ORIGINAL RESEARCH

BCL11B Regulates Arterial Stiffness and Related Target Organ Damage

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RATIONALE: BCL11B (B-cell leukemia 11b) is a transcription factor known as an essential regulator of T lymphocytes and neuronal development during embryogenesis. A genome-wide association study showed that a gene desert region downstream of BCL11B, known to function as a BCL11B enhancer, harbors single nucleotide polymorphisms associated with increased arterial stiffness. However, a role for BCL11B in the adult cardiovascular system is unknown.

OBJECTIVE: Based on these human findings, we sought to examine the relation between BCL11B and arterial function.

METHODS AND RESULTS: Here we report that BCL11B is expressed in the vascular smooth muscle where it regulates vascular stiffness. RNA sequencing of aortas from wild-type and Bcl11b null mice (BSMKO) identified the cGMP (cyclic guanosine monophosphate)-cGMP-dependent protein kinase G (PKG) as the most significant differentially regulated signaling pathway in BSMKO compared with wild-type mice. BSMKO aortas showed decreased levels of PKG1, increased levels of Ca++-calmodulin-dependent serine/threonine phosphatase calcineurin (PP2B) and decreased levels of their common phosphorylation target, phosphorylated vasodilator-stimulated phosphoprotein (pVASPS239), a regulator of cytoskeletal actin rearrangements. Decreased pVASPS239 in BSMKO aortas was associated with increased actin polymerization (filamentous/globular actin ratio). Functionally, aortic force, stress, wall tension, and stiffness, measured ex vivo in organ baths, were increased in BSMKO aortas, and BSMKO mice had increased pulse wave velocity, the in vivo index of arterial stiffness. Despite having no effect on blood pressure or microalbuminuria, increased arterial stiffness in BSMKO mice was associated with increased incidence of cerebral microbleeds compared with age-matched wild-type littermates.

CONCLUSIONS: We have identified vascular smooth muscle BCL11B as a crucial regulator of aortic smooth muscle function and a potential therapeutic target for vascular stiffness.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: actins □ blood pressure □ calcineurin □ phosphorylation □ vascular smooth muscle □ vascular stiffness

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Novelty and Significance

What Is Known?

- Arterial stiffness is a strong, independent risk factor for cardiovascular disease; however, genetic and molecular determinants of arterial stiffness are not fully understood, hampering the discovery of potential therapeutic targets to prevent cardiovascular disease.
- Single nucleotide polymorphisms in a genetic locus on chromosome 14, upstream of the gene BCL11B (B-cell leukemia 11b), have been shown to be significantly associated with increased pulse wave velocity, the gold standard measure of aortic wall stiffness ($P<5.6 \times 10^{-11}$ for the top single nucleotide polymorphism, rs1381289C>T).

What New Information Does This Article Contribute?

- We identified BCL11B in the vascular smooth muscle (VSM) as a novel and crucial regulator of vascular stiffness.
- VSM BCL11B contributes to maintaining aortic compliance via the cGMP (cyclic guanosine monophosphate)/protein kinase G (PKG)/pVASP S239 (phosphorylated vasodilator-stimulated phosphoprotein) signaling pathway, whose impairment, in absence of Bcl11b, leads to increased nonmuscle actin polymerization in VSM cells and increased VSM stiffness.
- Increased aortic stiffness is associated with increased cerebral microbleeds, underscoring the pivotal role of large elastic arteries in preventing microvascular damage and associated target organ damage.

