Interindividual Transcriptional Regulation of the Human biglycan Gene Involves Three Common Molecular Haplotypes

Boris Schmitz, Andrea Salomon, Alois Rötrige, Martin Ritter, E. Bernd Ringelstein, Jens W. Fischer, Martin Paul, Eva Brand, Stefan-Martin Brand

Objective—The extracellular matrix proteoglycan biglycan (BGN) is involved in cardiovascular disease pathophysiology, as it mediates the subendothelial retention of atherogenic apolipoprotein B-containing lipoproteins, affects adaptive remodeling after myocardial infarction, and exerts proinflammatory effects in macrophages. In a cardiovascular disease-related setting of vascular endothelial cells and human monocytes, we examined the molecular mechanisms of common molecular haplotypes affecting human BGN transcriptional regulation.

Approach and Results—After the molecular characterization of the BGN promoter, we determined the prevalence of BGN promoter variants (1199 base pair portion) in 87 individuals of European ancestry, and identified 3 molecular haplotypes by subcloning and sequencing of subjects’ single DNA strands: MolHap1 [G^{578},G^{151},G^{903}] MolHap2 [G^{578},A^{151},T^{604}] and MolHap3 [A^{578},G^{151},G^{903}]. By 5′ rapid amplification of cDNA-ends, we detected 1 additional upstream transcription start site at position −46 in EA.hy926 endothelial cells. Reporter gene assays located the BGN core promoter to the region spanning positions −39 and +162. Strongest promoter activity was mapped to the region between −1231 and −935. The introduction of MolHap2 and MolHap3 into the active BGN promoter led to a significant loss of transcriptional activity (all probability values <0.05), compared with MolHap1. By use of electrophoretic mobility shift assays, chromatin immunoprecipitation assays, and cotransfection of transcription factors, we identified specificity protein 1, v-ets erythroblastosis virus E26 oncogene homolog (ETS) family members, and an activator protein-1 complex to interact differentially with the BGN promoter in the context of each individual MolHap.

Conclusions—Our results indicate that molecular haplotypes within the BGN promoter may contribute to the molecular basis of interindividually different transcriptional BGN regulation, possibly modulating the predisposition to cardiovascular disease-related phenotypes. (Arterioscler Thromb Vasc Biol. 2013;33:871-880.)

Key Words: BGN ■ cardiovascular disease ■ genetics ■ molecular haplotypes ■ mutation ■ transcription factor

The pathophysiological basis of cardiovascular disease (CVD) in most instances is arteriosclerosis, a progressive, multifactorial, and proinflammatory disease.1 The development of acute coronary events depends on the composition and vulnerability of the atherosclerotic plaque.2 Stable plaques are marked by accumulation of the small leucine-rich proteoglycan biglycan (BGN) and versican, whereas erosive plaques are characterized by aggregation of hyaluronan and absence of BGN.3 BGN is also involved in early atherothrombotic lesion development, mediating subendothelial low-density lipoprotein retention and oxidation.4 BGN-deficient mice (bgn−/−) present increased mortality after myocardial infarction compared with bgn+/+ mice as a result of left ventricular ruptures,5 pointing to a pivotal function of BGN in adaptive cardiovascular remodeling. On release from the extracellular matrix (ECM) or macrophages, BGN acts as an effector of inflammatory signaling via toll-like receptors 2 and 4, leading to an increased expression of tumor necrosis factor-α.6 The human BGN represents a single-copy gene on the long arm of the X-chromosome (Xq28). Its 5′-flanking region lacks both a CAAT and TATA box, but is rich in GC content.7 BGN is ubiquitously expressed with increased mRNA levels in lung and liver, whereas vascular smooth muscles cells and cardiac fibroblasts are a major source for BGN in the cardiovascular system.6,7 Transforming growth factor β-1 (TGF-β1) has been identified as a positive regulator of BGN expression,8-10 with
a SMAD4-dependent regulation in pancreatic tumor cells. A negative feedback loop for BGN-regulating TGF-β1 activity has been suggested. In human chondrocytes, tumor necrosis factor-α has been shown to decrease BGN steady-state mRNA levels (~62%) and BGN transcription rate (~18%). BGN is 1 of at least 9 small leucine-rich proteoglycans, which are secreted ECM components with different localization, expression, and function. Grouped into 3 different classes, they can be described as either glycoproteins containing N-linked oligosaccharides or as proteoglycans containing chondroitin/dermatan sulfate or keratin sulfate chains. Class I comprises BGN and decorin, which are highly homologous (57%) and have been proposed to originate from an ancestral gene-by-gene duplication. Both contain 10 leucine-rich repeats flanked by cystein-rich regions, and either 1 (decorin) or 2 (BGN) glycosaminoglycan chains at their N-terminal domain. BGN self-aggregates into dimers and hexamers under physiological conditions and undergoes complex posttranslational modifications, as shown in rat cardiac fibroblasts, including increase of glycosaminoglycan chain length, induced by growth factors such as TGF-β1. Although the role of BGN protein has been extensively explored in different pathophysiological processes, the molecular characteristics of BGN gene expression are less comprehensively understood. We therefore investigated the influence of common molecular haplotypes (MolHaps) on BGN expression regulation, and functionally characterized cis-active promoter elements and transacting factors.

**Materials and Methods**

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

**Results**

Localization of BGN Promoter Transcriptional Activity

We initially investigated the localization of BGN promoter transcriptional activity to identify cis-active regulatory elements. Because the 5’-untranslated region (UTR) may harbor potential regulatory promoter elements and 5’ transcript heterogeneity by alternative transcription start site (TSS) utilization is a common feature of eukaryotic genes, we determined BGN expression in different cell lines followed by cell-specific TSS analysis. We were able to detect BGN mRNA expression in HEK293T, EA.hy926, human primary coronary artery smooth muscle cells (CaSMCs), and human monocyte cell line (THP-1) cells (Figure 1) by PCR, using a combination of several sense primers. Stimulation with cAMP, phorbol-myristate-acetate, and TGF-β1 was performed to account for potential alterations of TSS under stimulatory conditions. In addition to TSS1 (designated position +1), a second strong PCR signal was observed for a transcript originating upstream of TSS1 in EA.hy926 cells. Transcript initiation from this upstream position was also prominent in TGF-β1-stimulated THP-1 cells and CaSMCs. A 5’ rapid amplification of cDNA-ends was performed for the exact localization of both TSS, using mRNA from unstimulated EA.hy926 and THP-1 cells. TSS2 was, thereby, located to position –46. TSS upstream of TSS2 were exclusively detected in CaSMCs.

