 3 times, with similar results. *P < 0.05 versus ActD control.

RNA. Taken together, these data cannot completely exclude transcriptional regulation but strongly suggest a posttranscriptional mechanism of regulation.

Mevastatin-Mediated Induction of BMPER Expression Is Dependent on Isoprenoid Intermediates

Statins inhibit the HMG-CoA reductase and cause a depletion of downstream isoprenoids, such as mevalonate, geranylpyrophosphate, FPP, or GGPP, in the cells.24 To determine which downstream isoprenoid in the cholesterol biosynthetic pathway regulates BMPER expression, HUVECs were exposed to mevastatin alone or in combination with mevalonate (A), FPP (B), geranylpyrophosphate (GPP) (C), and GGPP (C) for 24 hours. Total RNA was harvested and assessed for BMPER expression by RT-PCR. Human RNA polymerase II (hRPII) was used as a loading control. One representative gel out of 3 independent experiments with similar results is shown. In addition, corresponding quantitative polymerase chain reactions were performed (C), and results are shown as a bar graph. *P < 0.05 versus control; #P < 0.05 versus mevastatin.
data underline that the statin-mediated BMPER upregulation is dependent on the cholesterol synthesis pathway and demonstrate that all tested isoprenoids are able to reverse the effect of mevastatin on BMPER.

**RhoA Is Involved in BMPER Regulation in Endothelial Cells**

The isoprenoids FPP and GGPP have regulatory roles in a number of signaling cascades, such as the Ras and Rho pathways. Because Rho is a major target of geranylgeranylation, inhibition of Rho and its downstream target, Rho kinase, mediates some of the pleiotropic effects of statins on the vascular wall.24,25 As a consequence, we hypothesized that activation of RhoA reverses mevastatin-induced BMPER expression. Therefore, we tested whether activation of RhoA using a direct and highly specific RhoA activator (cytotoxic necrotizing factor of Yersinia pseudotuberculosis [CNFY]) would reverse the statin effect.26 As expected, mevastatin increased the BMPER RNA level to 241% compared with basal level, whereas CNFY alone reduced the BMPER RNA level to 71%. Cotreatment of cells with mevastatin and CNFY completely reversed the upregulation of BMPER RNA by mevastatin (Figure 4A). These findings indicate that the statin-mediated inhibition of RhoA contributes to the increased BMPER expression.

To confirm these results, we tested the effect of RhoA inhibition on BMPER. Therefore, HUVECs were coincubated with clostridium botulinum C3 transferase, an exotoxin that inactivates Rho by ADP-ribosylation.27 Indeed, as shown in Figure 4B, treatment of HUVECs with the C3 toxin for 24 hours augmented BMPER RNA level to 140%. To increase the specificity, we overexpressed a dominant-negative RhoA mutant (RhoA19N) in HUVECs (Figure 4C). Specific inhibition of RhoA resulted in upregulation of BMPER RNA. These findings demonstrate that RhoA inhibition increases BMPER expression to an extent similar to inhibition of Rho GTPases by mevastatin. Taken together, these findings indicate that RhoA is an important regulator of BMPER RNA levels in endothelial cells and that the statin-mediated inhibition of geranylgeranylation of RhoA is responsible for the increased BMPER expression.

**Inhibition of Rock Increases BMPER RNA in Endothelial Cells**

Rho-associated coiled-coil containing protein kinase Rho kinase (Rock) is an important downstream target of RhoA activity.24,28 To determine the involvement of Rock in BMPER expression, endothelial cells were incubated with the specific Rock inhibitor fasudil for 24 hours. Confirming our hypothesis, fasudil upregulated BMPER RNA levels (246%) and protein levels (134%) compared with control (Figure 4D and 4E). These findings support the notion that the RhoA/Rock pathway is involved in the regulation of BMPER RNA levels.

