BubR1 Insufficiency Inhibits Neointimal Hyperplasia Through Impaired Vascular Smooth Muscle Cell Proliferation in Mice

Ryoichi Kyuragi, Takuya Matsumoto, Yui Harada, Satoru Saito, Mitsuho Onimaru, Yoshimichi Nakatsu, Teruhisa Tsuzuki, Masatoshi Nomura, Yoshikazu Yonemitsu, Yoshihiko Maehara

Objective—BubR1, a cell cycle–related protein, is an essential component of the spindle checkpoint that regulates cell division. Mice with BubR1 expression reduced to 10% of the normal level display a phenotype characterized by progeria; however, the involvement of BubR1 in vascular diseases is still unknown. We generated mice in which BubR1 expression was reduced to 20% (BubR1<sup>L/L</sup>) mice of that in wild-type mice (BubR1<sup>+/+</sup>) to investigate the effects of BubR1 on arterial intimal hyperplasia.

Approach and Results—Ten-week-old male BubR1<sup>L/L</sup> and age-matched wild-type littermates (BubR1<sup>+/+</sup>) were used in this study. The left common carotid artery was ligated, and histopathologic examinations were conducted 4 weeks later. Bone marrow transplantation was also performed. Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta to examine cell proliferation, migration, and cell cycle progression. Severe neointimal hyperplasia was observed after artery ligation in BubR1<sup>+/+</sup> mice, whereas BubR1<sup>L/L</sup> mice displayed nearly complete inhibition of neointimal hyperplasia. Bone marrow transplantation from all donors did not affect the reconstitution of 3 hematopoietic lineages, and neointimal proliferation was impaired in BubR1<sup>L/L</sup> mice. p38 mitogen–activated protein kinase–inhibited VSMCs showed low expression of BubR1, and BubR1-inhibited VSMCs showed low expression of p38.

Conclusions—BubR1 may represent a new target molecule for treating pathological states of vascular remodeling, such as restenosis after angioplasty. (Arterioscler Thromb Vase Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.304737.)

Key Words: Bub1b protein, mouse ± cell cycle

Arteries respond to damaging stimuli, such as injury, inflammation, and stretch, by activating a remodeling mechanism that leads to neointimal hyperplasia. Accumulating evidence has shown that the underlying causes of neointimal hyperplasia include the invasion and proliferation of vascular smooth muscle cells (VSMCs), via processes triggered and controlled by various growth factors. Excessive proliferation and migration of VSMCs from the arterial media to the neointima are the major causes of neointimal hyperplasia. Furthermore, leukocyte and macrophage infiltration to the neointima are key components of atherosclerosis. These contribute to the development and progression of vascular disorders, including atherosclerosis and restenosis after angioplasty for coronary artery disease or endovascular treatment of peripheral arterial disease.

The spindle assembly checkpoint is a surveillance mechanism that prevents chromosome missegregation during the division of eukaryotic cells by delaying the metaphase-to-anaphase transition until all chromosomes become properly attached to the spindle microtubules and align at the metaphase plate. Core components of the spindle assembly checkpoint include the evolutionarily conserved proteins Bub1, Bub3, Mad1, Mad2, BubR1 (Mad3 in yeast), Mps1, and Aurora B. The cell cycle–related protein BubR1 is an essential component of the spindle assembly checkpoint and has important roles in cell division. Previous studies revealed that mice with BubR1 expression reduced to 10% of the normal level, termed BubR1 hypomorphic mice, develop progressive aneuploidy, cataracts, lordokyphosis, loss of subcutaneous fat, impaired wound healing, severely shortened lifespan, and infertility, together with early aging–associated vascular phenotypes, including a reduced number of smooth muscle cells, reduced elasticity, impaired endothelial–dependent relaxation, and increased production of superoxide anions. However, it is difficult to evaluate smooth muscle proliferation in vivo because the initial number of smooth muscle cells in these mice is low. Therefore, we newly generated mice in which BubR1 expression was reduced...
to 20% of the normal level. These mice do not display significant abnormalities, such as progeria, infertility, shortened lifespan, and abnormal vascular structures during growth and development under normal conditions. These mice, with a low BubR1-expressing mutant genotype (termed BubR1<sup>L/L</sup> mice), are particularly suitable for investigating the precise roles of BubR1 in the vasculature. Here, we describe the effects of low BubR1 expression on arterial neointimal hyperplasia in mice.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Generation of BubR1<sup>L/L</sup> Mice

Targeted insertion of the neo gene into an intron of an endogenous mouse gene, with reverse orientation, produced a mutant allele that generated pre-mRNA harboring a cryptic neo exon (Figure 1A). During pre-RNA splicing, this exon was either spliced into the mRNA product or spliced out. The mRNAs carrying the cryptic exon were translated into truncated proteins because neo sequences introduce stop codons in all 3 reading frames. Consequently, the small amount of normal

![Figure 1](http://link.to/figure1)

