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\textsuperscript{−578},G\textsuperscript{−151},G\textsuperscript{+94}] MolHap2 [G\textsuperscript{−578},A\textsuperscript{−151},T\textsuperscript{+94}] and MolHap3 [A\textsuperscript{−578},G\textsuperscript{−151},G\textsuperscript{+94}]. 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 atherosclerosis, 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 atherosclerotic lesion development, mediating subendothelial low-density lipoprotein retention and oxidation.\(^4\) BGN-deficient mice (\textit{bgn}\textsuperscript{−/−}) present increased mortality after myocardial infarction compared with \textit{bgn}\textsuperscript{+/+} 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 \textit{BGN} expression,\(^8\) 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 heterogeneous by alternative transcription start site (TSS) activity and functionally characterized cis-active regulatory elements and transacting factors.

Materials and Methods

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 monocytic 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] ≈24%, BGN–MolHap2 [G−578,A−151,T+94] =36%, and BGN–MolHap3 [A−578,G−151,G+94] =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−578,G−151,G+94; Figure III in the online-only Data Supplement). To determine the effect of each MolHap on transcriptional activity of the BGN promoter, we introduced BGN–MolHap2 and BGN–MolHap3 in a 1025 base pair portion (position −893 to +132) promoter background. Although BGN–MolHap1 displayed sufficient transcriptional activity (Figure 2C and 2D) in EA.hy926 and THP-1 cells, MolHap2 led to a significant decrease of transcriptional activity (P<0.001, unpaired Student t test), whereas MolHap3 resulted in total abrogation of transcriptional activity compared with MolHap2 (P<0.05, unpaired Student t test) in both cell lines. Similar results were obtained, when BGN–MolHap2 and BGN–MolHap3 were introduced into deletion construct −1231/+162 (data not shown). To link the identified BGN promoter MolHaps with BGN mRNA level in circulating monocytes of patients with extensive carotid artery plaque using real-time PCR, 22 patients were randomly selected from the Carotid Samples for Molecular Analysis (CARMA) database. BGN mRNA level differed significantly between patients (Figure 2 in the online-only Data Supplement). Mean 1/ΔCt value of the 22 patients was 0.137 with lowest BGN expression in patient #10 (1/ΔCt=0.063), whereas patient #9 presented a 4-fold higher expression (1/ΔCt=0.255; P=0.0023). The intravariability difference in BGN expression was independent of classical risk factors (Table II in the online-only Data Supplement). However, statistical analysis of neither individual BGN promoter variants (Table IV in the online-only Data Supplement) nor BGN MolHaps (Table III in the online-only Data Supplement) revealed a association with BGN mRNA level in this study population.

**SP1 Is a Potent Enhancer of BGN Transcriptional Activity in Cotransfection Experiments**

To investigate which transcription factors (TF) are involved in BGN expression regulation, we analyzed changes in transcriptional activity of BGN reporter constructs during overexpression of potential transcriptional regulators. Web-based algorithms were used for in silico DNA sequence analyses and revealed an overall GC-content >60% for the BGN promoter, including a CpG-island spanning positions −198 to +138 (336 base pairs). A screen for conserved transcription factors binding sites using the 2 algorithms AliBaba2.1 and PROMO, both accessing the database TRANSFAC, which comprises data on eukaryotic TFs, was performed. Four highly homologous SP1 consensus sites (matrix dissimilarity rate <3%) were detected within the proximal promoter region (Figure 2B). Overexpression of SP1 in EA.hy926 cells and cotransfection of BGN promoter constructs resulted in the significant transcriptional activation of all promoter deletion constructs compared with pCMV vector shuttle control (Figure 3A; all probability values <0.01, unpaired Student t test). SP1 cotransfection increased transcriptional activity by an average of ≈2.8-fold, whereas the strongest effect was seen for the 5′-UTR–deficient 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).

**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 ≈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 G151A 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).
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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).

**Figure 4.** Transcription factor (TF) complex AP-1 binds at site G+94T. Immunoblot of gel shift experiments (electrophoretic mobility shift assay [EMSA]) using a 3'-biotinylated probe resembling position +94 (wild-type +94G or mutated A allele) and EA.hy926 or THP-1 nuclear protein extracts. A and B, The presented probes were detected with an antibiotin antibody. The allele-specific shift is marked by an arrow. EMSA reactions contained 40 fmol of probe and an unspecific competitor (poly[dI•dC]). The sequence-specific competitor was applied at 200-fold excess (8 pmol). C, The unlabelled oligonucleotide was detected with an anticFOS antibody (open arrow). An unlabeled AP-1 consensus sequence was used as positive control. Lanes without oligonucleotide served as control for unbound protein separation in native gels (neg.).

