ORIGINAL RESEARCH

Inhibition of 5-Hydroxytryptamine Receptor 2B Reduced Vascular Restenosis and Mitigated the β-Arrestin2–Mammalian Target of Rapamycin/p70S6K Pathway

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***Background*—**As a monoamine neurotransmitter, 5-hydroxytryptamine (5-HT) or serotonin modulates mood, appetite, and sleep. Besides, 5-HT also has important peripheral functions. 5-HT receptor 2B (5-HT2BR) plays a key role in cardiovascular diseases, such as pulmonary arterial hypertension and cardiac valve disease. Percutaneous intervention has been used to restore blood flow in occlusive vascular disease. However, restenosis remains a significant problem. Herein, we investigated the role of 5-HT2BR in neointimal hyperplasia, a key pathological process in restenosis.

***Methods and Results*—**The expression of 5-HT2BR was upregulated in wire-injured mouse femoral arteries. In addition, BW723C86, a selective 5-HT2BR agonist, promoted the injury response during restenosis. 5-HT and BW723C86 stimulated migration and proliferation of rat aortic smooth muscle cells. Conversely, LY272015, a selective antagonist, attenuated the 5-HT–induced smooth muscle cell migration and proliferation. In vitro study showed that the promigratory effects of 5-HT2BR were mediated through the activation of mammalian target of rapamycin (mTOR)/p70S6K signaling in a β-arrestin2–dependent manner. Inhibition of mammalian target of rapamycin or p70S6K mitigated 5-HT2BR–mediated smooth muscle cell migration. Mice with deficiency of *5-HT2BR* showed significantly reduced neointimal formation in wire-injured arteries.

***Conclusions*—**These results demonstrated that activation of 5-HT2BR and β-arrestin2–biased downstream signaling are key pathological processes in neointimal formation, and 5-HT2BR may be a potential target for the therapeutic intervention of vascular restenosis. **(*J Am Heart Assoc*. 2018;7:e006810. DOI: 10.1161/JAHA.117.006810.)**

Key Words: 5-HT receptor 2B • mammalian target of rapamycin • migration • restenosis • β-arrestin

Serotonin or 5-Hydroxytryptamine (5-HT) is a monoamine neurotransmitter formed by the hydroxylation and decarboxylation of tryptophan. In the central nervous system, it modulates behavioral and neuropsychological processes, such as mood, appetite, and sleep. In peripheral tissues, 5-HT also regulates critical physical processes, such as cardiovascular and metabolic functions.[1] Most of the peripheral 5-HT is stored in platelets, when released, to exert vasoconstrictive and prothrombotic effects. Emerging evidence has suggested that the dysregulated serotonin system is implicated in the pathogeneses of several cardiovascular diseases. As an important vasoconstrictor, 5-HT elicited hyperresponsiveness in hypertension, pulmonary arterial hypertension (PAH), and injured coronary arteries.[2–6] 5-HT also induced mitogenic response and was implicated in the remodeling of cardiovascular tissues, such as cardiac hypertrophy, fibrosis, valve hyperplasia,[7] PAH, and hypertension.[8] Circulating 5-HT was elevated in patients with hypertension, PAH,[9,10] coronary and peripheral artery disease and was positively correlated with intima-media thickness of the carotid arteries.[11–16] Plasma level of 5-HT in coronary circulation was locally increased after angioplasty, and the release of 5-HT was thought to be from the activated platelets.[17,18]

5-HT acts through its 7 major types of cognate receptors. The vascular effects of 5-HT are mediated primarily by 5-HT1 and 5-HT2 receptors, which belong to the G-protein–coupled receptor family.[3] 5-HT receptor 2B (5-HT2BR) has a key role in

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Accompanying Figures S1 through S7 are available at http://jaha.ahajournals.org/content/7/3/e006810/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- 5-Hydroxytryptamine (5-HT) receptor 2B was significantly increased in wire-injured arteries and promoted neointimal hyperplasia through regulating smooth muscle cell migration, proliferation, and phenotypic switch.
- β-Arrestin2 over canonical Gαq protein mediated the 5-HT receptor 2B–activated mammalian target of rapamycin/p70S6K signaling pathway in vascular smooth muscle cells.

What Are the Clinical Implications?

- 5-HT receptor 2B was identified to be the receptor for vascular remodeling that accounts for 5-HT–induced vascular injury reaction.
- Because of the diverse effects of 5-HT in the peripheral system, 5-HT receptor 2B was taken advantage of being the therapeutic target for vascular restenosis.

the previously mentioned 5-HT–related diseases, including cardiac hypertrophy, valve hyperplasia, and fibrosis.[19–21] 5-HT2BR induced hypersensitivity of arteries to 5-HT in hypertension and PAH[22–27] and was required for vascular remodeling in PAH.[22]

Percutaneous interventions, including balloon angioplasty and stenting, have been widely used for the treatment of patients with blocked coronary, carotid, and peripheral arteries, but the in-stent restenosis remains a difficult problem.[28] Neointimal hyperplasia is a key pathological feature of restenosis. Upon injury, medial smooth muscle cells (SMCs) were mobilized and migrate into the subendothelial intima, where they undergo proliferation and matrix remodeling, leading to the renarrowing of the lumen.[29,30] However, the mechanism by which 5-HT promotes vascular restenosis remains largely unknown. In particular, the specific type of 5-HT receptor involved in this process needs to be explored. In this study, by using the femoral artery wire injury model, we aimed to investigate the pathological role of 5-HT2BR in restenosis as well as the underlying signal transduction.

Methods

Cell Culture and Reagents

Rat or mouse aortic SMCs were isolated and grown in DMEM supplemented with 10% fetal bovine serum and antibiotics. SMCs were starved with serum-free DMEM for 24 hours before drug treatments. BW723C86, LY272015, SP600125, SB202190, PD98059, LY294002, and U73122 were from Tocris Bioscience (Bristol, UK). 5-HT, platelet-derived growth factor-BB (PDGF-BB), 5-bromo-2′-deoxyuridine (BrdU), and Pluronic F-127 powder were from Sigma-Aldrich (St. Louis, MO). Rapamycin was from Cell Signaling Technology (Beverly, MA). Antibodies against mammalian target of rapamycin (mTOR), phosphorylated mTOR (Ser2448), p70S6 kinase, phosphorylated p70S6 kinase (Ser389), α-smooth muscle actin (α-SMA), and SM22α were from Cell Signaling Technology. Antibody against 5-HT2BR was from BD Bioscience Pharmingen (San Diego, CA). Antibodies against 5-HT receptor 2A (5-HT2AR) and vimentin were from Abcam (Cambridge, MA). Antibodies against BrdU and CD31 were from Santa Cruz Biotechnology (Dallas, TX). Antibody against Mac-2 was from Celdarlane (Burlington, ON, Canada).

Animals

All animal care and experimental procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (eighth edition), with the approval by the Animal Research Committee of Peking University Health Science Center. 5-HT2BR knockout (5-HT2BR$^{-/-}$) mice were generated from the C57BL/6J background using a CRISPR/Cas9 protocol (Institute of Model Animal of Wuhan University, Wuhan, China). Their genotypes were confirmed by sequencing of polymerase chain reaction (PCR) products of mouse genomic DNA, and primers were 5′-GTCCCATCTTCGAGAGCCTG-3′ (forward) and 5′-CACCGCGAGTATCAGGAGAG-3′ (reverse). Male 5-HT2BR$^{-/-}$ mice and their wild-type littermates (12–16 weeks old) were used in experiments, and each group contains 6 to 8 mice at one time point.

Femoral Artery Injury and Morphometry

Wire injury of the mouse femoral artery was performed as described by Takayama et al.[31] Mice were anesthetized with IP injection of pentobarbital sodium (50 mg/kg). Left femoral arteries were exposed by a longitudinal groin incision and monitored under a surgical microscope. A wire (0.38 mm in diameter) was inserted via the deep femoral artery and left in place for 1 minute to denude the artery. After the wire was removed, the deep femoral artery was ligated and blood flow was restored. The skin incision was closed with a 5-0 silk suture. The uninjured right femoral arteries served as negative controls. For investigating the effect of 5-HT2BR agonist BW723C86 on restenosis, mice underwent femoral wire injury and received a pluronic gel (30%) with or without BW723C86 (10 mg/kg),[32,33] which was applied to the external surface of the injured femoral artery. Three days to 4 weeks after surgery, the femoral arteries were harvested, embedded in paraffin wax, and cut into 5-μm sections for hematoxylin and eosin and Masson staining (Service Biological Technology, Wuhan, China), then observed using a light microscope (Leica

DM3000B). The intima and media areas were measured by computerized morphometry with ImageJ software. Intimal hyperplasia was defined as the formation of the neointimal layer within the internal elastic lamina. The medium area was calculated as the area encircled by the external elastic lamina minus the area encircled by the internal elastic lamina. The intima/media ratio was calculated as the intimal area/the medial area. Additional vessels were also harvested and snap frozen for quantitative reverse transcription (RT)–PCR and Western blot analysis.

BrdU Incorporation Assay

Labeling of proliferating cells was performed using a BrdU-based assay. Cultured SMCs were seeded on coverslips in 6-well plates and were made quiescent in serum-free DMEM for 24 hours. Cells were incubated with BrdU (10 μmol/L) while treated with drugs for 24 hours. Wire-injured mice received an IP injection of BrdU solution (100 mg/kg) 24 hours and 1 hour before euthanasia. Cultured SMCs and paraffin sections of femoral arterial segments were denatured with 1 mol/L HCl, neutralized with 0.1 mol/L $Na_2B_4O_7$ (pH 8.5), and then incubated with antibody against BrdU. Immunofluorescence assay was used for detection of BrdU incorporation. The number of BrdU-stained nuclei was counted. The results were reported as a percentage of BrdU-labeled cells to the total amount of cells in cultured SMCs or BrdU-positive cells per femoral artery.

Immunostaining

Immunohistochemistry was performed on paraffin sections of femoral arteries by using antibodies against 5-HT2BR, Mac-2, and 5-HT2AR. The sections were incubated with horseradish peroxidase–conjugated secondary antibodies, developed with 3,3′-diaminobenzidine chromogen substrate, and counterstained with hematoxylin. Images were captured with a Leica DM3000B photomicroscope. For immunofluorescence staining, paraffin sections of femoral arteries were fixed and incubated with the 5-HT2BR, BrdU, α-SMA, CD31, and vimentin antibodies, followed by detection with the Alexa Fluor–conjugated secondary antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Digital images were acquired using a Leica TCS SP8 microscope.