Nonstandard Abbreviations and Acronyms

| Acronym | Description |
|---------|-------------|
| BCL11B  | B-cell leukemia 11b |
| BSMKO   | tamoxifen-inducible VSM-specific Bcl11b null mice |
| CAIP    | calcineurin autoinhibitory peptide |
| CTIP2   | COUP-TF interacting protein-2 |
| CVD     | cardiovascular disease |
| GC      | guanylate cyclase |
| HFHS    | high-fat, high-sucrose |
| ND      | normal diet |
| PKA     | cAMP-dependent protein kinase |
| PKG1    | PKG, isoform 1 |
| PP2B    | Ca2+/calmodulin-dependent serine-threonine phosphatase calcineurin |
| pVASP   | phosphorylated vasodilator-stimulated phosphoprotein |
| PWV     | pulse wave velocity |
| SNPs    | single nucleotide polymorphisms |
| VASP    | vasodilator-stimulated phosphoprotein |

VRK1  vaccinia-related kinase 1
VSM  vascular smooth muscle
WT  wild-type

Single nucleotide polymorphisms in a genetic locus on chromosome 14, known to function as an enhancer for the transcription factor BCL11B, are significantly associated with increased pulse wave velocity, the gold standard measure of aortic wall stiffness. Here we report, for the first time, that BCL11B, previously known uniquely for its role in T cell and neuronal lineage commitment, is present in VSM where it regulates vascular stiffness. Specifically, BCL11B modulates VSM cytoskeletal actin polymerization via the cGMP/PKG/pVASP S239 signaling pathway thereby regulating aortic stiffness. Notably, increased arterial stiffness in mice lacking VSM Bcl11b as well as in obese mice, a mouse model of arterial stiffness we previously described, is associated with increased incidence of cerebral microbleeds, suggesting a crucial role of large elastic arteries in preventing microvascular damage in downstream organs. Interestingly, despite profound effects on the aorta, BCL11B has a dispensable role in VSM contraction in resistance arteries. The present study strongly supports BCL11B or its downstream-regulated pathways as potential therapeutic targets against arterial stiffness and related target organ damage, to prevent overt cardiovascular disease, which remains a major cause of morbidity and mortality worldwide.
in this locus may be causally linked to an increased risk of subsequently developing major CVD events.

The aortic stiffness locus spans 2 Mb between coding genes B-cell leukemia 11b (**BCL11B**) and vaccinia-related kinase 1 (**VRK1**). By performing a detailed analysis of this locus, we found an ≈1.9 kb sequence, located ≈850 kb downstream (3′) of **BCL11B**, known to function as an enhancer for **BCL11B**, but not **VRK1**. Enhancers are DNA regulatory elements that activate the expression of target genes independently of distance or orientation. In the present study, we sought to elucidate the mechanistic basis of the association of the chromosome 14 locus with aortic stiffness and a possible cause-effect relation between **BCL11B** and vascular function.

**BCL11B**, also known as CTIP2 (COUP-TF [chicken ovalbumin upstream promoter transcription factor] interacting protein-2), is a transcription factor best known for its critical role in T cells and innate lymphoid cells, as well as neuronal development during embryogenesis; however, a role of **BCL11B** in the cardiovascular system has never been described. Here we report for the first time that **BCL11B** is expressed in the vascular smooth muscle (VSM) where it regulates vascular stiffness by increasing nonmuscle actin polymerization in VSM cells via the cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)/phosphorylated vasodilator-stimulated phosphoprotein (pVASP) signaling pathway. Notably, despite **Bcl11b** deletion having profound effects on the aorta, contractile properties of resistance vessels, blood pressure, and microalbuminuria in tamoxifen-inducible VSM-specific **Bcl11b** null mice (BSMKO) remained comparable with wild-type (WT) mice. In contrast, we found that increased aortic stiffness in BSMKO was correlated with enhanced cerebral microbleeds compared withagematched WT mice. Taken together, our study identifies **BCL11B** as a novel and crucial regulator of VSM cytoskeletal assembly in large arteries affecting aortic wall stiffness and suggests that **BCL11B** or its downstream signaling targets are promising candidates for the translational development of therapies against arterial stiffness and related target organ microcirculatory damage.

**METHODS**

**Data Availability**

A detailed description of materials and methods can be found in the Data Supplement. All data and supporting material are available upon request.