To identify which portions of the BGN promoter region were transcriptionally active, we designed serial promoter deletion constructs (based on NC_000023.10), which were inserted into the pGL3–Basic luciferase vector and transiently transfected into EA.hy926 and THP-1 cells. For further experiments, we chose endothelial cells (EA.hy926) and monocytes (THP-1) because of their potential role in CVD pathophysiology. Transcriptional activity of the generated reporter constructs depended on the potential of the inserted BGN promoter fragment to drive expression of the firefly luciferase. The complete 5’-UTR (position +1 to +162) was included in all BGN reporter vectors. The pGL3-Control vector, where transcription is driven by a powerful Simian vacuolating virus 40 promoter and enhancer, was used as positive control for transfection efficiency.

The shortest generated BGN reporter construct –39/+162, representing the complete 5’-UTR in EA.hy926 cells, displayed sufficient transcriptional activity compared with fragments –701/+162 and –386/+162 (P<0.005, unpaired Student t test), which showed only moderate transcriptional activity. Reporter constructs harboring the region between –1231 and –935 of the promoter displayed strong transcriptional activity,
at the level of pGL3-Control ($P<0.001$, unpaired Student $t$ test, compared with the 5'-UTR alone; Figure 2A), in EA.hy926 cells. Further truncation, represented by construct −701/+162, resulted in a considerable reduction of transcriptional activity ($P<0.001$, unpaired Student $t$ test). Interestingly, the promoter construct −1318/+162 did not show any transcriptional activity in EA.hy926 cells ($P<0.001$ compared with −1231/+162, unpaired Student $t$ test), whereas deletion of the entire 5'-UTR region resulted in partial reconstitution of transcriptional activity ($P<0.001$, unpaired Student $t$ test).

Two Common MolHaps Significantly Reduce Transcriptional Activity of the BGN Promoter

We detected 3 BGN promoter variants, 1 in the 5'-UTR (G+94T [rs5945197]) and 2 in the proximal promoter region (G-578A [rs11796997] and G-151A [rs112151463]; Figure 2B), by sequencing of 87 individuals of European ancestry (28 women [32.2%] and 59 men [67.8%]). This sample size yielded a >90% detection rate for genetic variants with a minor allele frequency of 1%. To define the commonly occurring combinations of alleles on a single DNA strand, we...
subcloned and resequenced individual DNA and determined 3 common MolHaps: BGN–MolHap1 [G\(^{–578}\),G\(^{–151}\),G\(^{+94}\)] \(\approx\)24\%, BGN–MolHap2 [G\(^{–578}\),A\(^{–151}\),T\(^{+94}\)] \(\approx\)36\%, and BGN–MolHap3 [A\(^{–578}\),G\(^{–151}\),G\(^{+94}\)] \(\approx\)40\%. The MolHaps of the cell lines used in this study were (G\(^{–578}\),G\(^{–151}\),G\(^{+94}\)) for EA.hy926 and THP-1 cells, whereas HEK293T cells were heterozygous at position \(–578\) (G/A, G\(^{–151}\),G\(^{+94}\); Figure III in the online-only Data Supplement). To determine the impact of each MolHap on transcriptional activity of the BGN promoter, we introduced BGN–MolHap2 and BGN–MolHap3 in a 1025 base pair fragment \(–1318/+1\) (4.1-fold). We also tested TFs of the CCAAT-enhancer-binding protein family and CREB-binding protein in cotransfections, because they are known activators of GC-rich promoters, but no effect on BGN transcriptional regulation was detected (data not shown).

**SP1 Binds BGN Promoter Regions in EA.hy926 Cells**

We then conducted chromatin immunoprecipitation assays in EA.hy926 cells (genotyped MolHap1 [G\(^{–578}\),G\(^{–151}\),G\(^{+94}\)]) to explore whether the observed transcriptional activation via SP1 was based on direct interaction of SP1 with the BGN promoter. Chromatin immunoprecipitation was performed for 3 regulatory promoter portions (Figure 3B), 2 of which were positioned in the distal region \((–1164 \text{ to } –1066\) and \(–918 \text{ to } –806\)) and a region covering TSS1, as well as part of the 5′-UTR (\(–28 \text{ to } +71\)). We used specific antibodies against SP1, phosphorylated cAMP response element-binding protein (cAMP response element-binding protein-P), and the erythroid-specific TF GATA-binding protein 1, which served as negative control. cAMP response element-binding protein and GATA-binding protein 1 antibodies did not result in pull-down of chromatin at any of the targeted positions, whereas application of SP1 antibody identified interaction of SP1 with the distal BGN promoter region comprising \(–918 \text{ to } –806\), as well as the 5′UTR position \(–28 \text{ to } +71\). Interaction of SP1 in EA.hy926 cells was also shown for the polymorphic sites \(–151\) and \(+94\), but not for the distal position \(–578\).

**TGF-β1 Stimulates BGN Expression in THP-1 Monocytes**

TGF-β1 has been shown to be involved in transcriptional and posttranslational regulation of BGN. We analyzed whether TGF-β1 affects BGN expression in EA.hy926 and THP-1 cells. BGN promoter deletion constructs displaying the highest transcriptional activity under basic conditions (Figure 2A) were stimulated up to 3-fold in THP-1 cells after treatment with TGF-β1 for 24 hours compared with mock-treated controls (Figure 3C). Subsequently, we detected a \(\approx\)2.5-fold increase of BGN mRNA level in THP-1 monocytes on stimulation with TGF-β1 for 24 hours (Figure 3D). Stimulation of CaSMCs with TGF-β1 resulted in comparable results (Figure 1 in the online-only Data Supplement), whereas no effect was seen for EA.hy926 cells (data not shown).