RNA Interference

SMCs were transfected at 70% confluency. The transfection solution was composed of Opti-MEM and Lipofectamine 2000 (Invitrogen, Carlsbad, CA), along with double-stranded siRNA (100 nmol/L) targeting rat β-arrestin2 (5′-GGCUCAGCUUGAACAAGAUTT-3′), p70S6K (5′-GCACCUGCGUAUGAAUCUATT-3′), or scrambled control.

Western Blot Analysis

Total protein lysates were prepared from SMCs and femoral arteries with the lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 15 mmol/L EGTA, 100 mmol/L NaCl, and 0.1% Triton X-100 supplemented with protease and phosphatase inhibitor cocktail) and resolved on SDS-PAGE. Western blot analysis was performed with appropriate primary antibodies and horseradish peroxidase–conjugated secondary antibodies, followed by enhanced chemiluminescence detection. Autoradiographs were scanned and quantified for band intensities.

Quantitative RT-PCR

Total RNA was isolated from SMCs and femoral arteries with TRIzol reagent and reverse transcribed by using M-MLV transcriptase and random primers. Quantitative RT-PCR was performed with the GoTaq qPCR Master Mix (Promega, Fitchburg, WI) and specific primers in the Stratagene Mx3000P qPCR System. The following primer pairs were used for RT-PCR analyses: 5-HT2BR, 5′-ACCTGATCCTGACTAACCGTT-3′ (forward) and 5′-TGGGTATTATCACCGCGAGTAT-3′ (reverse); 5-HT2AR, 5′-AGCTCTGTGCGATCTGGATT-3′ (forward) and 5′-CCCCTCCTTAAAGACCTTCG-3′ (reverse); β-actin, 5′-CCACCATGTACCCAGGCATT-3′ (forward) and 5′-AGGGTGTAAAACGCAGCTCA-3′ (reverse); α-SMA, 5′-GCTGCTCCAGCTATGTGTGA-3′ (forward) and 5′-CTGTCCTTTTGGCCCATTCC-3′ (reverse); and SM22α, 5′-AGATGGAACAGGTGGCTCAA-3′ (forward) and 5′-GCCCAAAGCCATTACAGTCC-3′ (reverse).

Migration Assay

For wound closure assay, confluent SMC monolayers were starved with serum-free DMEM for 24 hours and scratched with a pipette tip to produce a wound. After treatments, wound closure was observed under inverted microscope, and the images were taken at different time points. Three wound sites along the wounding scratch were examined. The width of the wounding gap was measured on the photographs (Leica microscopy imaging software). For Boyden chamber assay, SMCs (10^4 cells/well) were seeded in the upper chamber containing an 8-μm-pore polycarbonate membrane (Costar, Cambridge, MA). SMCs were starved in serum-free DMEM for 24 hours. The reagents were added in the lower chamber in the presence of PDGF-BB (10 ng/mL). Cells were incubated for 24 hours at 37°C. Non-migrated SMCs were removed with a cotton swab from the top filter surface. SMCs that migrated to the bottom surface were fixed in 4% paraformaldehyde and

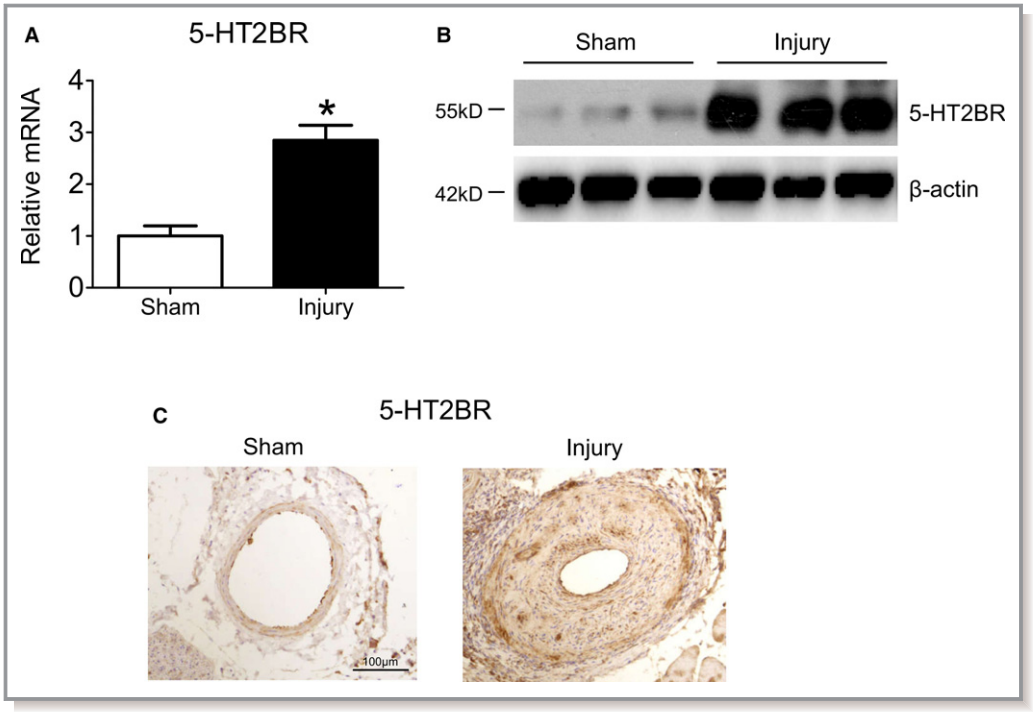

Figure 1. 5-Hydroxytryptamine receptor 2B (5-HT2BR) was increased in femoral arteries after vascular injury. A, Quantitative reverse transcription–polymerase chain reaction analysis of 5-HT2BR expression in wire-injured and sham–operated femoral arteries at 28 days after injury. Results are mean±SEM from 4 mice in each group. B, Western blot analysis of 5-HT2BR expression in femoral arteries. Representative results from 3 mice in each group were shown herein. C, Immunohistochemical staining of injured femoral arteries with antibody against 5-HT2BR. **P*<0.05 vs sham group from paired *t* test.

stained with crystal violet. Quantification of the migrated cells was expressed as the mean number of cells seen in 6 random high-power fields.

Statistical Analysis

Results were expressed as mean±SEM. The statistical differences between 2 groups were analyzed using Student *t* test. One-way ANOVA with post hoc tests was performed for multiple-group analysis. Time course experiments were analyzed using repeated-measures 2-way ANOVA, including time-treatment interaction. The differences between groups at different time points were assessed by post hoc tests. All post hoc tests were corrected for multiple comparisons. $P<0.05$ was considered statistically significant. All data were analyzed by using Prism 5.00.

Results

5-HT2BR Was Upregulated in Femoral Arteries After Vascular Injury

To evaluate 5-HT2BR expression level during vascular remodeling, we performed femoral artery wire injury in C57BL/6J mice. Quantitative RT-PCR and Western blot analysis showed that mRNA and protein levels of 5-HT2BR were elevated in femoral arteries at 28 days after wire injury (Figure 1A and 1B). The immunohistochemistry staining showed an increase of 5-HT2BR after vascular injury, primarily in media and neointimal layers (Figure 1C). To further examine the specific cell types in which 5-HT2BR was expressed, we performed immunofluorescence staining in wire-injured femoral arteries of C57BL/6J mice with antibodies against 5-HT2BR and the markers for the different types of vascular cells, including α-SMA (SMCs), vimentin (adventitial cells), and CD31 (endothelial cells). As shown in Figure S1, most of the 5-HT2BR expression was associated with the medial part of the arteries. However, 5-HT2BR expression was also expressed, to a lesser extent, in the neointima, adventitia, and endothelia.

5-HT2BR Agonist Promoted Vascular Remodeling After Wire Injury

We examine the effects of a selective agonist of 5-HT2BR, BW723C86, on vascular remodeling in C57BL/6J mice at 3, 7, and 14 days after wire injury. BW723C86 (10 mg/kg) was perivascularly delivered with 30% pluronic gel. After vascular injury, normally contractile SMCs dedifferentiated to a state of