**RESULTS**

**BCL11B Is Expressed in VSM and Is Down-Regulated in Animal Models of Arterial Stiffness**

A detailed analysis of the genetic locus on chromosome 14 with genome-wide association with elevated PWV revealed a highly conserved 550 bp sequence (>95% homology among species) within a **BCL11B** enhancer, in proximity of the highest significant SNP variant associated with increased PWV (rs1381289>G>T), which has recently been shown to correlate with **BCL11B** expression. By using Basic Local Alignment Search Tool (BLAST), we identified this highly conserved 550 bp sequence in a locus named Al060616 (National Center for Biotechnology Information [NCBI] nomenclature) and amplified one specific polymerase chain reaction (PCR) band of expected 356 bp molecular size, confirmed by sequencing, in aortic extracts (Figure 1A).

We next sought to determine whether **BCL11B**, a downstream target of this genomic locus, is present in the vasculature. By using double mutant mice expressing a red fluorescent protein (mTomato) upon removal of **Bcl11b** after tamoxifen administration (ER-Cre-**Bcl11b**<sup>fl/fl</sup>-mTomato mice), we were able to indirectly visualize **Bcl11b**'s localization in aortic sections and, specifically, in the tunica media (Figure 1B). These findings were confirmed by immunostaining human aortic smooth muscle cells with an antibody specific to **BCL11B** (Figure 1C), Western blotting of murine aortas (Figure 1D), and performing qRT-PCR of human aortas (Figure 1E). In addition to the aorta, **Bcl11b** was visualized in VSM of arteries and arterioles in heart, lung, and kidney of tamoxifen-treated **ER-Cre-Bcl11b**<sup>fl/fl</sup>-mTomato mice (Figure 1 in the Data Supplement). Last, a pG3 luciferase reporter construct containing **Bcl11b** promoter region Chr12: 108004359-108003144 (GRCm38.p6), demonstrated that VSM cells are transcriptionally competent to sustain **Bcl11b** expression compared with an empty (control) plasmid (Figure 1F).

To determine whether **Bcl11b** expression in the vasculature was linked to arterial function, we measured **Bcl11b** in aortas of high-fat, high-sucrose (HFHS)-fed obese mice, a model of arterial stiffness we previously described. We found that **Bcl11b** mRNA and protein levels were significantly decreased in aortas of HFHS-fed mice, with normal diet (ND)-fed mice (Figure 2A and 2B, quantitation in the Data Supplement). To further elucidate a functional role of **Bcl11b** in vivo, we measured PWV in mice with an inducible global **Bcl11b** knockout (**ER-Cre-Bcl11b**<sup>fl/fl</sup> treated with tamoxifen). We found that PWV was significantly increased in 10-month-old **Bcl11b** null mice compared with WT littermate controls (area under the curve: 260.3±4.7 m/s/mm Hg in WT versus 285.6±2.8 m/s/mm Hg in **Bcl11b**<sup>−/−</sup>; P=6.0×10<sup>−4</sup>; over a range of mean arterial pressures, Figure 2C). Notably, PWV in **Bcl11b**<sup>−/−</sup> mice increased to a similar extent as HFHS-fed obese mice (shadowed box in Figure 2C corresponding to PWV values in HFHS-fed mice, in a similar mean arterial pressure range, adapted from our previous publication). Taken together, our novel findings demonstrate that **BCL11B** is present in the VSM of the aortic wall, and that aortic **BCL11B** downregulation may increase aortic stiffness.
Based on our novel observation that BCL11B is expressed in VSM and its decreased aortic levels are associated with increased arterial stiffness, we generated mice with tamoxifen-inducible Bcl11b deletion in VSM (BSMKO; Figure II in the Data Supplement). Bcl11b deletion in VSM did not affect gross aortic morphology, as indicated by comparable aortic media thickness (54.5±1.0 μm in WT, n=5 versus 55.1±1.1 μm in BSMKO, n=7; P=6.9×10⁻¹) and diameters (unloaded dimensions: 0.74±0.02 mm in WT, n=5 versus 0.75±0.01 mm in BSMKO, n=7; P=8.1×10⁻¹; and Figure III in the Data Supplement for in vivo measurements) between WT and BSMKO mice. However, force, wall tension, and stress, generated by BSMKO aortic rings (n=7) in organ baths were significantly increased compared with WT (n=5; Figure 3A–3D). Likewise, aortic stiffness measured by PWV in vivo or derived from ex vivo elastic modulus with the Moens-Korteweg equation, was significantly increased in BSMKO mice (n=17) 2 months after VSM Bcl11b removal, compared with WT littermate.
controls (n=14; Figure 3E and 3F). Interestingly, aortic KCl- and phenylephrine-induced stress were not significantly affected by Bcl11b deletion (Figure 3G and 3H) indicating that Bcl11b regulates VSM force generation but not via KCl- and phenylephrine-stimulated pathways.