**Polymorphic 5′-UTR Site G+94T Is Bound by TF Complex AP-1**

We used band shift assays to characterize binding of TFs to BGN promoter sequences in vitro, and to reveal the molecular basis of TGF-β1 activation at the promoter level. Biotinylated probes representing the flanking portions of the 3 identified promoter variants were analyzed toward their interaction with nuclear extracts from EA.hy926 and THP-1 cells. Minor alleles were introduced to assess allele-specific binding of each probe. Application of consensus oligonucleotides, target-specific probe mutations, and specific antibodies against selected TFs were used to identify interacting nuclear proteins.
When EA.hy926 and THP-1 nuclear extracts were incubated with the probe bearing position G+94T, we observed a prominent and sequence-specific band shift (Figure 4A and 4B, black arrow) with significantly higher binding affinity for the +94T allele. A second specific shift of lower intensity (open arrow) appeared to be allele-specific exclusively for THP-1 extracts. In silico analyses for site G+94T predicted binding of the AP-1 TF complex to this promoter sequence. We used unlabelled oligonucleotides harboring either the +94G or T allele, and an AP-1 consensus sequence to bind EA.hy926 nuclear extracts with subsequent separation in a native PAGE to reveal AP-1 binding and identify potential AP-1 family members. Bound proteins were, therefore, detected with specific antibodies against JUN-B, c-JUN, and c-FOS. We subsequently detected a strong signal for the AP-1 control oligonucleotide when the c-FOS antibody was used, demonstrating AP-1 complex formation involving c-FOS under the applied electrophoretic mobility shift assay conditions (Figure 4C). The signal appeared at the same position in the gel, as the band shift obtained using the biotinylated probe (open arrow). Binding of c-FOS at the G+94T site was irrespective of the present allele. For this band shift, both detection methods, using the antibiotin or antic-FOS antibody, did not show any difference in signal intensity.

**Promoter Site G-151A Interacts With SP1**
Band shift experiments with probes harboring G-151A and EA.hy926 nuclear extracts revealed 2 specific bands for both alleles (Figure 5A). In silico predictions for position –151 identified 2 SP1 consensus motifs flanking this polymorphic site. A mutated probe was designed by altering either 2 nucleotides at both ends of the probe essential for SP1 binding. Application of this altered oligonucleotide as specific competitor did not result in band competition (Figure 5B). Accordingly, the biotinylated, mutated probe featured a completely different binding pattern (Figure 5C). The most considerable and strongest signal (black arrow, Figure 5A) was absent using the mutated probe. To determine the presence of SP1 in the detected DNA–protein complex, a SP1-specific antibody was used for detection, revealing the binding of SP1 to both alleles at site G-151A with identical signal intensity (Figure 5D).
oligonucleotide missing perfect SP1 consensus sites did not bind to SP1. Band shift experiments using THP-1 nuclear extracts, compared with nuclear extracts derived from EA.hy926 cells (Figure 5E), resulted in a different electrophoretic mobility shift assay binding pattern. Two specific and allele-independent interactions were observed (grey arrows). Application of a SP1 consensus site (Figure 5F) as sequence-specific competitor resulted in incomplete signal competition, whereas the SP1-mutated probe showed identical band shifts compared with the unaltered G-151 probe (Figure 5G).

**Binding of SP1 and the Hematopoietic ETS TF PU.1 to the G-578A Site Is Enhanced After TGF-β1 Stimulation**

Band shift analyses of position –578 using EA.hy926 nuclear extracts revealed a prominent allele-specific pattern for the
probe harboring the G allele (Figure 6A, black arrow), whereas no specific binding was observed for the A allele. Application of nuclear extracts obtained from TGF-β1–stimulated EA.hy926 cells changed the observed pattern of DNA–protein bands, resulting in a shift with high specificity for the A allele (Figure 6B, open arrow) and loss of the interaction with the G allele.

Signal detection using a SP1-specific antibody did not reveal any DNA–protein interaction of SP1 and the G-578A site under unstimulated conditions (Figure 6C), whereas SP1 was positively identified to bind G-578A when nuclear extracts from TGF-β1–treated cells were applied.

We further investigated in silico binding predictions for both alleles, with special respect to cell-specific regulatory factors. Computational analysis revealed a consensus site for the hematopoietic ETS TF PU.1 at the –578G site. The binding was predicted to be impaired by introduction of the A allele.

Band shift with nuclear extracts from THP-1 cells presented a different binding pattern compared with EA.hy926 protein extracts with 1 sequence-specific band (Figure 6E, grey arrow) for both alleles. An additional specific band shift (Figure 6F, angled arrows) emerged on stimulation with TGF-β1. In THP-1 cells as well, no interaction with SP1 was detected under basic conditions, whereas the emerging band under TGF-β1 conditions was identified to involve SP1 binding.

We further investigated in silico binding predictions for both alleles, with special respect to cell-specific regulatory factors. Computational analysis revealed a consensus site for the hematopoietic ETS TF PU.1 at the –578G site. The binding was predicted to be impaired by introduction of the A allele.

Discussion

The current study provides a molecular analysis of the transcriptional organization of the polymorphic human BGN promoter, including previously unpublished BGN MolHaps. We were able to demonstrate that (1) MolHaps had a significant impact on BGN transcriptional activity; (2) TGF-β1 signaling, mediated through TF SP1, significantly increased BGN...
expression; (3) TFs AP-1 and PU.1 interacted differentially with the BGN promoter, dependent on the present MolHap.

BGN transcription has been reported to initiate from multiple TSS, which results in different lengths of transcripts’ 5′-UTR. We used a 5′ rapid amplification of cDNA-ends approach and confirmed a common TSS (TSS1) in HEK293T, EA.hy926, CaSMCs, as well as THP-1 cells, whereas a second TSS, TSS2 located 46 base pairs upstream, was detected in EA.hy926 cells and CaSMCs. Transcript initiation from position −46 was also detected in TGF-β1–stimulated THP-1 cells. Transcript initiation from multiple TSS has explicitly been reported for CpG island promoters similar to the BGN promoter.