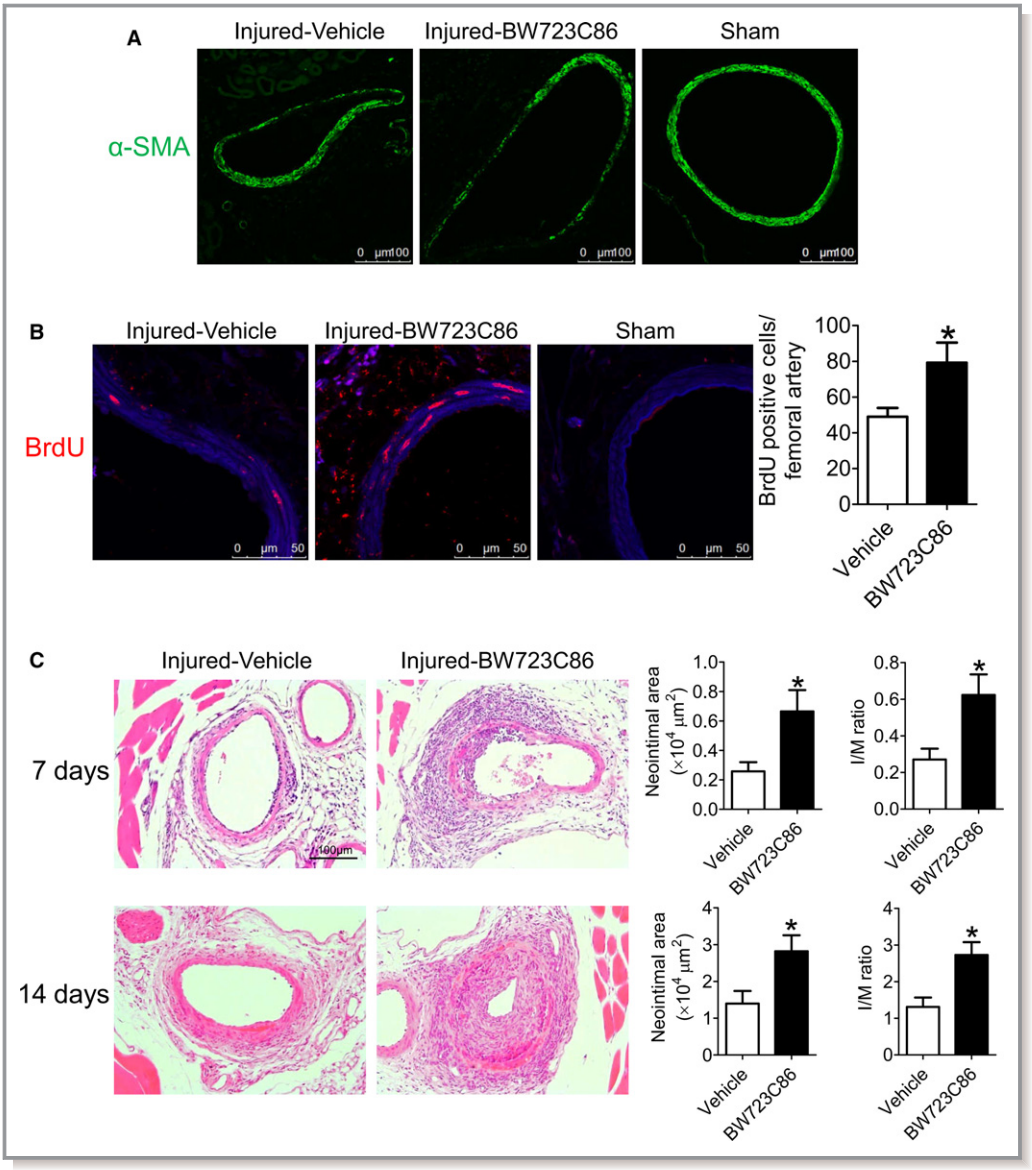

Figure 2. 5-Hydroxytryptamine receptor 2B agonist BW723C86 promoted vascular remodeling process after wire injury. A, Immunofluorescence staining of α-smooth muscle actin (α-SMA) on paraffin sections of wire-injured femoral arteries treated with or without BW723C86 at 3 days after injury. B, 5-Bromo-2′-deoxyuridine (BrdU) immunostaining photomicrographs of injured femoral arteries 3 days after injury. Quantification of BrdU-positive cells in injured arteries. Results are mean±SEM from 4 mice in each group. C, Hematoxylin and eosin staining of neointimal formation at 7 and 14 days after vascular injury in vehicle- and BW723C86-treated mice. Quantification of neointimal area and neointima/media (I/M) ratio of femoral arteries. Results are mean±SEM from 4 to 6 mice in each group. **P*<0.05 vs vehicle from *t* test.

proliferation, migration, and extracellular matrix secretion, a process referred to as phenotypic change. A key feature of this process is the loss of expression of SMC-specific gene products, such as α-SMA.[34,35] We found that BW723C86 further decreased α-SMA expression in wire-injured arteries compared with the vehicle group at 3 days after injury (Figure 2A). In addition, BrdU staining showed that BW723C86 promoted cell proliferation in wire-injured femoral arteries (Figure 2B). Then, we investigated neointimal formation in BW723C86-treated mice at 7 and 14 days after vascular injury. The data showed that BW723C86 increased neointimal hyperplasia compared with vehicle-treated mice (Figure 2C). Hematoxylin and eosin staining also showed a large amount of inflammatory cell infiltration after BW723C86 treatment. In conclusion, BW723C86, the selective 5-HT2BR agonist, exacerbated the injury response during restenosis.

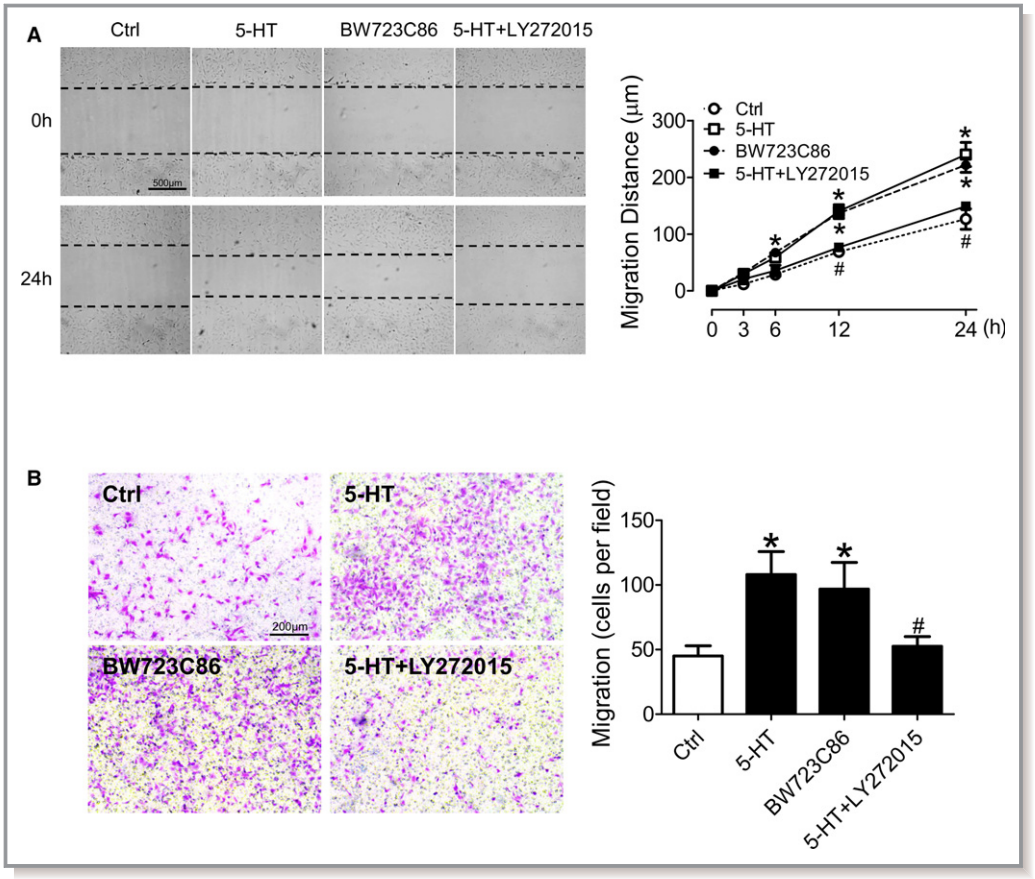

Figure 3. 5-Hydroxytryptamine receptor 2B (5-HT2BR) mediated 5-HT–induced smooth muscle cell (SMC) migration and proliferation. A, Aortic SMC migration was stimulated with 5-HT (1 μmol/L) with or without 5-HT2BR antagonist LY272015 (100 nmol/L) or 5-HT2BR agonist BW723C86 (1 μmol/L) for 24 hours. Cell migration was assessed with wound closure assay by measuring the distances between the migratory edges (dotted lines) at different time points after wounding. The unit of relative distance is pixel, measured using ImageJ software. Results are mean±SEM from 4 to 6 experiments. Repeated-measures ANOVA, followed by post hoc tests, was used. B, Transwell assay of the migration activity of SMCs. Results are mean±SEM from 4 to 6 experiments. One-way ANOVA, followed by post hoc tests, was used. C, Aortic SMCs in coverslips were starved for 24 hours and then treated with 5-HT (1 μmol/L) with or without 5-HT2BR antagonist LY272015 (100 nmol/L) or 5-HT2BR agonist BW723C86 (1 μmol/L) for 24 hours. Cell proliferation was assessed with 5-bromo-2′-deoxyuridine (BrdU) incorporation. Results were expressed as percentages of BrdU-labeled cells/the total amount of cells. Results are mean±SEM from 3 experiments. One-way ANOVA, followed by post hoc test, was used. **P*<0.05 vs control (Ctrl), #*P*<0.05 vs 5-HT. DAPI indicates 4',6-diamidino-2-phenylindole.

5-HT2BR Mediated 5-HT–Induced SMC Migration and Proliferation

We examined the effects of 5-HT2BR on the migration of SMCs. As shown in the wound closure assays, 5-HT and BW723C86, a selective agonist for 5-HT2BR, both significantly enhanced the SMC migration. However, pretreatment with LY272015, a 5-HT2BR antagonist, mitigated the 5-HT–induced migration (Figure 3A). Because PDGF-BB is a well-known promigratory cytokine in SMCs,[36] we further investigated the effects of 5-HT2BR on the PDGF-BB–induced migration. As shown with the Boyden chamber assay, 5-HT and BW723C86 both enhanced PDGF-BB–induced SMC migration. However, the enhancing effect of 5-HT was largely abrogated by LY272015 (Figure 3B). Consistent with the antimigratory effect of 5-HT2BR antagonist, deficiency of 5-HT2BR in SMCs also inhibited 5-HT–induced migration (Figure S2). As shown in the BrdU incorporation assays, 5-HT and BW723C86 significantly enhanced the SMC proliferation. Moreover, pretreatment with LY272015 mitigated the 5-HT–induced proliferation (Figure 3C). These results indicated that 5-HT2BR mediated the promigratory and proproliferative effects of 5-HT in SMCs.

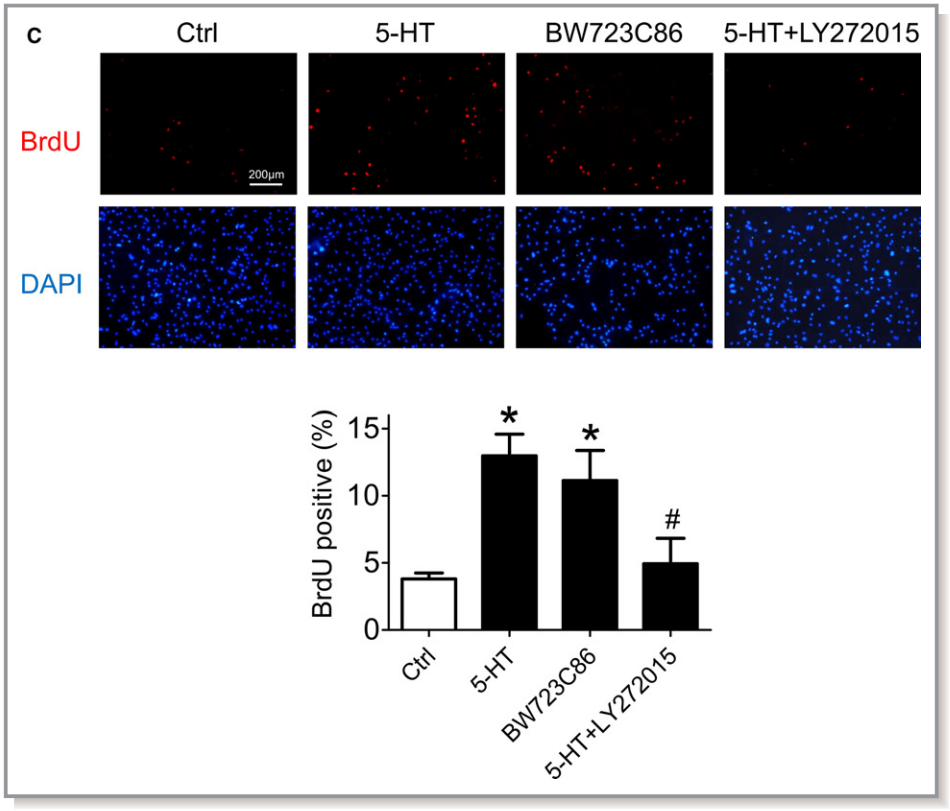

Figure 3. Contined.