**Disruption of the Endothelial Actin Cytoskeleton Increases BMPER Expression**

Rock phosphorylates various targets and mediates a range of cellular responses that involve the assembly of the actin cytoskeleton. The number of actin stress fibers and the reorganization of the cytoskeleton are mediated in part by

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**Figure 4.** Mevastatin-mediated upregulation of BMPER depends on RhoA and Rho kinase activity. A, Specific activation of the Rho pathway by CNFY prevents mevastatin-induced BMPER upregulation. HUVECs were treated with CNFY (400 ng/mL) and mevastatin (15 μmol/L) alone or in combination. Reverse transcription–quantitative polymerase chain reaction was performed to quantify BMPER RNA levels. Human RNA polymerase II (hRPII) was used as a loading control. *P<0.05 versus control; #P<0.05 versus mevastatin. B–E, Inhibition of the Rho pathway enhances BMPER expression. B, The Rho inhibitor C3 toxin augments BMPER RNA levels. HUVECs were treated with C3 toxin (250 ng/mL) for 24 hours. C, Overexpression of the dominant-negative RhoA (RhoN19) mutant in HUVECs increases BMPER RNA levels compared with control (empty vector). D and E, The Rock inhibitor fasudil upregulates BMPER RNA and protein expression in endothelial cells. HUVECs were treated with fasudil (50 μmol/L) for 24 hours before cells were harvested for RNA or protein analysis. F, Disruption of actin cytoskeleton increases BMPER RNA level in endothelial cells. HUVECs were incubated with the actin cytoskeleton disruptor cytochalasin D (5 μmol/L) for 8 hours. *P<0.05 versus control.

Rocks. To address the question of whether this downstream step of RhoA/Rock signaling is also involved in BMPER regulation, we treated endothelial cells with cytochalasin D, a well-characterized disruptor of the actin cytoskeleton, for 8 hours (Figure 4F). Cytochalasin D causes a strong increase in BMPER RNA levels to 338% compared with control, suggesting that the actin cytoskeleton is indeed involved in the regulation of BMPER expression.

**Mevastatin Downregulates Proinflammatory BMP4 in Endothelial Cells**

Having demonstrated that mevastatin upregulates BMPER, we asked whether other BMP pathway members are also

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regulated by statins. We decided to focus on BMP4 because we had shown earlier that BMPER interacts directly with BMP4 and because BMP4 is a known inducer of vascular inflammation.\(^{17}\) In contrast to BMPER, BMP4 was markedly downregulated by mevastatin (Figure 5A and 5B). This finding is consistent with the notion that downregulation of BMP4 contributes to the antiinflammatory effect of statins.

**BMPER Modulates ICAM-1 Expression**

Next, we asked whether regulation of BMPER by statins is also involved in vascular inflammation. To quantify vascular inflammation we chose ICAM-1 expression as a surrogate marker. When we added BMPER to HUVECs, ICAM-1 RNA was downregulated (Figure 5C). To investigate whether BMPER was capable of antagonizing proinflammatory stimuli, we coincubated HUVECs with tumor necrosis factor-\(\alpha\), a strong inducer of endothelial inflammation, and with increasing concentrations of BMPER. Indeed, BMPER inhibited tumor necrosis factor-\(\alpha\)-induced ICAM-1 expression in a concentration-dependent manner (Figure 5D). To confirm these findings, we silenced BMPER in HUVECs. Two different siRNAs designed to target BMPER were used in these experiments. Consistent with an inhibitory role of BMPER on ICAM-1 expression, we found that ICAM-1 RNA and protein are increased in BMPER silenced endothelial cells (Figure 5E and 5G). In our hands, BMPER-specific small interfering RNA (siBMPER) II consistently reached higher knockdown efficiencies than siBMPER I, and consequently, more pronounced effects on ICAM1 were observed (Figure 5F). After 8 hours, cells were harvested and used for Western blotting.

Taken together, these data suggest that statins reduce vascular inflammation by interfering with the BMP pathway at 2 ends. First, they downregulate proinflammatory BMP4, and second, they increase BMPER, for which we suggest an
antiinflammatory role by its suppressing activity on ICAM-1 expression.

**Discussion**

In this study, we characterize the BMP modulator BMPER as a novel pleiotropic target of statins in endothelial cells and present novel findings with regard to the regulation of BMPER and the BMP pathway. First, HMG-CoA reductase inhibitors upregulate BMPER expression in vitro and in vivo. Second, upregulation of BMPER expression by mevastatin is a posttranscriptional event. Third, the effect of mevastatin on BMPER involves inhibition of the RhoA/Rock/actin pathway. Fourth, mevastatin differentially regulates BMPER and BMP4 and thereby inhibits vascular inflammation as reflected by ICAM-1.