We based our promoter analysis on the major TSS1, including the complete 5′-UTR of exon 1. Serial deletion constructs were designed to identify relevant promoter portions involved in BGN transcriptional regulation. By systematic construct truncation, we simulated inactivation through nuclear histone packaging of cis-active DNA elements. Whereas, the deletion construct −1231/+162 displayed strong promoter activity, extension of the fragment by 87 nucleotides (construct −1318/+162) led to total abrogation of transcriptional activity. This effect may be explained by the distal localization of a repressive element that is potentially masked by chromatin formation in vivo. Deletion of the 5′-UTR from the inactive fragment −1318/+162 caused partial restoration of transcriptional activity. This highlights the remote interaction of a distal promoter element located within the 87 nucleotides and the 5′-UTR. A transcription factors binding site region suggested a conserved ETS motif within this defined promoter region.

We assessed the influence of genetic variation on BGN transcriptional activity and functionally analyzed MolHaps in the identified BGN promoter, including the 5′-UTR. Of note, compared with MolHap1, introduction of BGN MolHaps2 and 3 significantly decreased transcriptional activities, suggesting that the polymorphic sites −578, −151, and +94 are located in cis-active promoter regions. As SP1 acted as an overall activating factor of BGN transcription in coexpression experiments, we hypothesized an altered SP1/MolHap interaction and analyzed DNA binding of SP1 in vivo by chromatin immunoprecipitation. Interaction of SP1 in EA.hy926 cells, which represent MolHap1, was detected for the distal promoter region (−918 to −806), as well as for the 5′-UTR and the polymorphic sites at positions −151 and +94, whereas SP1 did not bind to position −578. Because chromatin immunoprecipitation also allows for detection of proteins that do not directly recognize DNA consensus motifs but are involved in heteromeric module formation and DNA bending, we analyzed each polymorphic site in separate in vitro band shift experiments, introducing the respective minor alleles. Electrophoretic mobility shift assay revealed allele-independent binding of SP1 to the G-151A site, but did not detect binding of SP1 at G+94T. Presumably, SP1 interaction at position +94 in vivo does not depend on DNA sequence recognition, but is attributable to remote protein–protein interaction with the G-151A site through DNA bending. This characteristic feature of SP1 has been reported previously in the concept of TATA box binding protein associated factor-dependent promoter activation. We also observed binding of the ETS family member PU.1 to the polymorphic site −578 under basic conditions and enhanced binding of PU.1 after TGF-β1 stimulation. ETS might colocalize with SP1 at this position, with a preference for ETS under basic conditions. Fostering SP1–chromatin interaction by TGF-β1 activation may simultaneously enhance ETS binding, resulting in increased transcriptional activation of the BGN promoter.

It has been reported that overexpression of both SMAD2 and SMAD3 potentiates the TGF-β1 effect on BGN expression. We suggest that this activation relies on the physical interaction of SMADs with SP1, which would result in enhanced interaction of SP1 and its G/C-consensus motifs. TGF-β signal transduction through the functional cooperation of SP1 with a complex of SMAD2, SMAD3, and SMAD4 has explicitly been shown for the induction of the p21 promoter. Different molecular mechanisms could be involved in this transcriptional activation process: (1) the oligomerization of SP1, (2) the recruitment of synergistic complementary factors, as well as (3) SP1 posttranslational modifications, such as phosphorylation, acetylation, and glycosylation, sumoylation, or ubiquitylation.

Furthermore, we observed altered binding affinity of PU.1 at positions −578 depending on the nature of the allele present. This may explain the reduced transcriptional activity of MolHap3 under basic conditions. Because PU.1 is predominantly expressed in hematopoietic lineages, we postulate that cell type–specific BGN expression is controlled by different ETS factors in other cells and tissues, controlling BGN promoter mode regulation. In addition, the importance of ETS TFs in the regulation of BGN expression is supported by the postulated ETS motif in the distal repressive promoter region. Because electrophoretic mobility shift assay did not reveal differential binding of proteins at position −151, the reduced transcriptional activity of MolHap2 can only be explained by the 5′-UTR site +94. At this obviously pivotal position, we identified AP1 complex formation involving c-FOS, whereas no binding of SP1 was detected in band shift experiments. The heterogeneous TF complex AP-1 might be alternatively assembled in the presence of the +94T allele, selectively including distinct subunits of JUN, FOS, activating transcription factor, or MAF, resulting in repression of BGN expression.

The ECM modulator TGF-β1 has been shown to be an important regulator of BGN transcriptional and posttranslational modifications, whereas promoter deletion analyses under TGFβ1 stimulation have been discussed controversially. In our experiments, in vascular endothelial cells and monocytes, the strongest transcriptional activity was observed in the distal region between positions −1231 and −935. Compared with our analyses, other investigators did not detect an impact of TGFβ1 on BGN transcriptional activity in their promoter constructs. Differences between our results and other reports on BGN promoter activity might be explained by different lengths of the included 5′-UTR. Furthermore, prior investigations did not account for genetic variation in the BGN promoter. All previous investigations were based on the 5′-flanking sequence provided by Ungefroren and Krull, which represented the particular MolHap3 and excluded the polymorphic 5′-UTR site G+94T.
In conclusion, we propose new structural features of the BGN promoter, including a model for a transcriptional module differentially interacting with polymorphic cis-active promoter portions (Figure 8), resulting in altered BGN expression. We identified and functionally characterized different MolHaps within the BGN promoter, which might importantly contribute to an understanding of the molecular basis of interindividually different BGN transcriptional regulatory properties and finally to the predisposition to CVD phenotypes. Interestingly, we detected significant differences of BGN expression level in 22 patients with carotid artery stenosis, which did not depend on classical factors. Altered BGN expression based on BGN MolHaps may affect onset, progression, or outcome of atherosclerosis at different stages. Persistent low level of BGN in the ECM could prevent early atherosclerotic lesion development by reduced retention and oxidation of subendothelial low-density lipoprotein. In turn, BGN is essential for controlled collagen deposition in the vasculature. Fibrous caps of stable aortic plaques are enriched in BGN and versican, and BGN affects scar formation and remodeling processes after myocardial infarction. Reduced BGN expression because of the presence of MolHaps2 and 3 could violate these processes, resulting in more severe outcomes. Our observation may be of particular relevance in vascular smooth muscle cells and cardiac fibroblasts, as these cells are the major contributors to BGN production in the cardiovascular system. We have shown that BGN is induced by TGF-β1 in CaSMCs, as it has also been reported for cardiac fibroblasts. Besides transcriptional regulation, degradation of proteoglycans is at least equally important in the pathogenesis of vascular disease. It has been reported that several proteolytic enzyme (soluble as well as membrane-associated) proteinases are capable of degrading BGN, including matrix metalloproteinases, such as matrix metalloproteinase 2, 3, 8, 9, 12, and 13, and aggrecanases, such as ADAMTS4 and ADAMTS5 (a disintegrin-like metalloproteinase with thrombospondin type 1 motif). Of note, ADAMTS4 and ADAMTS5 are also capable of degrading versican, another important proteoglycan involved in vascular lesion development, prominently expressed in early and late atherosclerotic plaques. BGN gene expression is only part of the complex processes affecting ECM composition. The impact of the here described MolHaps on BGN synthesis might be a sufficient, although not necessary condition, to affect the subtle and concerted cellular processes in the vasculature.