**Figure 1.** Mechanism involved in the reduction of protein expression by low-expression alleles generated by cryptic splicing. **A**, Schematic diagram. The neo cassette was inserted with reverse orientation. **B**, PCR of genomic DNA confirmed the presence of wild-type (475-bp fragment) and mutant (938-bp fragment) BubR1 genomic DNA. **C**, Real-time PCR confirmed that BubR1 expression was decreased in BubR1<sup>L/L</sup> mice. Thymus BubR1 expression in BubR1<sup>L/L</sup> mice was reduced to 20.9% of that in BubR1<sup>+/+</sup> mice. *P*<0.01 vs BubR1<sup>+/+</sup> mice; n=6 per group. **D**, Comparison of body weight of male BubR1<sup>+/+</sup> and BubR1<sup>L/L</sup> mice. Images of representative 39-week-old male mice from the indicated genotypes are shown. cont indicates control; FWD, forward; PCR, polymerase chain reaction; and REV, reverse.
mRNA generated from the mutant allele was translated into wild-type proteins. Tail DNA from BubR1+/−/−, BubR1−/−/−, and BubR1+/−/+ mice was subjected to polymerase chain reaction (PCR) genotyping using 3 primers that anneal to the neo gene and the upper and lower sides of the neo gene. Wild-type (BubR1+/−/+), DNA amplification was by primers targeting the intron, which yielded a 475-bp fragment (Figure 1B). By contrast, DNA from BubR1+/−/+ mice was amplified by primers targeting the intron and the neo gene, which yielded a 938-bp fragment. DNA from heterozygous mice (BubR1−/+/−) was amplified by primers targeting the intron and neo gene, yielding 475- and 938-bp fragments.

Next, we performed quantitative real-time PCR to determine the relative expression of BubR1 in the thymus and confirmed that its expression in BubR1−/−/− mice was 20.9% of that in BubR1+/−/+ mice (Figure 1C). BubR1+/−/+ mice had a normal appearance and size at birth but showed slow postnatal growth (Figure 1D).

Effects of Low BubR1 Expression on Neointimal Hyperplasia After Carotid Ligation

In response to an interruption of carotid blood flow, neointimal hyperplasia occurs via the proliferation and migration of VSMCs from the medial layer of the artery. Before carotid ligation, there was no difference in common carotid arterial size (vascular area), containing cell numbers and cell density in the vascular area of mice with either genotype (Figure IA in the online-only Data Supplement). Furthermore, no intimal thickening was present in either genotype (Figure IB in the online-only Data Supplement). There was no statistically significant difference between the body weight of male BubR1+/−/+ and BubR1+/−/+ mice at the age of 10 weeks (Figure 1D), as well as female mice (Figure IC in the online-only Data Supplement). In addition, the lifespan of male and female BubR1+/−/+ and BubR1+/−/+ mice was similar (data not shown). No progeroid phenotype appeared in either male (Figure 1D) or female BubR1+/−/+ mice (Figure IC in the online-only Data Supplement). In arterial tissues, such as the aorta and carotid artery, we evaluated that the expression level of BubR1 derived from BubR1+/−/+ mice was reduced to 17.9% (aorta) and 20.0% (common carotid artery) compared with that of BubR1+/−/+ mice. In other organs, such as the heart, liver, spleen, kidney, testis, and small intestine, BubR1 expression levels in male BubR1+/−/+ mice were reduced to 9.2, 19.2, 25.1, 17.6, 20.4, 9.5, and 35.5%, respectively, compared with those in BubR1+/−/+ mice, and organ weights were comparable except for the spleen. The weight of the spleen in BubR1+/−/+ mice was superior to that of BubR1+/−/+ mice (data not shown). In this study, we used 10-week-old male mice for analysis of the carotid ligation model.

We evaluated the time-dependent change of BubR1 expression in ligated common carotid arteries. Expression levels of BubR1 in the ligated arteries derived from BubR1+/−/+ mice were reduced to 21.1% (preoperative), 50.7% (3 days), 64.2% (1 week), 36.8% (2 weeks), and 15.7% (4 weeks) compared with that of BubR1+/−/+ mice, which were significantly lower preoperatively, 2 and 4 weeks after ligation (Figure 2A). BubR1 expression 3 days after ligation was significantly higher preoperatively, 2 and 4 weeks after ligation (Figure 2A).

Next, we evaluated the ligated arteries using morphological analysis. Significant neointimal hyperplasia was found 4
weeks after carotid ligation in BubR1<sup>+/+</sup> mice, whereas neointimal hyperplasia was almost completely inhibited in BubR1<sup>L/L</sup> mice (Figure 2B, top). Quantitative morphometry confirmed that the intimal area (4180±314 versus 23.56±4.107 μm<sup>2</sup>; P<0.01) and intima:media ratio (0.12±0.01 versus 0.68±0.10; P<0.001) were significantly lower in BubR1<sup>L/L</sup> mice than those in BubR1<sup>+/+</sup> mice (Figure 2B, bottom). These results indicate that reduced BubR1 expression is associated with a decrease of intimal thickening caused by carotid ligation.

Some previous studies have revealed that vascular smooth muscle progenitor cells derived from bone marrow are involved in the neointimal hyperplasia induced by vascular injury.13 14 So, we next investigated the effects of bone marrow transplantation on neointimal hyperplasia.