**Figure 5.** SP1 binding at the G-151A site in EA.hy926 cells is independent of the present allele. Electrophoretic mobility shift assay (EMSA) immunoblot with probes resembling position –151 (wild-type –151G or mutated A allele) and EA.hy926 or THP-1 nuclear protein extracts. A and E, The presented probes were detected with an antibiotin antibody. The specific shifts are marked by arrows. EMSA reactions contained 40 fmol of probe and an unspecific competitor (poly[dI•dC]). The sequence-specific competitor was applied at 200-fold excess (8 pmol). B, Two SP1 motifs flanking the –151G competitor were deleted by mutation of either 2 nucleotides (SP1M). C, Application of the mutated biotinylated probe SP1M with EA.hy926 extracts. D, Detection of the bound unlabelled oligonucleotide with an anti-SP1 antibody (black arrow). SP1M served as negative control for SP1 binding. Lanes without oligonucleotide served as control for unbound protein separation in native gels (neg.). F, Application of a SP1 consensus oligonucleotide as specific competitor in THP-1 G-151A band shifts. G, Application of the mutated, biotinylated probe SP1M with THP-1 protein extracts.
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. 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. Subsequently, gel shifts using nuclear extracts from untreated and TGF-β1–stimulated THP-1 monocytes were detected with a specific PU.1 antibody (Figure 7A). PU.1 was identified at the specific band shift (Figure 6E and 6F, lower grey arrow), whereas increased signal intensity of PU.1 was observed when TGF-β1–stimulated protein extracts were used. Potential differences in protein binding affinity for probes harboring the G or A allele were assessed using oligonucleotide serial dilutions and the PU.1 antibody (Figure 7B). In these experiments, under identical signal-detection conditions, a first signal for the –578G allele appeared at probe concentrations of 0.25 pmol, whereas the band representing the –578A allele appeared at concentrations of 1.0 pmol.

**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,21 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.22

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 search 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.23–25 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.11,26 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.27 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,28 sumoylation, or ubiquitylation.29

Furthermore, we observed altered binding affinity of PU.1 at position −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 module formation. 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,30 resulting in repression of BGN expression.

The ECM modulator TGF-β1 has been shown to be an important regulator of BGN transcriptional and post-translational modifications,17,31 whereas promoter deletion analyses under TGFβ1 stimulation have been discussed controversially.20,32 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.18 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,20,31,32 which represented the particular MolHap3 and excluded the polymorphic 5′-UTR site G+94T.
BGN affects scar formation and remodeling processes after stable aortic plaques are enriched in BGN and versican,3 and low-density lipoprotein.4 In turn, BGN is essential for development by reduced retention and oxidation of subendothelial in the ECM could prevent early atherosclerotic lesion development at different stages. Persistent low level of BGN expression based on BGN expression level in we detected significant differences of BGN finally to the predisposition to CVD phenotypes. Interestingly, 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.33,36 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.

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.4 In turn, BGN is essential for controlled collagen deposition in the vasculature. Fibrous caps of stable aortic plaques are enriched in BGN and versican,1 and BGN affects scar formation and remodeling processes after myocardial infarction.5 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.37 Besides transcriptional regulation, degradation of proteoglycans is at least equally important in the pathogenesis of vascular disease.33 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 13,34 and aggrecanases, such as ADAMTS4 and ADAMTS5 (a disintegrin-like metalloproteinase with thrombospondin type 1 motif).35 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.33,36 BGN gene expression is only part of the complex processes affecting ECM composition. The lack of significant association of BGN with CVD phenotypes until today could be due to 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.37 The latest GWA on multiple CVD-related traits by Middelberg and colleagues38 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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Figure 8. A proposed model for the individual transcriptional regulation of biglycan (BGN). MolHap1, A transcriptional module combined of SP1, ETS family members, and a selective AP-1 complex binds to MolHap1 [G-578-G-151-G-94]. Local BGN expression regulation is established by binding of cell type–specific ETS transcription factors (TFs). SP1 binding in the proximal promoter region and 5′-UTR initiates subsequent DNA bending, leading to convergence of the transcriptional module and interaction of distal ETS TFs and the AP-1 complex. MolHap2, An altered composition of the AP-1 complex for MolHap2 [G-578-A-151-T+94] leads to impaired interaction within the transcriptional module. MolHap3, Modified binding affinities for cell-specific ETS family TFs in presence of MolHap3 [A-578-G-151-G-94] result in the loss of transcriptional transactivation. The corresponding transcriptional activity is indicated by an angled arrow, weaker transcriptional activity is indicated by dotted lines.
Disclosures

None.