Bcl11b deletion in VSM did not significantly affect blood pressure or heart rate, measured by radiotelemetry in 4- or 15-month old, conscious mice over 8 consecutive days, compared with age-matched WT littermates (Table I in the Data Supplement). Aging comparably increased systolic and mean arterial pressures in both groups (Table I in the Data Supplement).

**pVASP** is Down-Regulated in Aortas of BSMKO Mice

We used RNA sequencing to identify molecular mechanisms that may contribute to increased VSM tone and stiffness in BSMKO aortas. Four hundred fifty-eight and 329 genes were differentially up- and down-regulated, respectively, in aortic mRNA extracts of BSMKO (n=5) compared with WT (n=5) mice (Figure 4A and 4B and Figure IV in the Data Supplement, in which the heatmap illustrates individual levels of expression for replicate mice for each of the top 40 differentially expressed genes; \( P = 2.7 \times 10^{-7} \) to \( 3.4 \times 10^{-4} \)). Pathway enrichment analysis (Database for Annotation, Visualization and Integrated Discovery [DAVID]) on differentially expressed genes revealed that cGMP-PKG signaling pathway was the most significantly down-regulated pathway in BSMKO compared with WT aortas (FDR; \( q = 9.4 \times 10^{-3} \); Figure 4C and Figures V and VI in the Data Supplement for a list of differentially regulated pathways as well as individual levels of expression for each gene within the cGMP-PKG pathway). We further validated RNA sequencing findings by analyzing levels of GC (guanylate cyclase) and cGMP-dependent PKG1 (PKG, isoform 1), the enzymes directly upstream and downstream of cGMP. We found that GC catalytic subunit isoforms (\( \alpha_1, \alpha_2, \beta_1 \)) mRNA (Figure 4D) and PKG1 protein levels (Figure 4E, quantitation in graph) were significantly down-regulated in VSM cells isolated from BSMKO aortas compared with WT. Similarly, downregulating Bcl11b with a validated siRNA, decreased PKG1 expression in VSM cells (Figure 4F, quantitation in graph).

As VASP (vasodilator-stimulated phosphoprotein) is a PKG1 phosphorylation target in VSM, we examined whether VASP phosphorylation was affected by Bcl11b deletion in VSM. Specifically, we analyzed VASP phosphorylation at serine 239, since we previously showed that VASP phosphorylation at this residue inversely correlates...
with arterial stiffness. Levels of pVASP\textsuperscript{S239} were dramatically decreased in BSMKO VSM cells compared with WT cells, cultured with or without fetal bovine serum (FBS) (Figure 5A; quantitation in graph), and in BSMKO aortas compared with WT controls (Figure 5B; quantitation in graph), while total VASP remained unchanged. Similar findings were obtained in aortas of HFHS-fed obese mice (ie, with decreased aortic Bcl11b, Figure 2B), compared with ND-fed mice (Figure 5C; quantitation in graph). Our findings of decreased pVASP\textsuperscript{S239} were corroborated in males (n=4) and females (n=4) of a second animal model in which constitutive Bcl11b removal in VSM was achieved with a Sm22\alpha (transgelin) promoter-driven Cre recombinase transgene (Figure VIIA in the Data Supplement). Last, no statistically significant differences were observed in tamoxifen- (n=3) compared with vehicle (oil)-treated Bcl11b\textsuperscript{fl/fl} mice (n=3), excluding the possibility that tamoxifen administration per se may have decreased pVASP\textsuperscript{S239} levels in the aorta (Figure VIIB in the Data Supplement).