5-HT2BR Stimulated the β-Arrestin2–mTOR/p70S6K Signaling

Activation of mTOR pathway mediated SMC migration and was implicated in vascular neointimal hyperplasia.[37–39] Thus, we examined the effect of 5-HT2BR on the activation of mTOR and its downstream serine/threonine kinase p70S6K. Treatment with 5-HT or BW723C86 led to rapid phosphorylation of mTOR (at serine^{2448}) and p70S6K (at threonine^{389}), the hallmarks of their activation (Figure 4A and 4B). The pretreatment with LY272015 antagonized the mTOR and p70S6K phosphorylation in response to 5-HT (Figure 4C), indicating that the 5-HT activated the mTOR/p70S6K pathway via 5-HT2BR in SMCs.

Phospholipase C (PLC) is known to couple with Gαq and mediate the intracellular signaling of 5-HT2Rs.[3] Thus, we pretreated SMCs with a PLC inhibitor, U73122, before the exposure to BW723C86. However, inhibition of PLC did not affect the BW723C86-stimulated activation of mTOR/p70S6K in SMCs (Figure 5A). Apart from canonical G-protein–mediated signaling, G-protein–coupled receptors also activate a β-arrestin–dependent noncanonical pathway.[40] β-Arrestins have been reported to be involved in motility of SMCs.[41] We found that silencing of β-arrestin2 with siRNA transfection in SMCs (Figure S3A) markedly reduced the phosphorylation of mTOR and p70S6K induced by BW723C86 (Figure 5B). These studies suggested that β-arrestin2 played a critical role in the 5-HT2BR–mediated activation of the mTOR/p70S6K pathway in SMCs.

Because β-arrestin can serve as a scaffold to assemble distinct signaling molecules for the functional selectivity of G-protein–coupled receptor agonists,[40] we further determined the specific roles of mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase, and protein kinase B pathways. As shown in Figure 5C, phosphatidylinositol 3-kinase inhibitor LY294002 attenuated BW723C86-induced phosphorylation of p70S6K and mTOR as potently as the mTOR inhibitor rapamycin. On the other hand, the MAPK inhibitors decreased the phosphorylation of p70S6K and, to a lesser extent, mTOR. Taken together, these results indicated that β-arrestin2 was involved in the serotonergic activation of mTOR/p70S6K after the 5-HT2BR agonism in SMCs.

mTOR/p70S6K Activation Was Required for the Promigratory Effect of 5-HT2BR

We used the mTOR inhibitor rapamycin and siRNA to examine the functional roles of mTOR and p70S6K in SMC migration in response to 5-HT and 5-HT2BR agonist. Western blot analysis showed that p70S6K significantly decreased with transfection of siRNA (Figure S3B). Wound closure

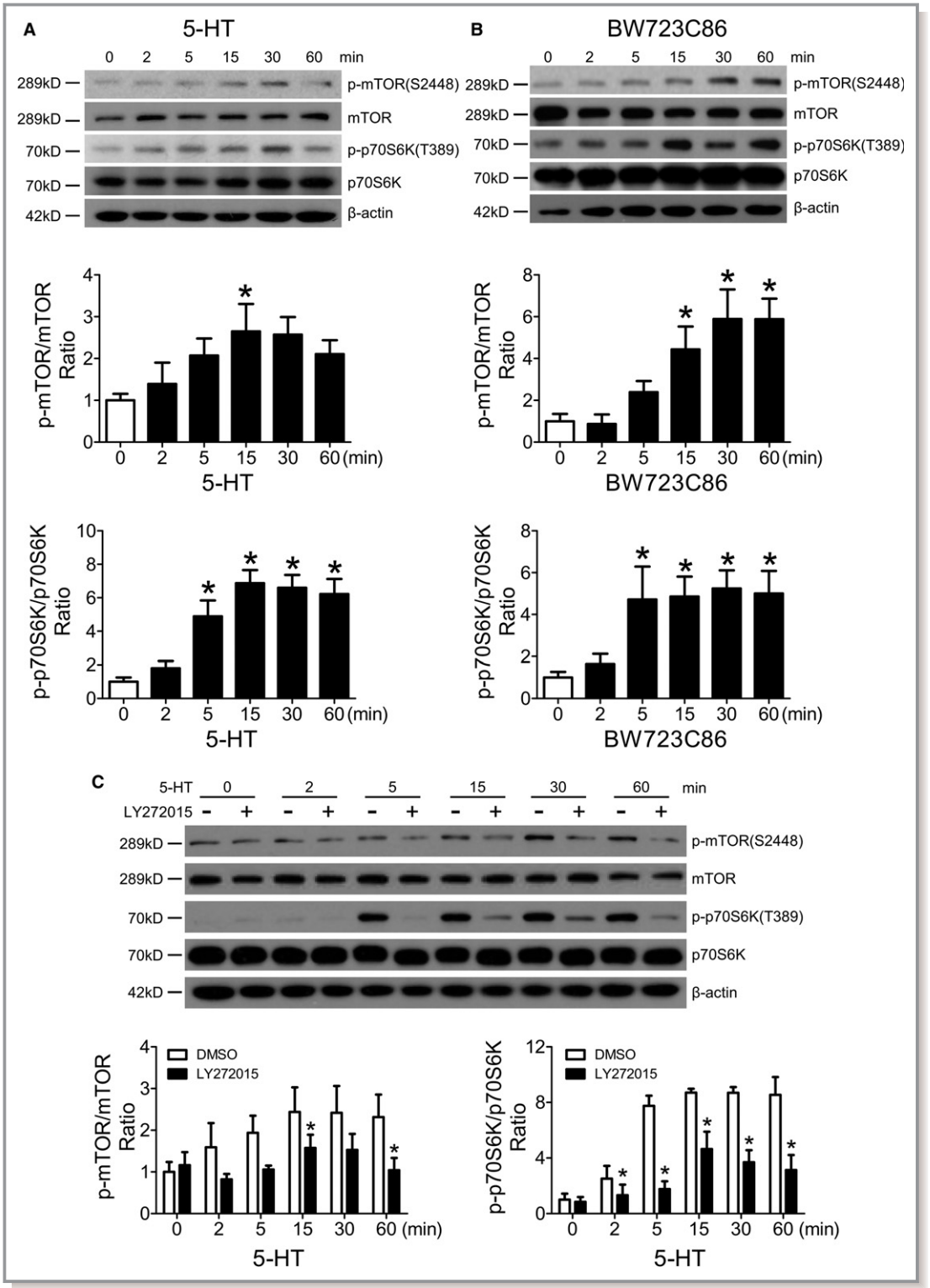

Figure 4. 5-Hydroxytryptamine receptor 2B (5-HT2BR) mediated the activation of mammalian target of rapamycin (mTOR)/p70S6K signaling. Western blot analysis of phosphorylated mTOR (p-mTOR), mTOR, p-p70S6K, and p70S6K in smooth muscle cells (SMCs) treated with 5-HT (1 μmol/L; A) or BW723C86 (1 μmol/L; B) for the indicated times. Quantitative results are mean±SEM from 6 to 8 experiments. **P*<0.05 vs 0 minutes from 1-way ANOVA, followed by post hoc tests. C, SMCs were treated with 5-HT2BR antagonist LY272015 (10 nmol/L) for 2 hours, followed by 5-HT (1 μmol/L) stimulation for the indicated times. mTOR/p70S6K activation was assessed by Western blot analysis. Results are mean±SEM from 6 experiments. **P*<0.05 vs dimethyl sulfoxide (DMSO) group of the same time point from *t* test.