Within the same cell, BubR1<sup>L/L</sup> mice had both wild-type BubR1 mRNA and low-expressed BubR1 mRNA (Figure 1A), which made it difficult to evaluate the bone marrow replacement rate by flow cytometric analysis. We, therefore, evaluated the bone marrow replacement rate using C57BL/6 (recipient) and green fluorescent protein (donor) mice in an identical manner, which revealed that the recipient mice had 91.7% of total blood cells derived from the donor mice (n=5). Bone marrow transplantation from all donors did not affect reconstitution of the 3 hematopoietic lineages (Table I in the online-only Data Supplement). Neointimal hyperplasia was observed in both BubR1<sup>+/+</sup> mice transplanted with bone marrow from BubR1<sup>L/L</sup> mice and in BubR1<sup>L/L</sup> mice transplanted with bone marrow from other BubR1<sup>+/+</sup> mice. Conversely, neointimal hyperplasia was still suppressed in BubR1<sup>L/L</sup> mice transplanted with bone marrow from BubR1<sup>+/+</sup> mice. Quantitative morphometry confirmed the suppression of neointimal hyperplasia in the BubR1<sup>L/L</sup> mice transplanted with bone marrow from BubR1<sup>+/+</sup> mice (Figure 2C). These results suggest that the vascular smooth muscle progenitor cells from bone marrow did not contribute to neointimal hyperplasia observed in this study.

**Migration and Proliferative Capacity Evaluated in Cultured VSMCs From BubR1<sup>L/L</sup> Mice**

Because neointimal hyperplasia after carotid artery ligation involves migration and proliferation of VSMCs from the medial layer of the artery (Figure IIA in the online-only Data Supplement) after infiltration of macrophages into the media (Figure IIB in the online-only Data Supplement), we determined the migration and proliferative capacity of primary cultured VSMCs. VSMCs were isolated by enzymatic dispersion as previously described.13 VSMCs from BubR1<sup>+/+</sup> and BubR1<sup>L/L</sup> mice were positive for smooth muscle cell markers, including α-smooth muscle actin, smooth muscle 22-α, and smooth muscle myosin heavy chain (Figure 3A). Using quantitative real-time PCR, we confirmed that BubR1 mRNA expression in BubR1<sup>L/L</sup> VSMCs was reduced to 25.8% of that in BubR1<sup>+/+</sup> VSMCs (Figure 3B).

First, we determined whether reduction of BubR1 affected the migration of VSMCs using transwell migration assays. However, exposure to serum and platelet-derived growth factor (PDGF)-BB increased the number of migrated VSMCs, and the number of migrated cells was not significantly different between BubR1<sup>+/+</sup> and BubR1<sup>L/L</sup> VSMCs (Figure 3C).

Next, we compared the proliferative capacities of BubR1<sup>+/+</sup> and BubR1<sup>L/L</sup> VSMCs by comparing growth curves. The proliferation of BubR1<sup>L/L</sup> cells was significantly slower than that of BubR1<sup>+/+</sup> cells in the presence of fetal bovine serum with or without exposure to PDGF-BB (10 ng/ml) at 3, 5, and 7 days after plating (Figure 3D). Immunohistochemical analysis of the ligated carotid arteries, to determine the proliferation capacity, was performed using Ki67-immunohistochemical staining. We found that medial VSMC proliferation was significantly reduced in BubR1<sup>L/L</sup> mice compared with that in BubR1<sup>+/+</sup> mice (Figure IIC in the online-only Data Supplement). Furthermore, siRNA-mediated knockdown of BubR1 impaired VSMC proliferation activity relative to that of BubR1<sup>+/+</sup> VSMCs (Figure III in the online-only Data Supplement), confirming that low BubR1 expression affects cell proliferation.

Taken together, these data indicate that reduced BubR1 expression impairs the capacity of proliferation, but not migration, of VSMCs.
Effects of Low BubR1 Expression on PDGF-BB–Stimulated Cell Cycle Progression

Figure 3 shows the low proliferative rate of VSMCs isolated from BubR1L/L mice. To determine whether BubR1 regulates the cell cycle of VSMCs, we performed cell cycle analysis of PDGF-BB–stimulated VSMCs by measuring the DNA content of cells stained with propidium iodide (Figure 4A). There were no significant differences between BubR1+/+ and BubR1L/L VSMCs in terms of proportions of cells in the G0/G1 and G2/M phases ≤ 24 hours or in the S phase ≤ 12 hours. However, BubR1L/L VSMCs showed delayed entry into the S phase at 18 hours after serum and PDGF-BB stimulation (Figure 4B). These results suggest that low BubR1 expression delays cell cycle progression through the S phase and suppresses PDGF-BB–stimulated VSMC proliferation.

Figure 4. Cell cycle progression analysis. A, Flow cytometric analysis of cell cycle activity. Vascular smooth muscle cells (VSMCs) were incubated in 1% serum for 48 hours and then stimulated with 10% serum and 10 ng/mL platelet-derived growth factor-BB (PDGF-BB) for 24 hours. Then, DNA content was analyzed by flow cytometry. B, VSMCs from BubR1+/+ showed delayed entry into the S phase at 18 hours after serum and PDGF-BB stimulation, with significant difference between BubR1L/L and BubR1+/+ VSMCs. *P < 0.01 vs BubR1+/+; n=4 per group.