In contrast to the aorta, pVASP\textsuperscript{S239} levels in mesenteric arteries isolated from the mesenteric plexus (Figure VIIA in the Data Supplement) were similar between WT (n=6) and BSMKO (n=6) mice (Figure VIIIB in the Data Supplement). Moreover, no statistically significant differences were observed in the contractile responses to phenylephrine or vasodilation responses to papaverine of mesenteric arteries from WT (n=8) and BSMKO (n=8; Figure VIIIC and VIIID in the Data Supplement), consistent with comparable blood pressures between WT and BSMKO mice (Table I in the Data Supplement). Taken together, these data suggest that the BCL11B–pVASP\textsuperscript{S239} axis is important for the regulation of large artery stiffness but is dispensable for vasoconstriction of resistance vessels.

**Figure 3.** Vascular smooth muscle (VSM) bcl11b (B-cell leukemia 11b) deletion increases contractile force of aortic rings and arterial stiffness.

A. Representative force tracings recorded ex vivo in organ baths in aortic rings from wild-type (WT; n=5) and tamoxifen-inducible VSM-specific Bcl11b null mice (BSMKO; n=7). Scale on graph. Scatter plots indicate individual values for (B) force (mg); *P=6.0×10\textsuperscript{-3}; (C) wall tension (N/m), *P=2.0×10\textsuperscript{-3}; (D) stress (kPa), *P=3.0×10\textsuperscript{-3}; (F) stiffness, expressed as pulse wave velocity (PWV) calculated via the Moens-Kortweg equation (PWVMK), *P=4.0×10\textsuperscript{-3}; (G) KCl, *P=7.5×10\textsuperscript{-3}; and (H) phenylephrine (PE)-induced stress (kPa), *P=2.9×10\textsuperscript{-3}. Each dot represents an aortic ring from one mouse; mean±SEM on graphs. E. Pulse wave velocity (m/s) measured in vivo by Doppler echocardiography in WT (n=14) and BSMKO (n=17) mice. *P=4.0×10\textsuperscript{-2} by unpaired t test. Details in Methods.
In addition to protein kinases PKG and PKA (cAMP-dependent protein kinase), VASP phosphorylation in VSM cells is finely regulated by protein phosphatases (PP1, PP2A, PP2B [Ca\(^{2+}\)/calmodulin-dependent serine-threonine phosphatase calcineurin], and PP2C) and Rho kinases (ROCK1 and ROCK2). Of interest, PP2B has been shown to directly interact with BCL11B to regulate gene expression in T cells. Therefore, we examined whether PP2B may also contribute to decreased pVASP S239 levels in BSMKO VSM cells. We found that protein levels of PP2B and NFAT2, a major PP2B phosphatase target, were significantly upregulated in BSMKO VSM cells compared with WT (Figure 6A). On the contrary, expression of ROCK1, a Rho-dependent kinase involved in cytoskeletal rearrangements and an upstream VASP regulator, was not significantly affected by Bcl11b deletion (Figure VIIC in the Data Supplement). Furthermore, overnight treatment with 2 PP2B inhibitors, cyclosporine A (1–10 \(\mu\)mol/L) or the more specific PP2B inhibitor CAIP (calcineurin auto-inhibitory peptide, 10–100 \(\mu\)mol/L), restored pVASP to control levels in BSMKO VSM cells in a dose-dependent manner (Figure 6B and 6C; quantitation in Figure IV in the Data Supplement).
graph), indicating that decreased pVASP^{S239} in BSMKO VSM is dependent, at least in part, on increased PP2B activity. Last, overexpressing Bcl11b in aortic media rings by transient transfection for 3 days was sufficient to significantly restore PKG, pVASP_{S239} and decrease PP2B to control levels (Figure 6D).