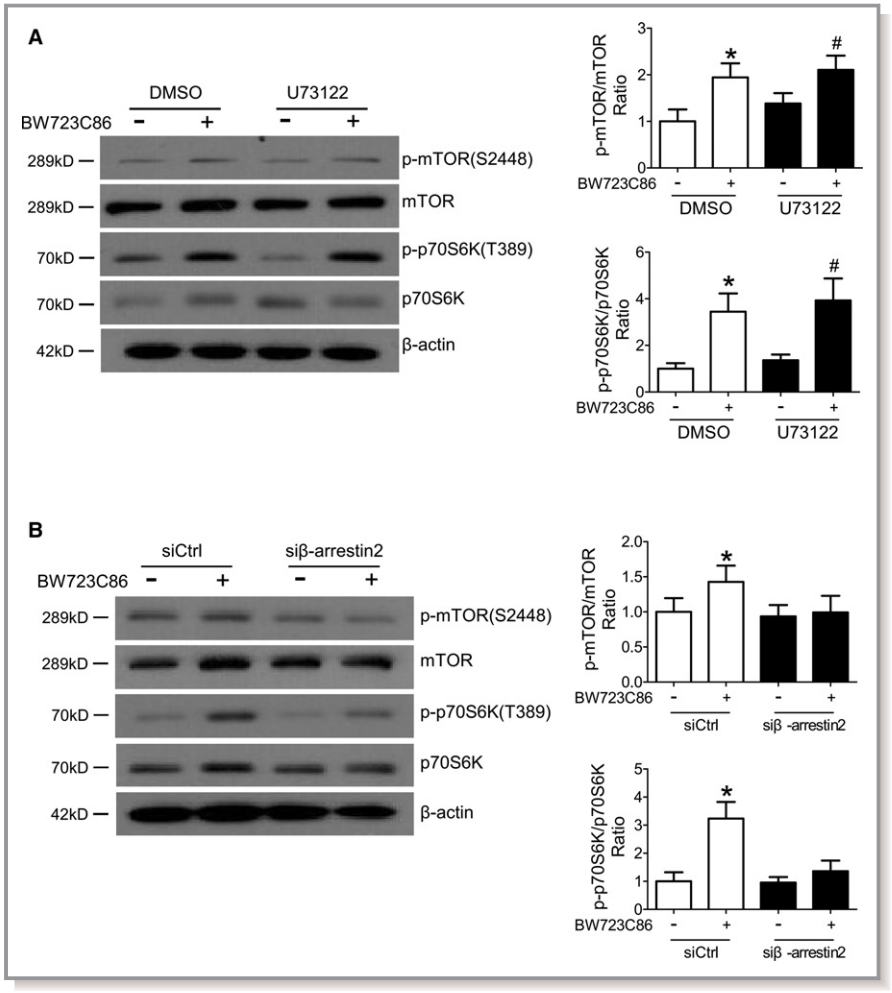

Figure 5. β-Arrestin2 signaling was involved in 5-hydroxytryptamine receptor 2B–mediated activation of the mammalian target of rapamycin (mTOR)/p70S6K pathway. A, Smooth muscle cells (SMCs) were treated with phospholipase C inhibitor U73122 (5 μmol/L) for 15 minutes, followed by BW723C86 (1 μmol/L) stimulation for 15 minutes. Western blot results were quantified and expressed as mean±SEM from 7 to 9 experiments. **P*<0.05 vs dimethyl sulfoxide (DMSO), #*P*<0.05 vs U73122 from *t* test. B, SMCs were transfected with β-arrestin2 small-interfering RNA (siRNA) and scramble control siRNA (100 nmol/L) for 24 hours, then cells were treated with BW723C86 (1 μmol/L) for 15 minutes. Western blot results were quantified and expressed as mean±SEM from 11 experiments. **P*<0.05 vs small-interfering control (siCtrl), #*P*<0.05 vs siβ-arrestin2 from *t* test. C, Effects of the inhibitors of mTOR (rapamycin, 100 nmol/L), phosphatidylinositol 3-kinase (LY294002, 10 μmol/L), p38 (SB202190, 10 μmol/L), JNK (SP600125, 10 μmol/L), and extracellular signal–regulated kinase (PD98059, 10 μmol/L) on mTOR/p70S6K activation. SMCs were treated with inhibitors for 2 hours, followed by BW723C86 (1 μmol/L) stimulation for 15 minutes. Western blot results were quantified and expressed as mean±SEM from 6 experiments. **P*<0.05 vs basal from *t* test, #*P*<0.05 vs DMSO group from 1-way ANOVA, followed by post hoc tests. p-mTOR indicates phosphorylated mTOR. JNK indicates c-Jun N-terminal kinase.

experiments (Figure 6A) and Boyden chamber assays (Figure 6B) demonstrated that the promigratory effects of 5-HT and BW723C86 were largely abrogated in SMCs treated with rapamycin or transfected with p70S6K siRNA. These results indicate that the activation of mTOR and p70S6K was required for the 5-HT2BR–mediated SMC migration.

Deficiency of 5-HT2BR–Attenuated Neointimal Formation After Femoral Artery Wire Injury

Given the increased level of 5-HT2BR in intimal hyperplasia in vivo, we used CRISPR/Cas9 technology for site-specific gene modification to generate 5-HT2BR knockout mice. One

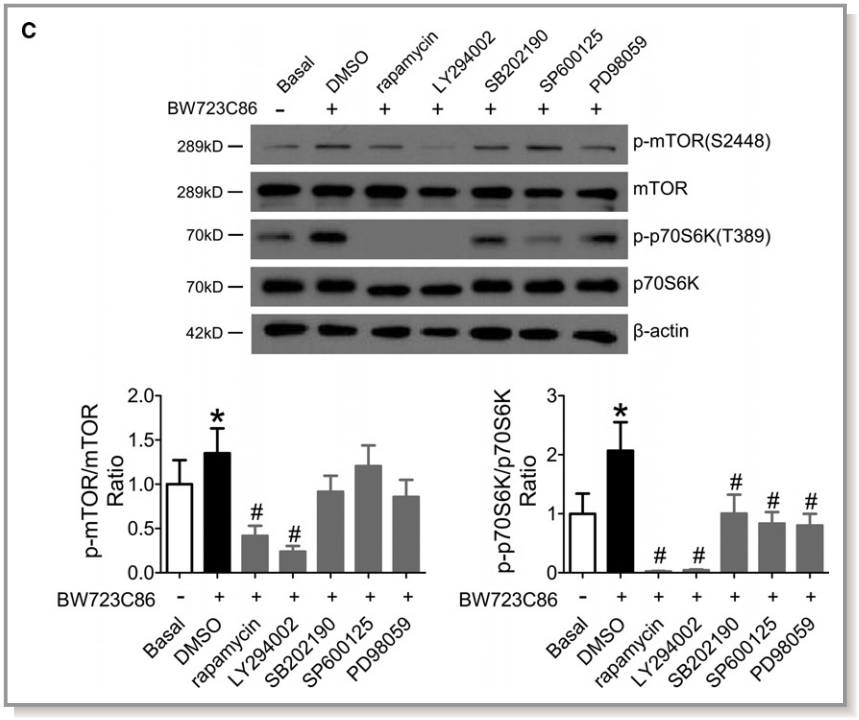

Figure 5. Continued.

single-guide RNA that targets 5-HT2BR exon 1 in mouse was designed and constructed. PCR products of 5-HT2BR$^{-/-}$ mouse genomic DNA were sequenced and showed a 4-bp deletion (Figure 7A). Quantitative RT-PCR and Western blot analysis showed that mRNA and protein of 5-HT2BR were absent in aortas of 5-HT2BR–knockout mice (Figure 7B). Subsequently, we evaluated neointimal formation in 5-HT2BR$^{-/-}$ mice at 28 days after femoral artery injury. Neointimal formation was significantly reduced in 5-HT2BR$^{-/-}$ mice compared with wild-type mice (Figure 7C). Quantitative analysis showed reduced neointimal area and intima/media ratio in 5-HT2BR$^{-/-}$ compared with wild-type mice. There were no significant differences between 2 groups in medial area (Figure 7D). Compared with wild-type mice, injured arteries from 5-HT2BR$^{-/-}$ mice also showed reduced extracellular matrix accumulation, as revealed by Masson staining of collagen fibers (Figure 7E). We also performed Mac-2 immunostaining on sections of wire-injured femoral arteries for investigating the inflammatory response. It showed decreased inflammatory cell infiltration after injury in the 5-HT2BR$^{-/-}$ group (Figure 7F, Figure S4). To determine the role of 5-HT2BR in the early response to vascular injury, we performed BrdU incorporation in injured femoral arteries at 7 days after the operation. The data indicated decreased BrdU-positive cells within the arterial walls in 5-HT2BR$^{-/-}$ mice (Figure 7G).

Discussion

Compared with other 5-HT receptor subtypes, 5-HT2BR displays a lower expression in the brain, but is more abundantly expressed in the cardiovascular system, gastrointestinal tract, bone, and liver,[25] suggesting that 5-HT2BR may play a greater role in peripheral tissues. Previous studies have revealed important roles of 5-HT2BR in the pathogeneses of vascular dysfunction and cardiac and valvular diseases.[22–27,42] More important, we found the expression of 5-HT2BR was induced by 5-HT itself in a feed-forward manner in pulmonary artery SMCs.[23] Inhibition of 5-HT2BR can reduce the 5-HT level.[32] However, the role of 5-HT2BR in neointimal formation and the underlying mechanisms were still unclear. Herein, we first demonstrated that inhibition of 5-HT2BR ameliorated restenosis by using the femoral artery injury model. Meanwhile, we provide a novel mechanism by which 5-HT2BR promoted SMC migration via stimulating β-arrestin2–dependent mTOR/p70S6K activation.

A 5-HT2R antagonist, sarpogrelate,[43] which inhibited platelet aggregation and thrombus formation[44–47] was introduced as a therapeutic agent for the treatment of vascular occlusive disease[48–51] and for patients undergoing percutaneous interventions.[52,53] In a porcine model, sarpogrelate inhibited 5-HT–induced coronary contraction and spam.[54,55] Sarpogrelate ameliorated the progression of atherosclerosis,