Effects of Low BubR1 Expression on p38 Expression in VSMCs

A previous report has shown that a p38 mitogen–activated protein kinase inhibitor, SB203580, inhibits angiotensin II–induced subdermal fat loss, impaired wound healing, and reduced aging-associated phenotypes, such as short lifespan, dwarfism, facial dysmorphism, cataract, sarcopenia, infertility, and impaired neointimal growth in the aortic root of BubR1 L/L mice. To determine whether BubR1 regulates the expression level of p38 in VSMCs, we performed quantitative real-time PCR to determine the relative expression of p38 in the VSMCs and confirmed that its expression in BubR1L/L mice was 44.7% of that in BubR1+/+ mice (Figure 5A). Next, we evaluated the effect of SB203580 on BubR1 expression. The BubR1 expression of SB203580-mediated VSMCs derived from BubR1+/+ mice was reduced to 37.1% of that in control VSMCs. *P < 0.05 vs control (cont) siRNA; n=5 per group. BubR1 knockdown was confirmed by quantitative real-time PCR, which revealed that its expression in siRNA-mediated VSMCs derived from BubR1+/+ mice was reduced to 60.5% of that in control VSMCs. *P < 0.05 vs control (cont) siRNA; n=5 per group.

Figure 5. Effects of low BubR1 expression on p38 expression in vascular smooth muscle cells (VSMCs). A, p38 expression in BubR1+/+ VSMCs was 44.7% of that in BubR1+/+ VSMCs. *P < 0.05 vs BubR1+/+ VSMCs. B, The BubR1 expression of SB203580-mediated VSMCs derived from BubR1+/+ mice was reduced to 37.1% of that in the control. *P < 0.05 vs p38 inhibition (−); n=5 per group. C, p38 expression in siRNA-mediated VSMCs derived from BubR1+/+ mice, which were BubR1 knockdown, was reduced to 60.5% of that in control VSMCs. *P < 0.05 vs control (cont) siRNA; n=5 per group.

BubR1 expression in human aortic smooth muscle cells.14 To determine whether BubR1 regulates the expression level of p38 in VSMCs, we performed quantitative real-time PCR to determine the relative expression of p38 in the VSMCs and confirmed that its expression in BubR1+/+ mice was 44.7% of that in BubR1+/+ mice (Figure 5A). Next, we evaluated the effect of SB203580 on BubR1 expression. The BubR1 expression of SB203580-mediated VSMCs derived from BubR1+/+ mice was reduced to 37.1% of that in control VSMCs by p38 inhibition (Figure 5B). BubR1 knockdown was confirmed by quantitative real-time PCR, which revealed that its expression in siRNA-mediated knockdown VSMCs was 31.6% of that in scrambled control siRNA–mediated VSMCs (data not shown). siRNA-mediated knockdown of BubR1 had an inhibitory influence on the expression of p38 (Figure 5C).

Taken together, these data indicate that BubR1 and p38 mutually affect each other, which has an influence on the impaired proliferation of VSMCs in BubR1L/L mice.

Discussion

Several lines of evidence suggest that low BubR1 expression is implicated in various disorders, especially progeroid- and aging-associated phenotypes, such as short lifespan, dwarfism, facial dysmorphism, cataract, sarcopenia, infertility, subdermal fat loss, impaired wound healing, and reduced
dermal thickness. In terms of vascular effects, it is previously reported that BubR1 hypomorphic mice display the following phenotypes: reduced arterial wall thickness, narrower inner diameter, low numbers of medial VSMCs, profound arterial wall fibrosis, reduced artery elasticity, reduced endothelium–dependent and endothelium–independent relaxation in response to nitric oxide, and reduced arterial compliance. Nevertheless, the precise role of BubR1 in vascular disorders is unknown. Until now, no reports have described the role of BubR1 in vascular remodeling or the involvement of migration or proliferation of VSMCs in this process. In the present study, we determined the role of the cell cycle–related protein BubR1 in the vascular response to blood flow cessation in BubR1+/− mice generated in our laboratory. We found that reduced BubR1 expression inhibited intimal hyperplasia after carotid artery ligation in a process that involved reduced proliferation of VSMCs through delayed cell cycle progression and impaired PDGF-BB–stimulated proliferation, without changes in VSMC migration.

BubR1 hypomorphic mice showed early aging–associated vascular disorders, including a reduced number of smooth muscle cells, reduced elasticity, impaired endothelial–dependent relaxation, and increased superoxide anion production. However, it was difficult to evaluate smooth muscle proliferation capacity in vivo because the number of smooth muscle cells in the vascular media was initially low in the BubR1 hypomorphic mice. Therefore, we generated mice with BubR1 expression that was ≈20% of that in BubR1+/+ mice, without major abnormalities, such as progeria, infertility, and shortened lifespan during development and growth, allowing us to investigate the role of BubR1 in vascular biology. Because the number of smooth muscle cells in the carotid artery media was not significantly different between BubR1−/− and BubR1+/− mice, we investigated the effects of low BubR1 expression on arterial neointimal hyperplasia.

Many studies have examined the responses of the arterial wall to a variety of stressors, including injury or blood flow cessation, and many growth factors have been implicated in neointimal hyperplasia. The proliferation and migration of VSMCs are thought to be essential components of neointimal hyperplasia in atherosclerosis and after mechanical arterial injury, for example, after angioplasty. PDGF plays a primary role in inducing VSMC proliferation and migration in vivo. In the present study, intimal hyperplasia was almost completely inhibited in BubR1−/− mice after carotid artery ligation (Figure 2B). The analysis for time-dependent change of BubR1 expression in ligated common carotid arteries showed that BubR1 expression was transiently elevated after ligation in both genotypes, which suggested that induction of BubR1 might contribute to neointimal hyperplasia (Figure 2A).