Actin Polymerization Is Dependent on VASP Phosphorylation in VSM Bcl11b Deleted Aortas

VASP is an important regulator of nonmuscle actin polymerization-dependent VSM tone\(^2^8\) whereas phosphorylation of VASP at serine 239 (pVASP^{S239}) inhibits actin polymerization in VSM cells,\(^2^9\) thereby regulating cytoskeletal actin assembly. We found increased filamentous to globular actin ratio, indicative of increased actin polymerization, in BSMKO aortas compared with WT (Figure 7A). Moreover, α-actinin, a major VASP-interacting protein during actin polymerization, was significantly upregulated in BSMKO (n=4) compared with WT (n=4) aortas (Figure 7B, quantitation in graph). Notably, preincubation with the specific PP2B inhibitor CAIP (10 \(\mu\)mol/L, 1 hour, 37°C), decreased both filamentous/globular actin ratio (Figure 7C) and aortic stiffness (from 245.7±4.3 kPa in BSMKO, n=4 to 184.0±17.2 kPa in BSMKO/CAIP, n=4; \(P=3.0 \times 10^{-2}\); Figure 7D) in BSMKO aortic rings. Taken together, our data indicate that lack of VSM BCL11B increases aortic stiffness, at least in part, because of decreased pVASP^{S239} levels and associated increased actin polymerization.

VSM Bcl11b Deletion Increased the Incidence of Cerebral Microbleeds

Emerging evidence strongly correlates measures of arterial stiffness (PWV and increased pulse pressure) with microcirculatory end-organ damage, including kidney disease\(^2^0,2^1\) and cognitive impairment.\(^2^2–2^4\) We sought to determine whether increased aortic stiffness in BSMKO mice is associated with increased indexes...
of end-organ microcirculatory injury (microalbuminuria, cerebral microbleeds and retinal vessel density). We found that the urinary albumin to creatinine ratio was not significantly affected by VSM\textit{Bcl11b} deletion compared with WT littermates (23.2±5.6 μg/mg in WT, n=12 and 12.0±5.3 μg/mg in BSMKO, n=8; \(P=1.9\times10^{-1}\); Figure IXA in the Data Supplement). In contrast, a significantly higher number of cerebral microbleeds was identified by magnetic resonance imaging mainly in the thalamus of BSMKO compared with WT mice (1.0±0.4 in WT, n=5 versus 6.5±1.7 in BSMKO, n=6; \(P=1.0\times10^{-2}\); Figure 8A). Histological staining of brain sections with Prussian blue confirmed an increased number of cerebral microbleeds in BSMKO compared with WT mice (5.7±0.05×10\(^3\) μm\(^2\) in WT, n=12 versus 11.2±2.2×10\(^3\) μm\(^2\) in BSMKO, n=12; \(P=3.0\times10^{-5}\); Figure 8B). Interestingly, cerebral microbleeds in BSMKO mice were comparable to HFHS-fed mice, in which we similarly observed significant increases in cerebral microbleeds compared with ND-fed control mice (7.0±1.4×10\(^3\) μm\(^2\) in ND, n=6 versus 12.6±2.2×10\(^3\) μm\(^2\) in HFHS, n=6; \(P=5.0\times10^{-2}\); Figure 8C). No statistically significant differences were observed between aged WT and BSMKO (24-months old; 13.8±4.2×10\(^3\) μm\(^2\) in WT, n=4 versus 12.0±8.0×10\(^3\) μm\(^2\) in BSMKO, n=4; \(P=3.9\times10^{-1}\)), indicating that VSM\textit{Bcl11b} deletion accelerated the development of aging-associated cerebral microbleeds, which then plateaued to levels comparable to aged WT. Independently of the presence of cerebral microbleeds, no statistically significant difference in cognitive function was detected between 5-month or 24-month old WT and BSMKO mice as assessed by a novel object recognition test (Figure IXB in the Data Supplement).