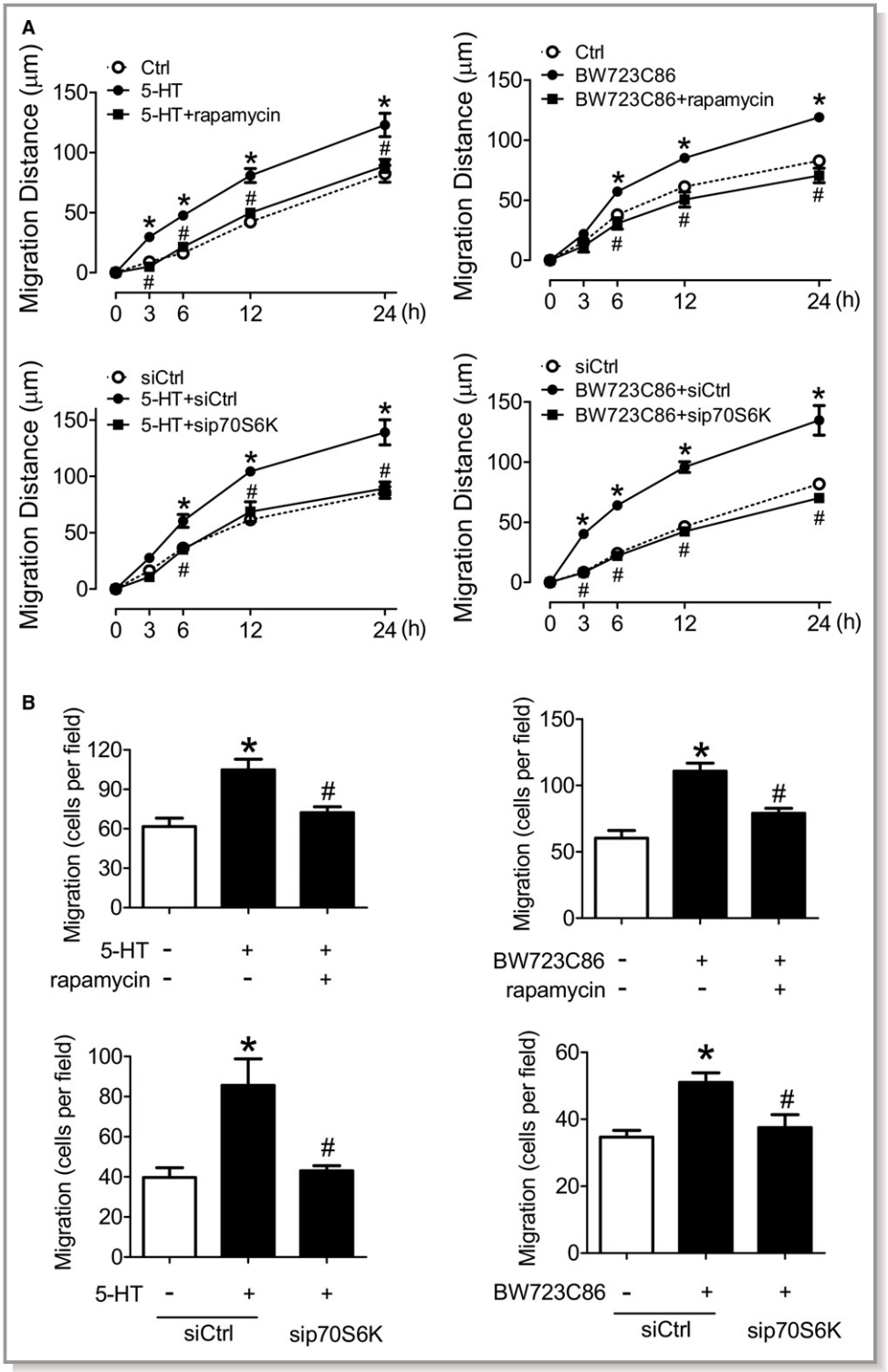

Figure 6. Mammalian target of rapamycin (mTOR)/p70S6K activation was required for 5-hydroxytryptamine (5-HT) receptor 2B–mediated smooth muscle cell (SMC) migration. A, SMC migration was stimulated with 5-HT (1 μmol/L) or BW723C86 (1 μmol/L) for 24 hours with or without mTOR inhibitor rapamycin (100 nmol/L). Alternatively, SMCs were transfected with p70S6K small-interfering RNA (siRNA) or scrambled control siRNA (siCtrl). Migration was measured with wound closure assay. Results are mean±SEM from 3 to 4 experiments. Repeated-measures ANOVA test, followed by post hoc tests, was used. B, Transwell assays for the SMC migration. Results are mean±SEM from 3 to 4 experiments. One-way ANOVA test, followed by post hoc tests, was used. $^{*}P<0.05$ vs control, $^{\#}P<0.05$ vs 5-HT or BW723C86 group.

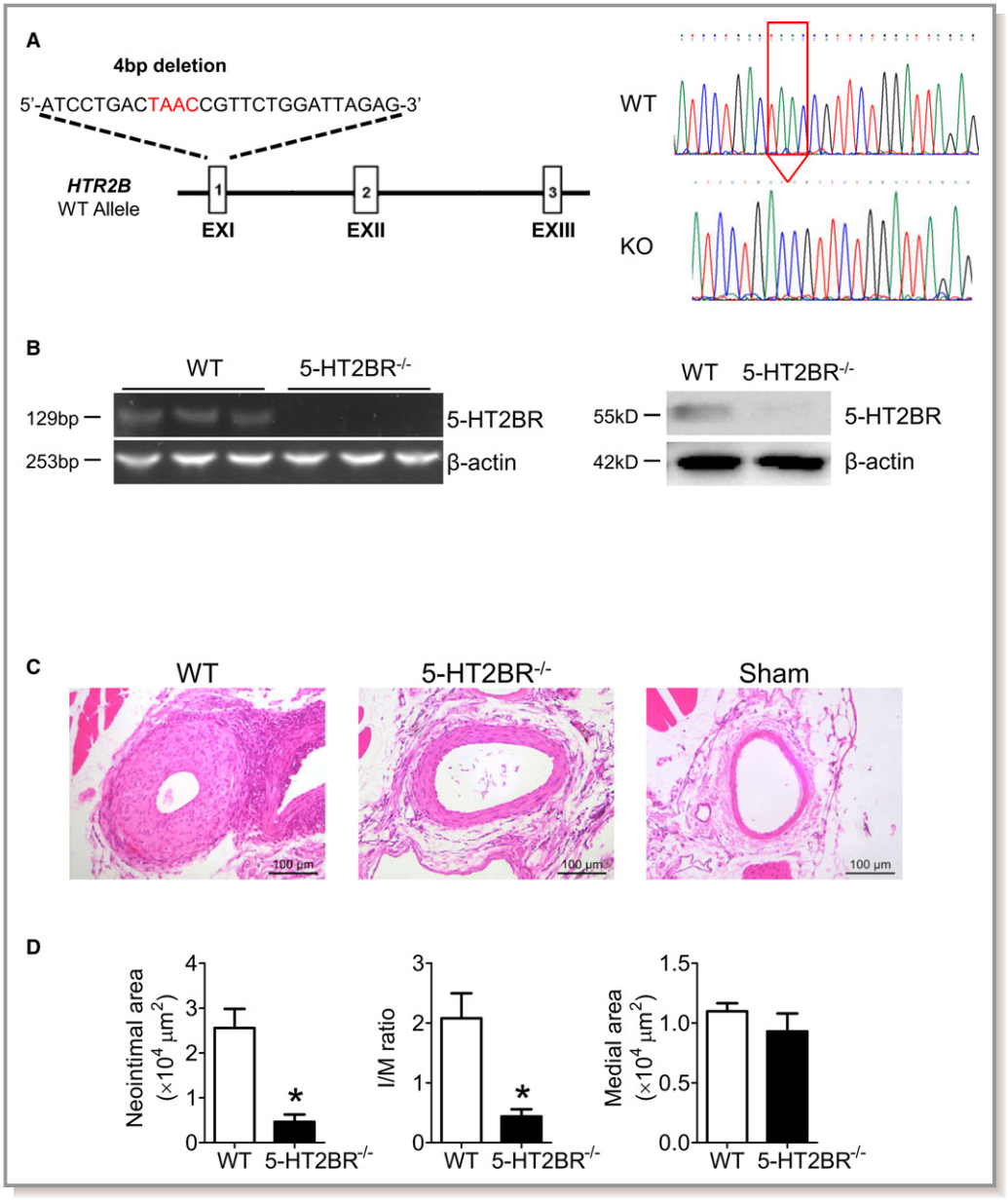

Figure 7. Deficiency of 5-hydroxytryptamine receptor 2B (5-HT2BR) reduced neointimal formation after vascular injury. A, Sequencing results of polymerase chain reaction products of 5-HT2BR$^{-/-}$ mouse genomic DNA showed 4-bp deletion in exon (EX) 1. B, 5-HT2BR transcript and protein levels in aortas of wild-type (WT) and 5-HT2BR$^{-/-}$ mice. C, Representative images of neointimal formation at 28 days after vascular injury in WT and 5-HT2BR$^{-/-}$ mice. D, Quantification of neointimal area, neointima/media (I/M) ratio, and medial area of femoral arteries. Results are mean±SEM from 6 mice in each group. E, Representative images and quantification of vascular extracellular matrix content stained by Masson's trichrome stain technique. Results are mean±SEM from 6 mice in each group. F, Immunohistochemical staining of Mac-2 in femoral arteries at 28 days after wire injury and quantification of Mac-2-positive areas. Results are mean±SEM from 6 mice in each group. G, 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay on wire-injured femoral arteries from WT and 5-HT2BR$^{-/-}$ mice at 7 days after injury. Results were expressed as BrdU-labeled cells per artery. Results are mean±SEM from 8 mice in each group. *P<0.05 vs WT from t test.

reduced intimal hyperplasia in vein graft, and inhibited thrombus formation on neointima in rabbit models.[56–58] These pathological effects of sarpogrelate are thought to be mediated through 5-HT2AR, whereas it also binds to 5-HT2BR.[59] According to the pharmacokinetic parameters analysis, plasma concentration of sarpogrelate can reach to 400 to 900 μg/L in humans,[60,61] which will have an inhibitory effect on 5-HT2BR (Negative logarithm of the inhibitory

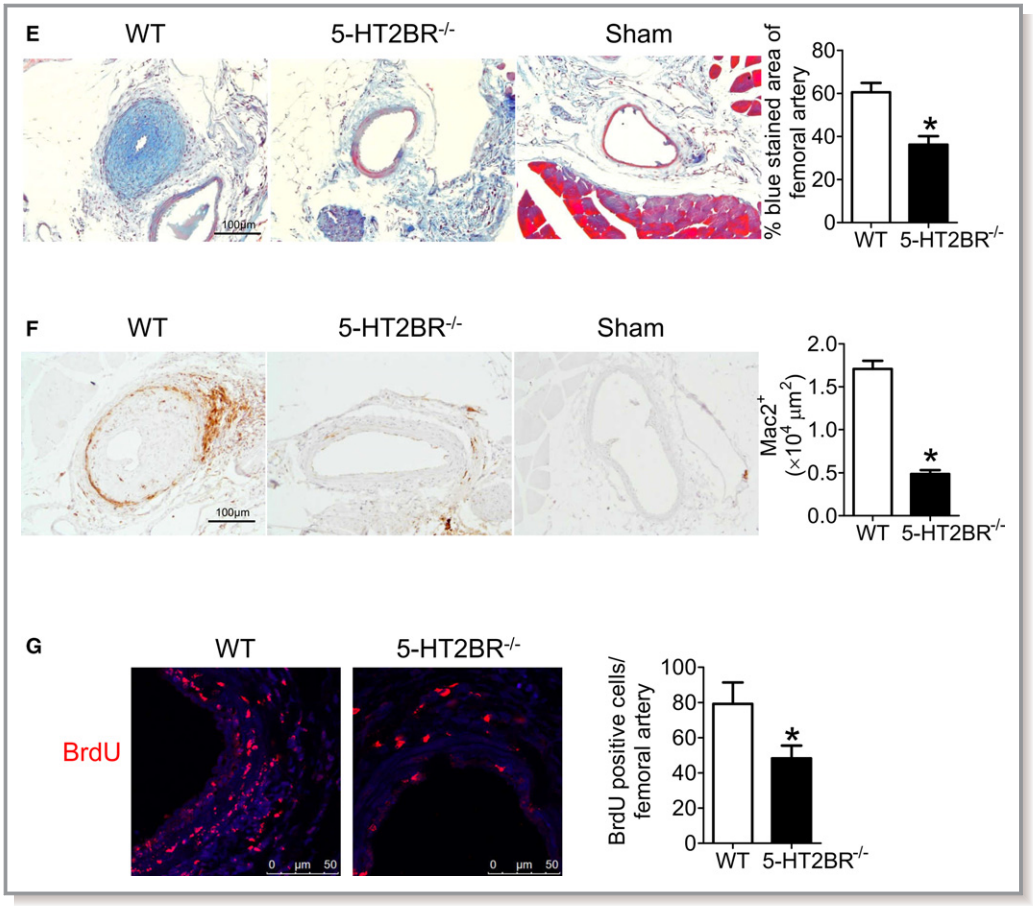

Figure 7. Continued.

constant (pKi)=6.57±0.12). It was postulated that the effect of sarpogrelate was partially mediated by 5-HT2BR in clinical trials. We also investigated the expression level of 5-HT2AR in wire-injured femoral arteries, and it showed no change in mRNA and protein levels (Figure S5).