The lack of significant association of BGN with CVD phenotypes until today could be because of the sparse inclusion of X-chromosomes (and also Y-chromosomes) in available genome-wide association studies. Only most recently, some genome-wide association studies have started to include sex chromosomes in their analysis for complex diseases. The latest GWA on multiple CVD-related traits by Middelberg and colleagues did include genetic variants on the X-chromosome, but did not identify any association of X-chromosomal loci with CVD.

Our results indicate that hemizygous men carrying a distinct BGN MolHap may more likely be affected by BGN-dependent changes in the vasculature compared with heterozygous women. This effect might be most prominent under the stimulatory regime of TGF-β1.

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Disclosures

None.

References

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### Supplemental material

#### Table SI

**Supplemental table 1 Sequences and positions of oligonucleotides used in this study**

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| **Oligonucleotide sequences for PCR** | | | |
| P-1 | CTCTTCACAAACTGCCCAGG | -1 | NC_000023.10 |
| P-46 | CCACCGCTGCCAGCCCTTTAGC | -46 | NC_000023.10 |
| P-78 | CGGCTTCCTCCTTGCCTTC | -78 | NC_000023.10 |
| P-110 | CCCACCACGGCCACTTCCCTCC | -110 | NC_000023.10 |
| Exon 4 AS | GAGCTGGGTAGGGTTGGGCGG | Exon 1 | NM_001711 |
| Exon 2 SS | TGACACCCCCGCTGCTCTGG | Exon 2 | NC_000023.10 |
| rp27 SS | CCAGGATAAGGAAGGAATTCCTG | Exon 3/4 | NM_00135592.2 |
| rp27 AS | CCAGGATAGGAGGAGGAGGAGG | Exon 6 | NM_00135592.2 |

| **Oligonucleotide sequences for real-time PCR** | | | |
| BGN RT SS | CTCTGCCCAGGCTGCTGCTAC | Exon 8 | NM_001711 |
| BGN RT AS | ATGAGGAGGAGGAACAGAACATG | Exon 8 | NM_001711 |
| GAPDH RT SS | CGGCTGAGGAGGAGGAAAGGAGGAGGAGG | Exon 6 | NM_002046 |
| GAPDH RT AS | GTGATGGCATGGACTGTGGTCATG | Exon 6/7 | NM_002046 |

| **Oligonucleotide sequences for generation of MolHap promoter constructs** | | | |
| BGN MolHap SS | GCTAGCCAGTGTCGTACTAAGGACG | Exon 1318 | NC_000023.10 |
| BGN MolHap AS | CCCGGGTGGAGAGGGAGGCGG | Exon 1395 | NC_000023.10 |

| **Oligonucleotide sequences for site-directed mutagenesis** | | | |
| BGN +94T | CTGAGGAGGCAGCTTGAAG | +162 | NC_000023.10 |
| BGN ChIP -578A | AAGGGAAGAAGTCTAGAGTGGAAGGGAGGG | -593 | NC_000023.10 |
| BGN ChIP -151A | GAAGCTGCCAGGGGGACCGGGAAGCCTGCCC | -136 | NC_000023.10 |
| BGN ChIP +94T | CACCACCCCAGCCCTCCAACTAGTCAGCCT | +79 | NC_000023.10 |

| **Oligonucleotide sequences for generation of promoter deletion constructs** | | | |
| BGN ChIP -386/+162 SS | GCAGTTTGGTCAAGGTGCCA | -386 | NC_000023.10 |
| BGN ChIP -39/+162 SS | TGCCCAGCCTTTAGCCTC | -39 | NC_000023.10 |
| BGN ChIP +162 AS | CACGAGGGAGCAGCTTGAAG | +162 | NC_000023.10 |

| **Oligonucleotide sequences for ChIP** | | | |
| BGN ChIP -28/+78 SS | AGCCTCCCCGGCGCCCGCGCCT | -28 | NC_000023.10 |
| BGN ChIP -28/+78 AS | CGGACGGCTATGCTGCAGGGTG | +78 | NC_000023.10 |
| BGN ChIP -918-806 SS | AGGGGACACTACGGGACAG | -918 | NC_000023.10 |
| BGN ChIP -1164/-1066 SS | CGGTTTTAGGATGCAGCT | -1164 | NC_000023.10 |
| BGN ChIP -1164/-1066 AS | GAGAGAGAGGGGTGGCTAGAG | -1066 | NC_000023.10 |
| BGN ChIP G-579A SS | GATCGCCGCTCTTCTTTAG | -610 | NC_000023.10 |
| BGN ChIP G-578A AS | TAGGTGTTGTGATTTTTCG | -511 | NC_000023.10 |
| BGN ChIP G-151A SS | CGTCTACAAAAATGTGCT | -191 | NC_000023.10 |
| BGN ChIP G-151A AS | GGGGAGGGAGAAGGAGG | -77 | NC_000023.10 |
| BGN ChIP G+94T SS | TGGCCAGAGGAGTGGATGCTC | +14 | NC_000023.10 |
| BGN ChIP G+94T AS | AGGCGAGGCGCTAGTTGG | +115 | NC_000023.10 |
**In silico analyses of putative transcription factor binding sites (TFBS)**

The following parameters were used for the identification of TFBS.