Consistent with cerebral microvascular damage, total and average vessel length and branching, measured in isolectin B4-stained retinal flat-mounts, trended to increase in BSMKO compared with WT as well as in HFHS-fed compared with ND-fed mice (Figure IXC in the Data Supplement), suggesting a stimulation of neovessel growth in a damaged retinal microvasculature.
Arterial stiffness, or loss of elastic compliance of large arteries, is a strong, independent risk factor for CVD. Elevated PWV, the gold standard measure of aortic wall stiffness, strongly associates with adverse cardiovascular outcomes. Moreover, mounting evidence correlates measures of arterial stiffness and pressure pulsatility (PWV, pulse pressure) to kidney disease and cognitive impairment/dementia. However, genetic and molecular cues of aortic wall stiffening are not fully understood hampering the discovery of therapeutic targets that can slow or reverse arterial stiffening thereby decreasing target organ damage and the risk of developing overt CVD.

Arterial stiffness trait loci are moderately heritable, but little is known about genetic determinants of arterial stiffness. A recent genome-wide association study demonstrated that SNPs in the vicinity of the BCL11B genetic locus are associated with increased arterial stiffness and subsequent risk of developing CVD. Interestingly, the most significant SNP variant (rs1381289 C>T) in this aortic stiffness locus falls in a highly conserved sequence within a BCL11B enhancer. We postulated that SNP variants in the 3'-BCL11B locus may alter the BCL11B gene enhancer function and may play a causal role in the pathogenesis of arterial stiffness by regulating BCL11B expression. A recent report showed that rs1381289 C>T genotype in the 3'-BCL11B locus inversely correlates with BCL11B mRNA expression in aortic rings from transplant donors, despite the authors not detecting any BCL11B protein expression in the same aortic rings. These observations prompted us to further examine whether BCL11B is present in the vasculature and whether there is a cause-effect relation between BCL11B and vascular function.

Our novel findings demonstrate that BCL11B, previously known solely for its role in T lymphocyte and neuronal lineage commitment, is expressed in the VSM of the aortic wall. Importantly, here we report for the first time that VSM BCL11B is a crucial regulator of VSM structural components and aortic stiffness, as corroborated by the following findings: (1) in vivo Bcl11b deletion in VSM (BSMKO mice) resulted in increased nonmuscle actin polymerization (filamentous/globular actin ratio); (2) mice lacking Bcl11b globally or specifically in VSM (BSMKO) have increased PWV, the in vivo index of arterial stiffness, compared with WT littermates; (3) aortic rings from BSMKO mice have increased force, stiffness, and decreased actin polymerization; and (4) treatment of BSMKO aortas with CAIP (calcineurin autoinhibitory peptide) decreased stiffness, measured ex vivo on aortic rings in organ bath.

**DISCUSSION**

Arterial stiffness, or loss of elastic compliance of large arteries, is a strong, independent risk factor for CVD. Elevated PWV, the gold standard measure of aortic wall stiffness, strongly associates with adverse cardiovascular outcomes. Moreover, mounting evidence correlates measures of arterial stiffness and pressure pulsatility (PWV, pulse pressure) to kidney disease and cognitive impairment/dementia. However, genetic and molecular cues of aortic wall stiffening are not fully understood hampering the discovery of therapeutic targets that can slow or reverse arterial stiffening thereby decreasing target organ damage and the risk of developing overt CVD.

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stress, wall tension, and stiffness compared with WT; and (4) Bcl11b is down-regulated in aortas of high fat, high sucrose-fed obese mice, a mouse model of arterial stiffness that we previously described. Although a major BCL11B interacting protein, COUP-TFII, has been previously shown to partake in atria and blood vessel development during embryogenesis, to the best of our knowledge, this is the first report of a functional role of BCL11B in the adult cardiovascular system.