Studies report that bone marrow–derived vascular smooth muscle progenitor cells are mobilized into the circulation from the bone marrow, whereby they home to sites of injury and differentiate into vascular smooth muscle–like cells, thereby contributing to neointimal hyperplasia. In the present study, bone marrow transplantation did not affect neointimal hyperplasia after carotid ligation, which indicates that the recipient phenotype was the primary determinant of the degree of neointimal hyperplasia. In BubR1+/− mice, the growth of splenic T cells, in which BubR1 expression was reduced to ≈40% of that in BubR1+/+ mice, was not significantly different from that of T cells from BubR1−/− mice (data not shown). The reduced influence on hematopoietic lineages may be because of the higher expression level in T cells (40% in T cells versus 20% in VSMCs). BubR1 plays an important role in cell cycle progression by means of a threshold mechanism.

VSMCs within the medial layer of arteries are normally in a quiescent state. However, in response to vascular stress, such as angioplasty, a subpopulation of medial VSMCs migrate to the neointima where they proliferate, ultimately resulting in neointimal hyperplasia. Growth factors, such as PDGF, that aggregate at the site of injury, are thought to be involved in this process. Therefore, we examined the role of BubR1 in PDGF-BB–stimulated cellular activities in VSMCs in vitro. However, we found that PDGF-BB–induced migration of VSMCs was not defective in BubR1−/− mice (Figure 3C), and PDGF-BB–stimulated VSMC proliferation was significantly reduced in BubR1−/− VSMCs (Figure 3D), which was consistent with our in vivo findings (Figure 2B). Thus, low BubR1 expression is responsible for a slower growth of VSMCs but does not significantly affect VSMC migration.

Cell proliferation is tightly controlled by a series of regulators that act at sequential points throughout the cell cycle. The G1→S phase transition is controlled by cyclin-dependent kinase complexes and is promoted by phosphorylation of the retinoblastoma protein. Ligand–induced activation of p38 mitogen–activated protein kinase is an upstream mediator of BubR1 activation in VSMCs. In this study, we found that reduced BubR1 expression also has an inhibitory influence on the transcription of the cyclin–dependent kinase inhibitor p16. Tissues in BubR1 hypomorphic mice, including skeletal muscle, adipose, and eye, are reported to have much higher levels of p16 compared with those in BubR1+/+ mice, but there are no significant differences in p16 expression levels in other tissues, including the aorta. Similar to this previous report, we found no significant difference in p16 mRNA expression between VSMCs derived from out BubR1+/− mice and BubR1−/− mice (data not shown). So, it seems that other mechanisms are concerned in the delayed VSMC proliferation of BubR1+/− mice.

We previously reported that the p38 mitogen–activated protein kinase inhibitor, SB203580, inhibits angiotensin II–induced BubR1 expression in human aortic smooth muscle cells. These findings support the hypothesis that p38 mitogen–activated protein kinase is an upstream mediator of BubR1 activation in VSMCs. In this study, we found that reduced BubR1 expression also has an inhibitory influence on p38 expression in VSMCs (Figure 5A). Furthermore, siRNA-mediated BubR1 knockdown repressed p38 expression (Figure 5C). This result indicates that impaired BubR1 expression is associated with decreased expression of p38. We also evaluated the influence of p38 inhibition on VSMCs. Exposure to SB203580 resulted in lower expression of BubR1 in VSMCs derived from BubR1−/− mice. Taken together, BubR1 and p38 mutually affected each other, which may be one of the reasons for the impaired proliferation of VSMCs in BubR1−/− mice compared with BubR1−/− mice. In another report, the interleukin-8 receptor B antagonist, SB225002, is shown to induce the phosphorylation of BubR1 in a
Conclusions

We generated BubR1L/L mice in which BubR1 expression was ≈20% of that in BubR1+/+ mice. The BubR1L/L mice did not display significant abnormalities of the vascular structures, including progeria, infertility, or shortened lifespan during growth and development. We, therefore, used these mice to examine the role of BubR1 in vascular biology. Low BubR1 expression almost completely inhibited intimal hyperplasia after carotid ligation by suppressing the proliferation of VSMCs, which was caused, in part, by delayed cell cycle progression. VSMCs derived from BubR1L/L mice might impair the proliferative capacity through the inhibition of p38 expression. These findings suggest that BubR1 may represent a potential target molecule for treating pathological vascular remodeling, including restenosis after angioplasty.

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Disclosures

None.

References

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Supplementary Figure I.

A

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B

BubR1^+/+

BubR1^+/+

BubR1^L/L

BubR1^L/L

C

body weight (g) vs. weeks after birth

- BubR1^+/+
- BubR1^L/L

40 weeks

BubR1^L/L 26.4g

BubR1^+/+ 30.9g
Supplementary Figure II.

A

HE

α-SMA (VSMC)

B

F4/80 (macrophage)

C

BubR1^{+/+}

BubR1^{L/L}

Ki67

Media

*P < 0.001
Supplementary Figure III.