BMPER is an extracellular BMP modulator that is expressed by endothelial cells. Data from *Drosophila*, *Xenopus*, zebrafish, chicken, and mouse reveal that BMPER is necessary to sharpen BMP gradients and that its activity is sensitive to dose changes. At low doses, BMPER is needed to enhance BMP4 activity, but at higher doses, BMPER increasingly inhibits BMP4 activity. Therefore, a detailed understanding of BMPER regulation is crucial to control BMP effects.

Here we present data demonstrating that mevastatin increases BMPER expression in endothelial cells (Figure 1). Until now, this is the first drug that has been shown to increase BMPER expression. Pleiotropic effects of statins are frequently controlled by posttranscriptional events rather than by control of gene promoter activity. This is also the case for BMPER. Although our data cannot completely rule out transcriptional modification of BMPER expression by statins, they strongly suggest a posttranscriptional effect, resulting in prolongation of BMPER RNA half-life (Figure 2). The isoprenoids downstream of HMG-CoA reductase are important modulators of small GTPases. For example, inhibition of Rho geranylgeranylation and membrane translocation of Rho by mevastatin lead to a greater accumulation of inactive Rho in the cytoplasm. Because supplementation of GGPP could reverse the statin effect on BMPER, we hypothesized that the Rho/Rock/actin pathway involved in BMPER regulation (Figure 3). Indeed, specific activation of this pathway by CNFY reduced BMPER expression, whereas pathway inhibition by either a specific RhoA inhibitor (C3-transferase toxin), a dominant-negative RhoA mutant (RhoA19N), or a Rock inhibitor (fasudil) results in upregulation of BMPER (Figure 4). Similarly, inhibition of downstream actin cytoskeleton assembly increases BMPER RNA. Taken together, this is compelling evidence that the RhoA/Rock/actin pathway plays a pivotal role in mevastatin-mediated BMPER expression. Remarkably, this is a very similar mechanism to the regulation of endothelial nitric oxide synthase by statins. The mechanistic data dissecating the RhoA/Rock/actin pathway of BMPER activation are of great value, as recently specific inhibitors of Rock have emerged as novel therapeutic strategies to treat vascular dysfunction and its long-term consequences such as atherosclerosis or pulmonary artery hypertension. Our data demonstrate that Rock inhibition, in addition to the well-described consequences for endothelial nitric oxide synthase, also modifies the BMP pathway.

Modification of the BMP pathway has a major impact on vascular inflammation. BMP4 exerts prooxidant, proinflammatory, and prohypertensive effects on endothelial cells and is involved in vascular calcification. By downregulation of BMP4 expression, statins decrease BMP activity, which mediates antiinflammatory, antiatherogenic, and vasculoprotective effects. Another new target of statins within the BMP pathway is BMPER. Here we show that high levels of BMPER modulate expression of adhesion molecules on endothelial cells. This is demonstrated by downregulation of ICAM-1, a marker of endothelial cell activation and inflammation (Figure 5). Thus, statins modulate the BMP pathway at different levels: they downregulate BMP4, and at the same time they upregulate the dose-dependent BMP4 modulator BMPER, together resulting in a strong antiinflammatory activity.

In conclusion, we demonstrate that BMPER is upregulated by mevastatin via posttranscriptional modification involving the RhoA/Rock/actin pathway. At the same time, BMP4 is downregulated by mevastatin. This dual modification of the BMP pathway results in decreased vascular inflammation and thus represents a hitherto unknown antiinflammatory effect of statins.

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**Disclosures**

None.

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Antibodies and Reagents

Monoclonal anti-human BMPER antibody was purchased from R&D Systems (MAB1956), anti-human ICAM-1 antibody from Cell Signaling (# 4915) and R&D Systems (BBA3), fasudil (Cat. No. 371970) from Calbiochem. Mevastatin (M2537), simvastatin (S6196), mevalonate (M4667), FPP (F6892), GPP (G6772), GGPP (G6025), DRB (D1916) and actinomycin D (A1410) were from Sigma-Aldrich. C3-toxin and CNFY-toxin were kind gifts from K. Aktories, Freiburg. The expression plasmid pZIP-RhoA19N was kindly provided by K. Burridge, UNC Chapel Hill.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) are cultured in endothelial cell growth medium (Provitro) with 10% fetal bovine serum (FBS). Cultures were kept at 37°C in a 5% CO₂ humidified atmosphere.