Furthermore, we uncovered a pivotal role of VSM BCL11B in the regulation of VSM cytoskeletal filaments, which form a coordinated system to efficiently transduce contractile forces to the extracellular matrix and among adjacent VSM cells, thereby sustaining aortic wall mechanics and compliance. In response to pressure or mechanical stretch, thin filament dynamic assembly, namely nonmuscle actin polymerization, become a major determinant of VSM contraction and basal tone, independently of myosin light chain 2 phosphorylation and actino-myosin cross-bridges cycles. Cytoskeletal actin polymerization can sustain VSM force generation and cytoskeletal stiffness to maintain vessel diameter in response to wall tension or stretch, as it may occur in the aorta exposed to cyclic strain induced by cardiac contraction, particularly in the proximal regions. Therefore, our finding of increased filamentous/globular actin ratio, indicative of increased actin polymerization, is consistent with increased force and wall tension in BSMKO aortas and increased PWV in BSMKO mice compared with WT controls.

At the molecular level, we found that the cGMP/PKG/pVASP signaling pathway was dramatically decreased in BSMKO aortas and VSM cells, while total VASP remained unchanged. VASP has emerged as an important mediator of actin polymerization-dependent VSM force generation. Specifically, VASP interacts with α-actinin, vinculin, zyxin, and other components of thin filament assembly along focal adhesion and dense bodies, which are important sites of VSM contractile filament attachment, cell-cell interactions and cell adhesion to the extracellular matrix, thereby contributing to VSM tone and stiffness independently of myosin light chain 2 phosphorylation. The drug cytochalasin D, commonly used to block actin polymerization in a variety of cell types, is known to interfere with VASP localization to nascent F-actin filaments underscoring the pivotal role of VASP in cytoskeletal actin rearrangement. Moreover, VASP overexpression has been shown to induce F-actin

Figure 8. Cerebral microbleeds in wild-type (WT) vs tamoxifen-inducible VSM-specific Bcl11b null mice (BSMKO).

A. Representative magnetic resonance imaging (MRI) of whole brains from WT (n=5) and BSMKO mice (n=6). A microbleed-rich region is highlighted with the white box. Quantitation in graph. **P=1.0×10−2 by unpaired t test.

B. Representative histological staining of WT (n=12) and BSMKO (n=12) brain sections (40x magnification); areas stained in blue are indicative of cerebral microbleeds. Quantitation in graph (µm²). Each dot represents the average of at least 5 sections, corresponding to a cumulative thickness of 150 µm, for each mouse. *P=3.0×10−2 by unpaired t test.

C. Representative histological staining of cerebral microbleeds in normal diet (ND) (n=6) and high fat, high sucrose (HFHS)-fed (n=6) mice. Quantitation in graph. **P=5.0×10−2 by unpaired t test.
that penetrates into the microcirculation. However, as the flow/low resistance organs, such as brain and kidneys. This intriguing finding on a differential role of BCL11B in the regulation of VSM tone in resistance vessels. This fact that smooth muscle along the adult vascular tree is not homogeneous but rather a mosaic of phenotypically and functionally distinct smooth muscle cell types.

Interestingly, despite having profound effects on the aorta, lack of VSM Bcl11b did not affect pVASP levels and contractile properties of resistance arteries, nor blood pressure in BSMKO mice suggesting a dispensable role of BCL11B in the regulation of VSM tone in resistance vessels. This study has uncovered a novel and crucial role for VSM BCL11B in aortic structural and functional integrity and strongly supports BCL11B or its downstream regulated pathways as potential therapeutic targets against arterial stiffness (illustrated in the graphic summary). Although, the cause-effect relationship as well as the temporal progression from arterial stiffness to target organ damage, including hypertension, remains to be fully unraveled, as we previously pointed out targeting arterial stiffness could represent a novel clinical approach to prevent the progression into target organ damage leading to cardiovascular complications and cognitive decline. Further studies in human populations from different ethnic groups are warranted to establish whether the genotype at the 3'-BCL11B locus may be used as diagnostic biomarker to identify individuals at increased risk of developing arterial stiffness and other vascular diseases.

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