Cell number (cells)

Control (n=2)
Control siRNA (n=2)
BubR1 siRNA (n=2)

Culture duration (hrs)
**Supplementary Materials and Methods**

**Immunohistochemical Analysis**

Standard immunohistochemical techniques were used with anti-αSMA (1:200; Millipore, Billerica, MA, USA), anti-F4/80 (1:50; AbD Serotec, Kidlington, Oxford, UK) and anti-Ki67 (1:25; DAKO, Produktionsvej, Denmark) antibodies and a biotinylated secondary antibody on perfusion-fixed, paraffin-embedded tissues. Streptavidin-biotin complex and horseradish peroxidase were applied, and the reaction products were visualized using diaminobenzidine.

**RNA Interference and Effects on Cell Proliferation**

*BubR1* gene silencing was performed by transfecting VSMCs using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. VSMCs were transfected with an siRNA targeting *BubR1* (sc-37543; Santa Cruz Biotechnology, Dallas, Texas, USA) or scrambled control (sc-37007; Santa Cruz Biotechnology) at a final concentration of 10 nmol/L in transfection reagent (dilution, 1:500; vol/vol). BubR1 knockdown was confirmed by quantitative real-time PCR, which revealed that its expression in VSMCs with siRNA-mediated knockdown of *BubR1* was 31.6% of that in the VSMCs siRNA-mediated scrambled control.

For proliferation studies, VSMCs were initially plated on six-well gelatin-coated plates at a density of 10,000 cells per well. Cells were incubated overnight in DMEM containing 10% FBS and 10 ng/mL PDGF-BB. VSMCs obtained from *BubR1*+/+ mice were transfected with *BubR1* siRNA, washed, and then incubated in DMEM containing 10% FBS with 10 ng/mL PDGF-BB. Cells were counted after incubation for 4, 7, 11, and 14 days. Additional batches of VSMCs were transfected with scrambled control (control siRNA) or without siRNA as controls.
Supplementary Table Legend
Complete blood counts of $BubR1^{+/+}$ and $BubR1^{L/L}$ mice following bone marrow transplantation. There were no significant differences in blood counts among any of the groups.

Supplementary Figure Legend

Supplementary Figure I. Morphological differences of the left common carotid artery before ligation in $BubR1^{+/+}$ and $BubR1^{L/L}$ mice. A, There were no differences in common carotid arterial size (vascular area), containing cell numbers in the arterial wall (cell number) and cell density in either genotype. N.S. indicates no significant difference. $n = 4$ per group. B, Hematoxylin/eosin-stained images of the common carotid arteries. Low- and high-power views of the left common carotid arteries from representative mice showed no intimal thickening. Scale bar = 200 $\mu$m (left panels) and 800 $\mu$m (right panels). C, Comparison of body weight of female $BubR1^{+/+}$ and $BubR1^{L/L}$ mice. Images of representative 40-week-old female mice from the indicated genotypes are shown.

Supplementary Figure II. Immunohistochemical analysis of the ligated left common carotid artery. A, Representative immunohistochemical analysis of the VSMC marker $\alpha$-smooth muscle actin ($\alpha$SMA), in the ligated artery of a $BubR1^{+/+}$ mouse. HE refers to hematoxylin/eosin staining. Scale bar = 100 $\mu$m. B, Representative immunohistochemical analysis of F4/80, a macrophage marker, in a ligated artery of a $BubR1^{+/+}$ mouse. Scale bar = 100 $\mu$m. C, Immunohistochemical analysis of the proliferative marker Ki67, in the ligated arteries of $BubR1^{+/+}$ and $BubR1^{L/L}$ mouse. Low- and high-power views of the ligated left common carotid arteries from representative mice. Scale bar = 100 $\mu$m (left panels) and 400 $\mu$m (right panels). The number of Ki67-positive cells divided by the number of total cells in the media was significantly suppressed in $BubR1^{L/L}$ mice ($n = 7$) compared with $BubR1^{+/+}$ mice ($n = 9$). $*P < 0.001$ vs. $BubR1^{+/+}$ mice.

Supplementary Figure III. Proliferation activity of VSMCs transfected with siRNA. Knockdown of $BubR1$ mRNA with siRNA reduced VSMC proliferative capacity relative to VSMCs transfected with control siRNA or control VSMCs. $n = 2$ per group.
Materials and Methods

Experimental Animals
Low-BubR1-expressing mutant mice (BubR1<sup>L/L</sup>) were newly generated in our laboratory as described below (Generation of BubR1<sup>L/L</sup> mice). Male BubR1<sup>L/L</sup> mice (6–10 weeks old) and age-matched wild-type littermates (BubR1<sup>+/+</sup>) raised on a mixed 129 and C57BL/6 background were used in all experiments. The mice were fed a regular pelleted diet and were housed in a room with a 12:12 h light–dark cycle. Experimental protocols and housing facilities were approved by the Animal Experimentation Committee of Kyushu University Health Science Center.