As a serine/threonine protein kinase, mTOR mediates growth factor–induced cell migration.[62] mTOR inhibitors were clinically approved to reduce restenosis after angioplasty for patients with vascular occlusion.[63,64] It has been reported that 5-HT activated mTOR and p70S6K in neurons.[65,66] In the pulmonary artery, activation of p70S6K by 5-HT induces SMC proliferation.[67] We demonstrated that mTOR/p70S6K pathway activation played a critical role in 5-HT2BR–promoted vascular SMC migration, which was a key process in the development of intimal hyperplasia.

We next explored how 5-HT2BR agonist activates mTOR/p70S6K signaling. The promigratory effect of 5-HT2BR appeared not to be mediated via coupling of Gαq to stimulate PLC activity. In fact, inhibition of PLC had little effect on the 5-HT2BR–triggered mTOR/p70S6K activation in SMCs (Figure 5A). Rather, the 5-HT2BR activated the mTOR/p70S6K via a β-arrestin2–biased signalsome (Figure 5B). β-Arrestins are known to mediate many G-protein–independent pathways. β-Arrestins can act as positive mediators for signaling, leading to activation of protein kinases, such as Src, MAPKs, and phosphatidylinositol 3-kinase–protein kinase B.[40] It was reported that β-arrestins can regulate myosin light chain phosphorylation to promote the motility of SMC.[68] β-Arrestin2 mediates angiotensin II–induced SMC migration in such a manner.[41] More important, ergolines, such as lysergic acid diethylamide and ergotamine, displayed bias for β-arrestin signaling at 5-HT2BR.[69] It is indicated that the β-arrestin–biased signalsome mediated the pathological effects of 5-HT2BR by converging the serotonergic signaling with the downstream mTOR/p70S6K pathways. Corroborating our results from the cultured SMCs, several previous studies also suggested the pathophysiological roles of these signaling molecules in vascular remodeling. These results include the following: (1) β-arrestin2, but not β-arrestin1, knockout mice had a reduced neointimal hyperplasia in wire-injured carotid arteries[70]; and (2) the roles of the mTOR pathway in neointima hyperplasia have been well established with the successful use of sirolimus (rapamycin)–eluting stents in angioplasty. We previously also showed that the activation of mTOR in the neointima in balloon-injured rat carotid arteries and perivascular delivery of rapamycin in

pluronic gel decreased neointima formation.[37,38] In addition, sirolimus ameliorated hypercontractility to 5-HT in balloon-injured porcine coronary arteries.[4]

It was reported that 5-HT2BR activated MAPKs in fibroblast cells, chronic liver disease, and myocardial remodeling.[71–73] We also investigated the effect of MAPKs in 5-HT2BR–induced mTOR/p70S6K activation. We found that MAPKs had little effect on 5HT2BR-induced mTOR activation. Interestingly, MAPK inhibitors significantly decreased p70S6K activity. Previous studies also showed that MAPKs exert a positive feedback on the p70S6K in neutrophils and T cells to promote the motility.[74,75] It was supposed that, in addition to mTOR, other kinases may also be involved in the phosphorylation of p70S6K.

An important role of 5-HT2BR in vascular remodeling is supported by the consolidating results from the experiments using 5-HT2BR loss-of-function and gain-of-function approaches. Although inhibition of 5-HT2BR using deficient mice or selective antagonist attenuated neointima formation and SMC migration, activation of the receptor with a selective agonist, BW723C86, exacerbated the processes. On the other hand, neointima formation after arterial injury also involves many other pathological processes, such as the infiltration of inflammatory cells, SMC phenotypic switching, proliferation, and extracellular matrix accumulation.[29] The 5-HT2BR deficiency mitigated neointimal hyperplasia, with significantly reduced inflammatory cells, proliferating SMCs, and extracellular matrix (Figure 7E through 7G), indicating that the 5-HT2BR may have pleiotropic effects beyond its promigratory action. Furthermore, 5-HT and selective 5-HT2BR agonist reduced the expression of α-SMA and SM22α in cultured SMCs and wire-injured arteries (Figure S6, Figure 2A). We also showed that the expression of SMC differentiation markers was still preserved in cultured SMCs before the treatment of 5-HT (Figure S7). It is suggested that activation of 5-HT2BR may promote SMC phenotypic switching. Given that medial SMC dedifferentiation and switching of contractile SMCs into a synthetic state are recognized as an important step in vascular remodeling, the functional significance and the specific signaling involving this progress warrant further study. Nevertheless, cell type–specific roles of 5-HT2BR in vascular remodeling await future studies using various tissue-specific knockout models. In addition, the decreased protein levels of the SMC differentiation marker genes detected with the use of Western blot analysis were also possibly attributable to the increased population of α-SMC and SM22 negative cells.

A multitude of effects of 5-HT have been demonstrated on the cardiovascular systems.[3] Plasma levels of 5-HT were increased in vascular remodeling diseases, such as PAH, hypertension, and atherosclerosis.[12,13,76,77] However, therapeutic interventions have been difficult because of the diverse functions and broad distribution of the 5-HT receptors. Sparse expression of 5-HT2BR in the central nervous system may be considered as an advantage in terms of a peripheral target for intervention. As such, a pathological role of 5-HT2BR in vascular restenosis may warrant further exploration.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

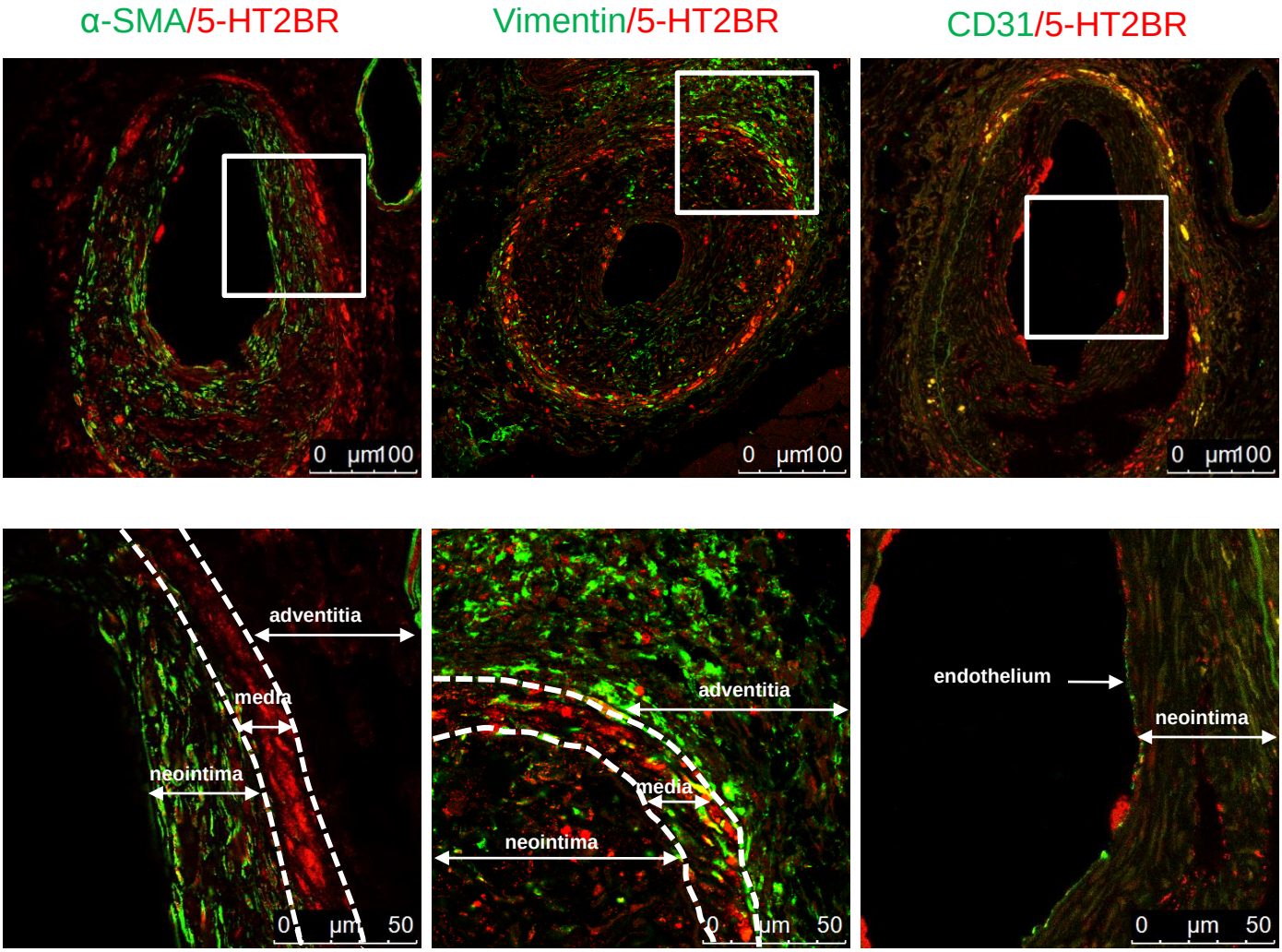

Figure S1. Expression of 5-HT2BR in wire-injured femoral artery.
Immunofluorescence staining of 5-HT2BR, α-SMA, vimentin and CD31 on paraffin sections of wire-injured femoral arteries of C57BL/6J mice at 28 days after operation.