**AliBaba2.1:**
Pairsim to known sites: 50; matrix width: 10 bp; minimum matrix conservation: 75%; factor class level: 3 (e.g. RAR) or 4 (e.g. RAR-b') or 5 (e.g. RAR-b'1).

**PROMO3.0.2:**
Only human factors/human sites; maximum matrix dissimilarity rate ≤6.

**Confirmation of transcription start sites (TSS) by PCR**

PCR reactions (25 µl) consisted of 1 µl cDNA, 200 nM of sense and antisense primer (Invitrogen, Karlsruhe, Germany), 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 1.4 M Betain (Sigma-Aldrich, Munich, Germany), 1 U GoTaq DNA Polymerase (Promega, Mannheim, Germany), 5 µl of 5x GoTaq DNA polymerase buffer (MgCl₂ at a concentration of 7.5 mM), add nuclease free water. A touch-down PCR (74°C to 58°C, progressive transition of 2°C every three cycles) with a final amplification of 25 cycles was used.

**Study population**

To link identified BGN promoter MolHaps with BGN mRNA level in circulating monocytes of patients with extensive carotid artery plaque, 22 patients were randomly selected from the “carotid samples for molecular analysis” (CARMA) Database. Patients admitted to the stroke unit of the department of Neurology of the University Hospital of Münster were enrolled between October 2011 and July 2012. All but one patient had suffered minor stroke (n=13), transitory ischemic attack (n=3), or transient (n=3) or persistent (n=2) monocular blindness attributable to the ipsilateral carotid artery as presenting symptom. One patient presented with asymptomatic carotid stenosis. Patients had a median age of 70 years (range: 50-85), 14 were male, 8 female. Median degree of stenosis (NASCET criteria) was 80% (range: 50-95). Eighteen patients (78%) were hypertensive, 11 (48%) had diabetes mellitus, 8 (35%) were smokers, 14 (61%) had hypercholesterolemia. Nine (39%) patients had a
history of coronary heart disease (CHD) and 7 (30%) had a history of peripheral vascular disease (PVD). Blood was sampled prior to surgery, which was performed at a median of 4 days (range: 1-14) after symptom onset. The study was approved by the ethics committee of the Medical Faculty (ethics committee number 2011 560-f-S), Westfalian Wilhelms-University of Münster and written informed consent was obtained from all study subjects. Patients were genotyped as described.

Supplemental results

Figure S1

Supplemental figure I Transforming growth factor beta-1 (TGF-β1) activates BGN expression in human primary coronary artery smooth muscle cells (CaSMCs). Relative BGN expression in TGF-β1-stimulated cells (n=2) increased up to 3-fold compared to unstimulated CaSMCs (mock control, CTR; n=2). BGN expression was determined via real-time PCR and normalized to the endogenous control GAPDH. ***p<0.001.
Supplemental figure II Spectrum of BGN mRNA level in peripheral blood mononuclear cells from patients of the CARMA (CARotid samples for Molecular Analysis) cohort. BGN mRNA level differed significantly between patients. Mean 1/∆Ct value of the 22 patients was 0.137. BGN expression was lowest in patient #10 (0.063), while patient #9 presented a 4-fold higher expression (0.255, p=0.0023). BGN expression was determined via real-time PCR and normalized to the endogenous control GAPDH.
### Table SII

**Supplemental table II Characteristics of the CARMA study population**

<table>
<thead>
<tr>
<th></th>
<th>High BGN (n=9)</th>
<th>Low BGN (n=13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (m)</td>
<td>4 (44%)</td>
<td>10 (77%)</td>
<td>0.0651</td>
</tr>
<tr>
<td>Age (&lt;70 years)</td>
<td>3 (33%)</td>
<td>7 (54%)</td>
<td>0.4214</td>
</tr>
<tr>
<td>ACI symptom (stroke)</td>
<td>5 (56%)</td>
<td>8 (62%)</td>
<td>0.2755</td>
</tr>
<tr>
<td>Hypertension</td>
<td>7 (78%)</td>
<td>11 (85%)</td>
<td>0.3255</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4 (44%)</td>
<td>7 (54%)</td>
<td>0.6784</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>5 (56%)</td>
<td>9 (69%)</td>
<td>0.5941</td>
</tr>
<tr>
<td>CHD</td>
<td>3 (33%)</td>
<td>6 (46%)</td>
<td>0.4168</td>
</tr>
<tr>
<td>PVD</td>
<td>3 (33%)</td>
<td>4 (31%)</td>
<td>0.8858</td>
</tr>
<tr>
<td>Smoking</td>
<td>3 (33%)</td>
<td>5 (38%)</td>
<td>0.7036</td>
</tr>
<tr>
<td>Days after symptom onset (&lt;4)</td>
<td>3 (33%)</td>
<td>7 (54%)</td>
<td>0.2555</td>
</tr>
</tbody>
</table>

Twenty-two patients of the CARMA study cohort were randomly selected after admittance to the stroke unit of the department of Neurology of the University Hospital of Münster. All but one patient had suffered minor stroke (n=13), transitory ischemic attack (n=3), or transient (n=3) or persistent (n=2) monocular blindness attributable to the ipsilateral carotid artery as presenting symptom. One patient presented asymptomatic carotid stenosis. Patients had a median age of 70 years (range: 50-85), 14 were male, 8 female. Median degree of stenosis (NASCET criteria) was 80% (range: 50-95). Eighteen patients (78%) were hypertensive, 11 (48%) had diabetes mellitus, 8 (35%) were smokers, 14 (61%) had hypercholesterolemia. Nine (39%) patients had a history of coronary heart disease (CHD) and 7 (30%) had a history of peripheral vascular disease (PVD). Blood was sampled prior to surgery, which was performed at a median of 4 days (range: 1-14) after symptom onset. RNA was extracted from buffy coats. Values are expressed as n (%). ACI, acute cerebral infarction; high $BGN$ expression was defined as $1/\Delta C_{\text{t}}>0.137$ with reference to the mean $BGN$ expression of the study cohort. Student’s $t$-test was used to determine level of significance for $BGN$ expression between groups.
Table SIII