Generation of BubR1<sup>L/L</sup> mice
A λ phage clone, which included the genomic functional domain area of BubR1, was isolated from a λTK library, as previously described. The λTK-BubR1 genome (14.8 kbp) was then isolated. A pMC1neo cassette was inserted in the XbaI site in intron 5 of the murine BubR1 gene with reverse orientation as previously described. By reversing the orientation of the cassette, a cryptic splice acceptor and a donor site were introduced, which led to production of mutant BubR1 pre-mRNA. This pre-mRNA is spliced abnormally, creating BubR1 mRNA that cannot generate BubR1 protein, thereby reducing cellular levels of BubR1. We electroporated the targeting vector into embryonic stem cells and screened drug-resistant clones for homologous recombination by Southern blotting. Targeted embryonic stem cell clones were isolated and incorporation of the pMC1neo cassette was verified. These clones were amplified and used to generate chimeric mice. F1 heterozygote mice derived from each clone were intercrossed to produce homozygous mutant offspring (termed BubR1<sup>L/L</sup>), as well as BubR1<sup>+/+</sup> and BubR1<sup>+/L</sup> mice. BubR1<sup>L/L</sup> mice were used on hybrid backcross between C57BL/6 and 129. Using reverse-transcription–polymerase chain reaction (PCR), we confirmed that the expected wild-type and mutant BubR1 mRNAs were produced by BubR1<sup>L/L</sup> cells. Mouse genotypes were routinely determined by PCR.

Genotyping
Pieces of mouse tail were digested for 30 min at 95°C with 0.05 M NaOH 500 µl. Tris-EDTA buffer (1 M Tris-HCl [pH 8.0], 10 mM EDTA) was added and the mixture was vortexed. After centrifugation (20000 × g, 5 min), the supernatant containing genomic DNA was used for genotyping and amplified with the following primers: forward primer 1, 5'-CCTTCCCGCTTCAGTGACAAC-3'; reverse primer 2, 5'-AGAATCCGCGATCTACC-3'; and forward primer 3, 5'-AGTTCTGGCCCTCAGGAAACC-3'. The amplification protocol involved initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were fractionated by electrophoresis on 1%
agarose gels and detected by ethidium bromide staining. The PCR products from $BubR1^{+/+}$ and $BubR1^{-/-}$ mice were 475 and 938 bp, respectively.

**Mouse Carotid Artery Ligation Model**
Ten-week-old male $BubR1^{+/+}$ and $BubR1^{-/-}$ mice underwent complete carotid artery ligation, as previously described. After mice were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital, the left common carotid artery was exposed through a small midline incision in the neck. The artery was completely ligated with a 6-0 silk suture just proximal to the carotid bifurcation to disrupt blood flow. A sham procedure was also performed in which $BubR1^{+/+}$ and $BubR1^{-/-}$ mice underwent surgery without artery ligation. The animals were allowed to recover for 4 weeks before further experimental procedures. All animals recovered completely and showed no neurological deficits.

**Time-dependent Change of BubR1 Expression in the Ligated Common Carotid Artery**
Ten-week-old male $BubR1^{+/+}$ and $BubR1^{-/-}$ mice underwent complete carotid artery ligation, as described above. The ligated left common carotid arteries were harvested 3 days, 1 week, 2 weeks and 4 weeks after ligation. Preoperative carotid arteries were also harvested. Total RNA from the harvested arterial tissues was extracted and reverse-transcribed to cDNA as described below (RNA Extraction and Real-Time PCR). Quantitative real-time PCR was performed to determine the expression levels of BubR1 as described below (RNA Extraction and Real-Time PCR).

**Morphometric Analysis**
Four weeks after carotid artery ligation, the animals were euthanized by an intraperitoneal injection of pentobarbital. The left ventricle was cannulated and perfused with phosphate-buffered saline, and then perfused and fixed with 10% phosphate-buffered formalin for 5 min. The left carotid artery was then removed. After incubation in 10% phosphate-buffered formalin overnight, the carotid arteries were excised and embedded in paraffin. Cross sections (5-µm thick) were taken starting at the carotid ligation site and processed for hematoxylin/eosin staining. Cross sections taken 1.5 mm proximal to the ligation site were obtained from each mouse. Four different regions (lumen, intima, media and total vascular area) of hematoxylin/eosin-stained cross-sections were analyzed using ImageJ software (NIH), as previously described. The areas surrounded by the luminal surface, internal elastic lamina, and external elastic lamina were then calculated. The intimal area was determined by subtracting the luminal area from the area defined by the internal elastic lamina, and the medial area was calculated by subtracting the area defined by the internal elastic lamina from the area defined by the external elastic lamina.
Irradiation and Bone Marrow Transplantation

Six-week-old male $BubR1^{+/L}$ and $BubR1^{+/+}$ mice underwent lethal irradiation (10 Gy) and were injected in the tail vein with $2 \times 10^6$ freshly prepared sterile bone marrow cells obtained from $BubR1^{+/L}$ and $BubR1^{+/+}$ mice, as previously described. Four weeks after bone marrow transplantation, the left carotid arteries of the mice were ligated, as described above (Mouse Carotid Artery Ligation Model). The mice were sacrificed 4 weeks later and the carotid arteries were obtained and processed, as described above (Morphometric Analysis).