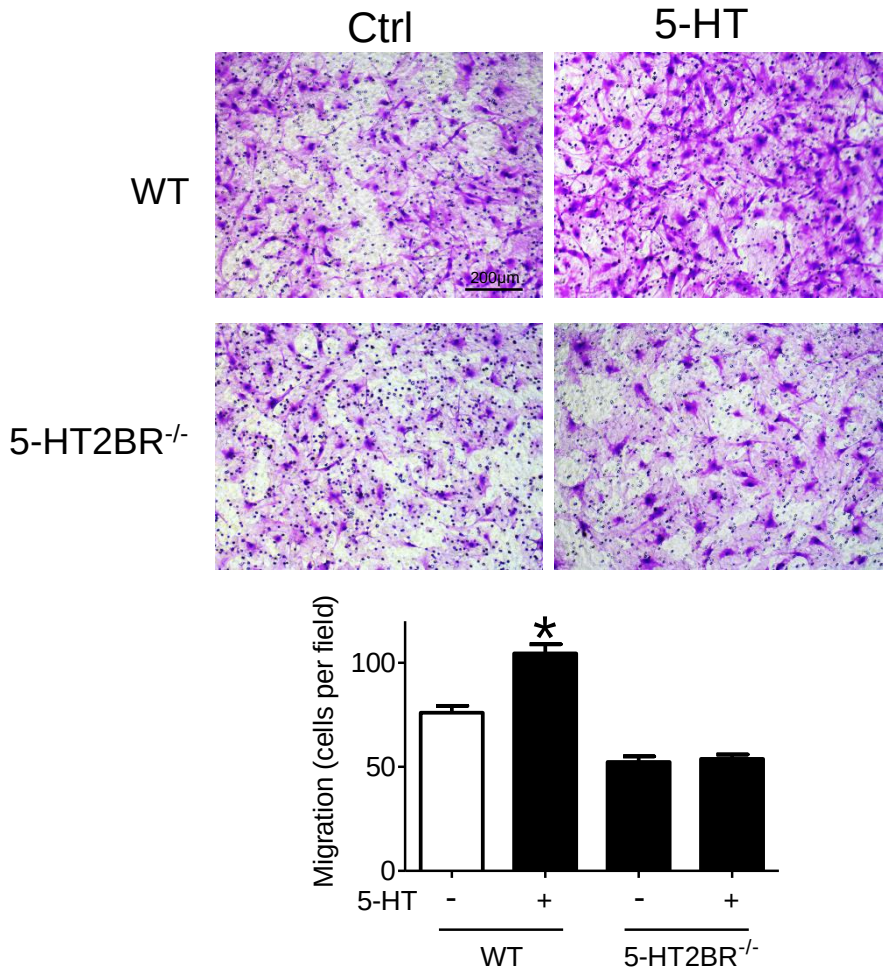

Figure S2. Deficiency of 5-HT2BR eliminated 5-HT-induced SMC migration.
(A) Aortic SMCs from WT and 5-HT2BR$^{-/-}$ mice was stimulated with 5-HT (1 μmol/L) for 24 h. Cell migration was assessed with transwell assay. Results are mean ± SEM from 6 experiments. $^{*}p<0.05$ vs. control from One-way ANOVA followed by post-hoc tests.

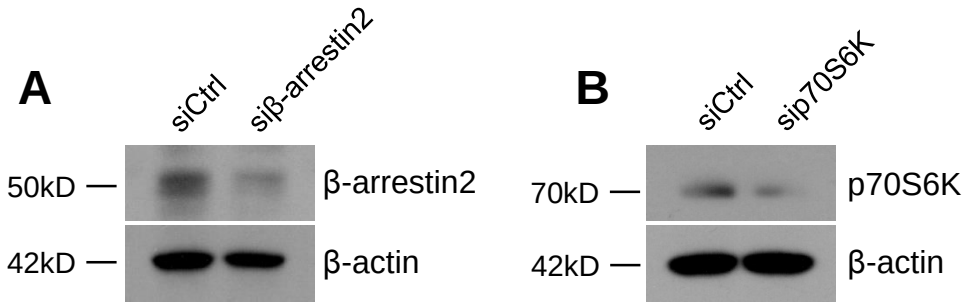

Figure S3. Knockdown of β-arrestin2 and p70S6K in SMCs.
Western blotting analysis showed the protein levels of (A) β-arrestin2 and (B) p70S6K following transfection of siRNA (100 nmol/L) or scramble control siRNA in SMCs.

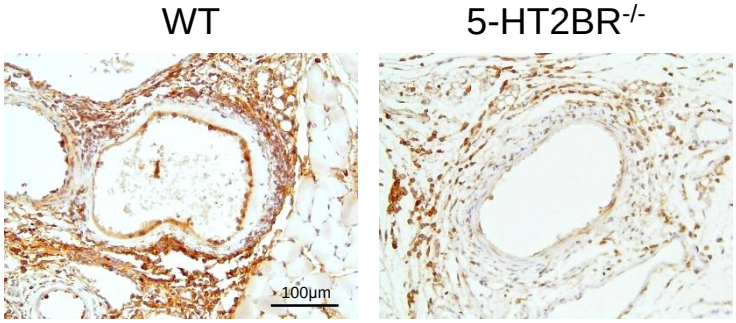

Figure S4. Deficiency of 5-HT2BR reduced macrophage infiltration. Immunohistochemical staining of Mac2 in femoral arteries of WT and 5-HT2B$^{-/-}$ mice at 3 days after wire injury.

Figure S5. Expression of 5-HT2AR was unchanged in femoral arteries after vascular injury. (A) Quantitative RT-PCR analysis of 5-HT2AR expression in wire-injured and control sham-operated femoral arteries. Results are mean ± SEM from 4 mice in each group. (B) Western blotting analysis of 5-HT2AR expression in femoral arteries. Representative results from 3 mice in each group were shown here. (C) Immunohistochemical staining of femoral arteries with antibody against 5-HT2AR.

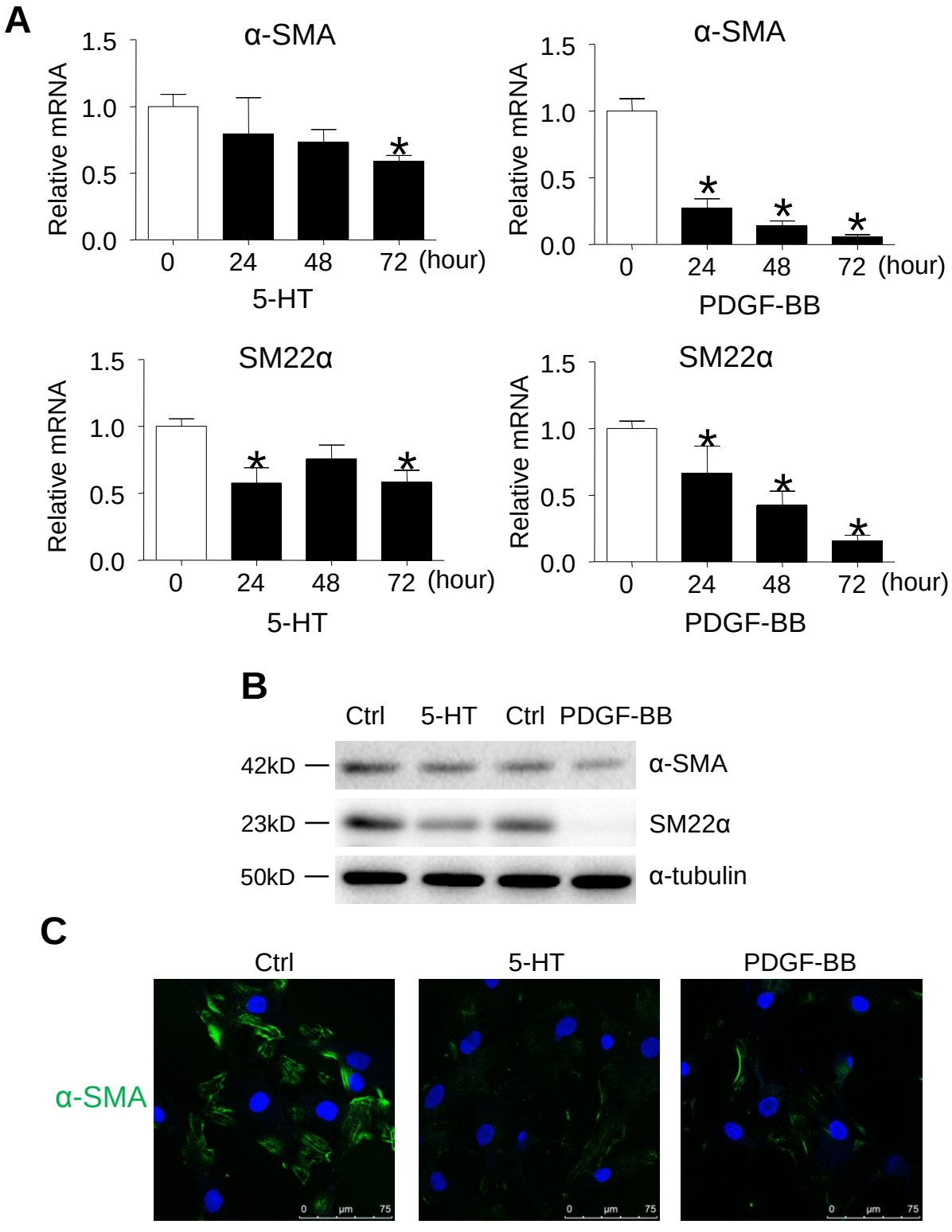

Figure S6. 5-HT decreased phenotypic markers for differentiated SMCs.
(A) Aortic SMC were starved for 24 h and then treated with 5-HT (1 μmol/L) for 24, 48 and 72 h. Quantitative RT-PCR analysis of the expression of vascular SMC differentiation markers. Results are mean ± SEM from 4 experiments. $^{*}p<0.05$ vs. control from One-way ANOVA followed post-hoc tests. (B, C) SMCs were treated with 5-HT for 72 h, and then was investigated SMC phenotypic switch by western blotting analysis and immunofluorescence staining.

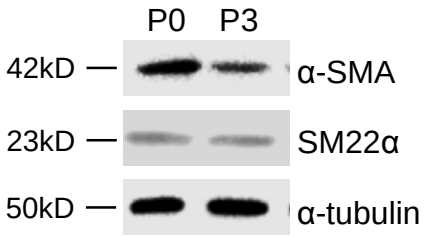

Figure S7. Differentiation markers of SMCs at different passages.
Western blotting analysis of protein levels of SMC differentiation markers in directly isolated aortic SMCs (passage 0) and cultured SMCs (passage 3).