Immunocytochemistry

HUVEC cells were seeded on glass coverslips and were stimulated for 24h with mevastatin (10µM). Cells were fixed in ice-cold methanol/acetone at -20°C for 10min and were blocked with 10% donkey serum for 30min at room temperature. Subsequently HUVECs were incubated with a primary BMPER antibody. After washing signals were detected using a donkey anti goat IgG-FITC secondary antibody and DAPI for nuclear staining. For detection of ICAM-1 we used an anti-human ICAM-1 antibody and an anti-mouse IgG FITC secondary antibody.

Promoter constructs

The 5’ flanking region of the mouse BMPER gene was cloned by PCR using genomic DNA (C57/BL6) as a template. The 5’ deleted fragments were cloned into promoterless pGL3-basic
vector (Promega). The sequences of all DNA constructs were confirmed by DNA sequencing 1.

**Transfection and luciferase assay**

BAEC were plated at a density of 1x 10^5/well in a 6-well dish. After 24 hours transient transfections were performed using Fugene 6 (Roche Molecular Biochemics) according to the instructions provided by the manufacturer. Cells were transfected using 2µg of luciferase reporter vector and 0.5µg of the pCMV-β-galactosidase (pCMV-β-Gal) as a transfection control. After 24 hours luciferase activity and β-galactosidase activity were measured. Data are shown as mean +/- SD of triplicates of at least three independent experiments.

**RT-PCR**

Total RNA was isolated from HUVECs using the Total RNA Mini Kit (BioRad) and equal amounts were reverse transcribed with iScript cDNA Synthesis Kit (BioRad). PCR was performed using the following primers: BMPER forward 5’-AGGACAGTGCTGCCCCAAATG-3’, BMPER reverse 5’-TACTGACACGTCCCTGAAAG-3’; hRPII forward 5’-GCACCACGTCCTGACAT-3’, hRPII reverse 5’-GTGCGGCTGCTTCCATAA-3’; KLF15-forward 5’-CTTCCAGCCTACCCCTGAGAG-3’ and KLF15-reverse 5’-TTGGGTGACATCCTTGCTGCAGCC-3’. PCR products were analyzed using an ethidium bromide stained 2% agarose gel. Gel bands were semi-quantified using the Quantity One 1D Analysis Software Version 4.4 (Bio-Rad). All RT-PCR experiments were repeated at least three times and representative results are shown.
Quantitative Real-Time PCR

Quantitative PCR (qPCR) analysis was performed using the real-time PCR detection system (Bio-Rad) and the MyiQ lightcycler software. Human RNA-polymerase II was used for normalization. Quantification was calculated using the $\Delta\Delta^{C_T}$ method.

siRNA transfection

SiRNA targeted against human BMPER and a non-specific control siRNA were purchased from Invitrogen. HUVECs were plated one day before transfection 4 x 10$^4$/well in a 12-well dish in HUVEC medium with 10% fetal bovine serum (FBS). HUVECs were transfected with siRNA using Lipofectamine RNAiMAX according to the manufacturer's protocol. Cells were harvested for RNA. Knock down efficiency was confirmed by Real-Time PCR.

Western Blot

HUVECs were stimulated for 24 hours with indicated concentrations of mevastatin in endothelial cell growth medium (Provitro) with 10% fetal bovine serum (FBS). Cell lysates were resolved on a reducing polyacrylamide gel, plotted onto a nitrocellulose membrane (Amersham Bioscience) and blocked with 5% non-fat milk in PBS/Tris with 0.1% Tween 20 for 2h at room temperature (20-22°C). The membrane was then incubated with primary antibody overnight at 4°C. After 1h of incubation with the secondary antibody, proteins were visualised using ECL reagent (Amersham). All western blots were repeated at least three times and quantified data are shown.

Animal procedures

C57/BL6 mice (18-22g, Charles River laboratories) were injected s.c. with 0.5 ml of activated simvastatin (20mg/kg) or with PBS once daily for 14 days. Afterwards mice were sacrificed; tissues were immediately isolated and snap-frozen. Total lung RNA was isolated, reverse
transcribed and quantified by qPCR for BMPER and RNA polymerase II as a housekeeping control. Animal experiments were performed according to the Animals Scientific Procedures Act of 1986 and local ethics protocols.

**Supplement References:**