Cell Culture

Mouse vascular smooth muscle cells (VSMCs) were isolated by enzymatic dispersion as previously described. Briefly, VSMCs were isolated from the aortas of 8-week-old male $BubR1^{+/L}$ and $BubR1^{+/+}$ mice. The aortas were excised, washed in phosphate-buffered saline containing heparin, and incubated for 45 min in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 2 mg/mL collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ). Under microscopic guidance, the adventitia was removed with fine forceps and the vessels were longitudinally incised. The blood vessel was cut into 2–3-mm square pieces. The tissue pieces were placed inside the aorta on a 60-mm gelatin-coated dish (IWAKI, Tokyo, Japan). Next, 10% fetal bovine serum (FBS) in DMEM was gently added and the cells were placed in an incubator, undisturbed for approximately 10 days. VSMCs were used at passage 4–8. Cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin, in a humidified incubator (95% air and 5% CO$_2$) at 37°C.

RNA Extraction and Real-Time PCR

Except for arterial tissue, total RNA was extracted using ISOGEN reagent (NIPPON GENE, Tokyo, Japan) according to the manufacturer’s protocol. For arterial tissue (aorta, carotid artery and ligated carotid artery), total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Venlo, the Netherlands) according to the manufacturer’s protocol. Total RNA was reverse-transcribed to cDNA using SuperScript III First-strand SuperMix (Invitrogen). For quantitative real-time PCR, 20 µL of amplification mixtures (LightCycler® 480 SYBR Green I Master; Roche, Penzberg, Upper Bavaria, Germany) were prepared, containing cDNA and primers. Reactions were run on a LightCycler DX400 (Roche). Each sample was analyzed in triplicate and normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The PCR protocol involved initial denaturation at 95°C for 5 min; 45 cycles of 95°C for 10 s, 60°C for 20 s (p38; 57°C), and 72°C for 10 s. We used the following forward and reverse primers: $BubR1$, 5’-CAGTCCCAGCACAGACAGTTCCA-3’ and 5’-GCTAGCGAGCTTCTCTGTTCA-3’; $GAPDH$, 5’-TCTGGAAAGCTGTGGCG-3’ and 5’-CCACGACGGACACATTG-3’; and $p38$, 5’-AACAGGATGCAAGCCATGA-3’ and
5′-GGGTCGTGGTACTGAGCAAA-3′. The PCR products were validated by electrophoresis on 2% agarose. The BubR1 forward primer annealed at the junction between exon 5 and exon 6 but did not anneal to cDNA derived from BubR1<sup>−/−</sup> mice (Figure 1C).

**Cell Proliferation Assay**
For proliferation measurements, VSMCs were initially plated in triplicate at a density of 10,000 cells per well on six-well gelatin-coated trays (IWAKI), as previously described. Cells were cultured in DMEM containing 10% FBS with or without 10 ng/mL of platelet-derived growth factor (PDGF)-BB (R&D System, Minneapolis, MN, USA), and were harvested after 3, 5, and 7 days of culture. Cell number was counted in triplicate for cells in each well using a Beckman Coulter automated cell counter (Beckman Coulter, Brea, CA, USA).

**Transwell Migration Assay**
VSMC invasion was evaluated using 6.5-mm-diameter Transwell plates with polycarbonate membrane filters containing 8-µm-diameter pores (Corning, New York, NY, USA). The lower chamber contained PDGF-BB (1 or 10 ng/mL) as a chemoattractant. After 12 h, the cells on the upper surface were removed by gentle abrasion with a cotton bud, and the cells on the lower surface were fixed with methanol and stained with hematoxylin/eosin staining. Migrated cells were quantified as the mean of four randomly chosen high-power fields of three independent experiments performed in duplicate.

**Cell Cycle Assay**
VSMCs synchronized by exposure to 1% serum for 48 h were treated with 10% FBS and 10 ng/mL PDGF-BB for 6, 12, 18, or 24 h. Cells were harvested, fixed and stained with propidium iodide (Sigma–Aldrich, St. Louis, MO, USA). DNA content was analyzed by flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ), as previously described.

**p38 Inhibition Assay**
VSMCs synchronized by exposure to 1% FBS for 24 h were pre-incubated with or without p38 mitogen-activated protein kinase inhibitor SB203580 (10 µM) for 30 min. Then VSMCs were treated with 10% FBS and 10 ng/mL PDGF-BB for 60 min and collected. Total RNA was extracted and reverse-transcribed to cDNA as described previously. BubR1 and p38 expression were evaluated with quantitative real-time PCR.

**siRNA-Mediated Knockdown of BubR1**
BubR1 gene silencing was performed by transfecting VSMCs derived from BubR1<sup>−/−</sup> mice using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. VSMCs were transfected with an siRNA targeting
BubR1 (sc-37543; Santa Cruz Biotechnology, Dallas, TX, USA) or scrambled control (sc-37007; Santa Cruz Biotechnology) at a final concentration of 10 nmol/L in transfection reagent (dilution, 1:500; vol/vol). Forty-eight hours after siRNA transfection, siRNA-mediated VSMCs were treated with 10% FBS and 10 ng/mL PDGF-BB for 24 h and collected. Total RNA was extracted and reverse-transcribed to cDNA as described previously. BubR1 and p38 expression were evaluated with quantitative real-time PCR.

Statistical Analysis
All data are expressed as the mean ± standard error of the mean and were statistically analyzed using one-way analysis of variance. The statistical significance of differences between groups was determined using Dunnett’s post hoc test. Values of P < 0.05 were considered statistically significant.

References
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