Supplemental table III Haplotype frequencies and BGN mRNA level in the CARMA study sample

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>n</th>
<th>Mean BGN expression 1/ΔCt (SEM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G&lt;sup&gt;-578&lt;/sup&gt;-G&lt;sup&gt;-151&lt;/sup&gt;-G&lt;sup&gt;+94&lt;/sup&gt;</td>
<td>4</td>
<td>0.1275 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>G&lt;sup&gt;-578&lt;/sup&gt;-A&lt;sup&gt;-151&lt;/sup&gt;-T&lt;sup&gt;+94&lt;/sup&gt;</td>
<td>17</td>
<td>0.1438 ± 0.02</td>
<td>0.6746</td>
</tr>
<tr>
<td>A&lt;sup&gt;-578&lt;/sup&gt;-G&lt;sup&gt;-151&lt;/sup&gt;-G&lt;sup&gt;+94&lt;/sup&gt;</td>
<td>7</td>
<td>0.1503 ± 0.02</td>
<td>0.6116</td>
</tr>
</tbody>
</table>

BGN haplotypes G<sup>-578</sup>-A<sup>-151</sup>-T<sup>+94</sup> and A<sup>-578</sup>-G<sup>-151</sup>-G<sup>+94</sup> were tested independently against haplotype G<sup>-578</sup>-G<sup>-151</sup>-G<sup>+94</sup>. A heterozygous female carrier of the very rare haplotype A<sup>-578</sup>-A<sup>-151</sup>-T<sup>+94</sup> (1/ΔCt = 0.155) was also identified in the CARMA study sample but not included in the statistical haplotype analysis. Student’s t-test was used to determine level of significance for BGN expression between groups. Mean BGN expression is given as 1/ΔCt ± standard error of the mean (SEM).

Table SIV

Supplemental table IV Allele frequencies and BGN mRNA level in the CARMA study sample

<table>
<thead>
<tr>
<th>Position</th>
<th>Allele</th>
<th>n</th>
<th>Mean BGN expression 1/ΔCt (SEM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-578</td>
<td>G</td>
<td>22</td>
<td>0.1435 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>8</td>
<td>0.1509 ± 0.02</td>
<td>0.7848</td>
</tr>
<tr>
<td>-151</td>
<td>G</td>
<td>12</td>
<td>0.1444 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>18</td>
<td>0.1470 ± 0.02</td>
<td>0.9166</td>
</tr>
<tr>
<td>+94</td>
<td>G</td>
<td>11</td>
<td>0.1420 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>19</td>
<td>0.1475 ± 0.01</td>
<td>0.8252</td>
</tr>
</tbody>
</table>

Student’s t-test was used to determine level of significance for BGN expression between groups. Mean BGN expression is given as 1/ΔCt ± standard error of the mean (SEM).
Figure SIII

Sequences of cell lines THP-1, EA.hy926 and HEK293T at polymorphic positions G-578A, G-151A and G+94T. Sequences of positions -151 and +94 are given in reverse orientation.
Methods

Cell culture

The human vascular endothelial cell line EA.hy926 (1) and HEK293T cells were maintained in DMEM (Sigma-Aldrich, Munich, Germany) with 10% conditioned fetal calf serum (FCS; PAA, Cölbe, Germany), penicillin (100 units/ml), streptomycin (100 ng/ml), and L-Glutamine (2 mmol/ml, all Sigma-Aldrich). THP-1 monocytes were grown in RPMI 1640 (Sigma-Aldrich) with 10% FCS, MEM amino acid mix (1%, Sigma-Aldrich), penicillin (100 units/ml), streptomycin (100 ng/ml), L-Glutamine (2 mmol/ml) and sodium pyruvate (1%, Sigma-Aldrich). Human primary coronary artery smooth muscle cells (CaSMCs, PromoCell, Heidelberg, Germany; #C-12511, Lot. #9052501.4) were maintained in Smooth Muscle Cell Growth Medium 2 (PromoCell) with 5% FCS, epidermal growth factor (0.5 ng/ml), basic fibroblast growth factor (2 ng/ml) and insulin (5 µg/ml, all PromoCell). Prior to TGF-β1 stimulation, cells were starved for 24h in DMEM (GIBCO/Life Technologies, Darmstadt, Germany) with penicillin (100 units/ml), streptomycin (100 ng/ml) and MEM amino acid mix (1%, all GIBCO). Differentiation of monocytes into macrophages was induced by 10 nmol/l phorbol myristate acetate (PMA; Sigma-Aldrich) for 72h. For stimulation experiments, cells were incubated with 0.5 mmol/l 8-Br-cAMP (Biolog, Bremen, Germany) or 10 nmol/l PMA for 24h. TGF-β1 (Merck, Darmstadt, Germany) was used at 5 or 10 ng/ml for 12 or 24h.

Identification of BGN promoter MolHaps

Genomic DNA was prepared from whole blood by use of a commercial kit (Qiagen, Hilden, Germany). To identify BGN promoter MolHaps, 1199 bp of the promoter region (NC_000023.10) were amplified, in case of female heterozygosity individually subcloned, and sequenced (both DNA strands) using an automated sequencing device (ABI PRISM 3770, Perkin Elmer, Foster City, USA). Eighty-seven individuals with European ancestry were consecutively selected from the Münster MolProMD study (2). The study was approved by the ethics committee of the Medical Faculty, Westfalian Wilhelms-University of Münster (ethics committee number 2007-211-f-S) and written informed consent was obtained from all study subjects.
**In silico analyses of putative transcription factor binding sites (TFBS)**

Web-based algorithms were used for TFBS prediction. DNA sequences were compared to the TRANSFAC database (3) using AliBaba2.1 (http://www.gene-regulation.com) (4) and PROMO3.0.2 (http://alggen.lsi.upc.es/) (5). The following parameters were used for the identification of TFBS. AliBaba2.1: Pairsim to known sites: 50; matrix width: 10 bp; minimum matrix conservation: 75%; factor class level: 3 (e.g. RAR) or 4 (e.g. RAR-b') or 5 (e.g. RAR-b'1). PROMO3.0.2: Only human factors/human sites; maximum matrix dissimilarity rate ≤6.

**Isolation of total RNA and cDNA generation**

Total RNA was extracted using the NucleoSpin RNA II (Macherey-Nagel, Düren, Germany). Up to 5 µg of total RNA was applied as template for cDNA synthesis by SuperScript II (Invitrogen, Karlsruhe, Germany).

**Real-time PCR**

BGN quantitative real-time PCR was performed using Power SYBR Green (Applied Biosystems) and primer 5’-CTCTGCCAGGGCTGCCGAC-3’ (sense) and 5’-ATGAGGAGGAGGAACAGAACA TG-3’ (antisense), specific for protein coding transcripts (ENSG00000182492), on an Applied Biosystems 7500 Fast real-time PCR system. Samples were run in duplicates under standard real-time PCR conditions. Relative quantification was calculated using the ∆Ct method. GAPDH (sense primer 5’-CTGCACCACCAACTGCTTAGCAC-3’; antisense primer 5’-GTGATGGGCATGGACTGTG GTCATGAG-3’) was used as endogenous control. The absence of non-specific amplification was confirmed by agarose gel electrophoresis of PCR amplicons and generation of melting curves using the Applied Biosystems real-time PCR system software. Statistical analysis was performed using Fisher’s exact test. Real-time PCR has been repeated at least twice.

**Rapid amplification of 5’ cDNA ends (5’-RACE)**

5’-RACE PCR was performed as described previously (6). RNA from unstimulated EA.hy926 and THP-1 cells was employed for first strand cDNA synthesis using a sequence-specific antisense primer.
and SuperScript II. PCR products were sequenced for identification of the exact transcription start site (TSS). TSS were confirmed by semi-quantitative PCR with GoTaq DNA-Polymerase (Promega, Mannheim, Germany). PCR reactions (25 µl) consisted of 1 µl cDNA, 200 nM of sense and antisense primer (Invitrogen, Karlsruhe, Germany), 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 1.4 M Betain (Sigma-Aldrich, Munich, Germany), 1 U GoTaq DNA Polymerase (Promega, Mannheim, Germany), 5 µl of 5x GoTaq DNA polymerase buffer (MgCl₂ at a concentration of 7.5 mM), add nuclease free water. A touch-down PCR (74°C to 58°C, progressive transition of 2°C every three cycles) with a final amplification of 25 cycles was used. PCR has been repeated at least three times.

**Reporter gene constructs**

Genomic DNA from an individual bearing MolHap1 was used as template in a PCR with HighFidelity proofreading polymerase (Fermentas, St. Leon-Rot, Germany) for the generation of all promoter deletion constructs. The pCR8/GW/TOPO TA Cloning Kit (Invitrogen) was applied for subcloning into the Gateway compatible pGL3-Basic vector (Promega). MolHap2 and MolHap3 were generated using the QuikChange Site-directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands). Sequences of primers used for deletion construct generation are shown in data supplement table I. All vectors were sequenced to ensure sequence accuracy and identity.

**Transient transfections**

EA.hy926 and THP-1 cells were transfected using Nanofectin (PAA) with a DNA:Nanofectin ratio of 1:3.2 (24h). For co-transfections, expression vector for TF SP1 and reporter gene plasmids were transfected with a 1:3 ratio. Transfection experiments were repeated at least three times. Standard deviation of transfection triplicates is shown as bars and was calculated by unpaired Student’s t-test.

**Preparation of nuclear protein extracts**

Nuclear protein extracts were harvested by a modified procedure of the protocol published by Schreiber et al. (7).
EMSA

Oligonucleotides (35 bp, HPLC-purified; IBA, Göttingen, Germany) were 3’-biotinylated with biotin-16-ddUTP (Roche, Mannheim, Germany). Per reaction, 5 µg nuclear protein extracts were incubated with 500 ng pre-sheared poly(dI•dC) (USB, Staufen, Germany) as non-specific competitor and a 200-fold molar excess of unlabeled oligonucleotides as specific competitor. AP-1 (8) and SP1 (9) consensus oligonucleotides were used as positive controls. Additionally, membranes were detected with selected antibodies (anti-SP1, Millipore, Bedford, USA; anti-cFOS, Santa Cruz, Heidelberg, Germany; anti-PU.1, Santa Cruz) after blotting to detect proteins interacting with the presented probes. EMSAs are representative for experiments and were repeated at least three times.

ChIP

ChIP was performed as previously described (10, 11). DNA was sonicated using a Bioruptor (Diagenode, Liège, Belgium) until the chromatin had an average size of 300 - 500 bp (≤45min, 0.5s interval, 200W, 4°C). ChIP was conducted using 3 µg of selected antibody (anti-SP1; anti-GATA1, Santa Cruz; anti-CREB-P, Nanotools, Teningen, Germany). ChIP experiments were repeated three times.

CARMA study population

To link identified BGN promoter MolHaps with BGN mRNA level in circulating monocytes of patients with extensive carotid artery plaque, 22 patients were randomly selected from the “carotid samples for molecular analysis” (CARMA) Database. Patients admitted to the stroke unit of the department of Neurology of the University Hospital of Münster were enrolled between October 2011 and July 2012. All but one patient had suffered minor stroke (n=13), transitory ischemic attack (n=3), or transient (n=3) or persistent (n=2) monocular blindness attributable to the ipsilateral carotid artery as presenting symptom. One patient presented with asymptomatic carotid stenosis. Patients had a median age of 70 years (range: 50-85), 14 were male, 8 female. Median degree of stenosis (NASCET criteria) was 80% (range: 50-95). Eighteen patients (78%) were hypertensive, 11 (48%) had diabetes mellitus, 8 (35%) were smokers, 14 (61%) had hypercholesterolemia. Nine (39%) patients had a
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Methods references