References


Significance

Molecular haplotypes (MolHaps) represent distinct individual allele combinations on a single DNA strand and can only be detected by subcloning of patients’ genomic DNA. Expression patterns of cardiovascular disease (CVD) candidate gene products may be differentially affected by these specific allele combinations in regulatory DNA regions. The extracellular matrix proteoglycan biglycan (BGN) is involved in CVD pathophysiology, as it mediates the subendothelial retention of atherogenic apoB-containing lipoproteins, affects adaptive remodeling after myocardial infarction and exerts proinflammatory effects in macrophages. By use of electrophoretic mobility shift assays, chromatin immunoprecipitation assays, and cotransfection of transcription factors, we identified SP1, ETS family members, and an AP-1 complex to interact differentially with the BGN promoter in the context of each individual MolHap. Our results indicate that individual MolHaps within the regulatory region of the human BGN gene may contribute to the molecular basis of interindividually different BGN expression. This might possibly modulate the individual predisposition to CVD-related phenotypes.
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## Supplemental material

### Table SI

#### Supplemental table 1

<table>
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<td><strong>Oligonucleotide sequences for generation of promoter deletion constructs</strong></td>
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**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 $1/\Delta C_t$ 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**

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<th>High BGN (n=9)</th>
<th>Low BGN (n=13)</th>
<th>P-value</th>
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<td>Gender (m)</td>
<td>4 (44%)</td>
<td>10 (77%)</td>
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<td>Age (&lt;70 years)</td>
<td>3 (33%)</td>
<td>7 (54%)</td>
<td>0.4214</td>
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<td>ACI symptom (stroke)</td>
<td>5 (56%)</td>
<td>8 (62%)</td>
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<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>
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<tr>
<td>CHD</td>
<td>3 (33%)</td>
<td>6 (46%)</td>
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<td>PVD</td>
<td>3 (33%)</td>
<td>4 (31%)</td>
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<tr>
<td>Smoking</td>
<td>3 (33%)</td>
<td>5 (38%)</td>
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<tr>
<td>Days after symptom onset (&lt;4)</td>
<td>3 (33%)</td>
<td>7 (54%)</td>
<td>0.2555</td>
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</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 $1/\Delta C_t > 0.137$ 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

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<th>Haplotype</th>
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<th>Mean BGN expression 1/ΔCt (SEM)</th>
<th>P-value</th>
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<td>G(^{-578})G(^{-151})-G(^{+94})</td>
<td>4</td>
<td>0.1275 ± 0.04</td>
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<tr>
<td>G(^{-578})-A(^{-151})-T(^{+94})</td>
<td>17</td>
<td>0.1438 ± 0.02</td>
<td>0.6746</td>
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<tr>
<td>A(^{-578})-G(^{-151})-G(^{+94})</td>
<td>7</td>
<td>0.1503 ± 0.02</td>
<td>0.6116</td>
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*BGN* haplotypes G\(^{-578}\)-A\(^{-151}\)-T\(^{+94}\) and A\(^{-578}\)-G\(^{-151}\)-G\(^{+94}\) were tested independently against haplotype G\(^{-578}\)-G\(^{-151}\)-G\(^{+94}\). A heterozygous female carrier of the very rare haplotype A\(^{-578}\)-A\(^{-151}\)-T\(^{+94}\) (1/ΔC\(_t\) = 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/ΔC\(_t\) ± standard error of the mean (SEM).

**Table SIV**

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

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<td>-578</td>
<td>G</td>
<td>22</td>
<td>0.1435 ± 0.01</td>
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<td></td>
<td>A</td>
<td>8</td>
<td>0.1509 ± 0.02</td>
<td>0.7848</td>
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<td>-151</td>
<td>G</td>
<td>12</td>
<td>0.1444 ± 0.02</td>
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<td></td>
<td>A</td>
<td>18</td>
<td>0.1470 ± 0.02</td>
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<td>+94</td>
<td>G</td>
<td>11</td>
<td>0.1420 ± 0.02</td>
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<tr>
<td></td>
<td>T</td>
<td>19</td>
<td>0.1475 ± 0.01</td>
<td>0.8252</td>
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Student’s t-test was used to determine level of significance for *BGN* expression between groups. Mean *BGN* expression is given as 1/ΔC\(_t\) ± standard error of the mean (SEM).
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-b1).
- **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'-CTCTGCCAGGCTGCCGCAC-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'-CTGACCACCACACTGCTTAGCAC-3'; antisense primer 5'-GTGATGGCATGGACTGTGGT CATGAG-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 (MgCl$_2$ 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
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.

**Methods references**

1. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA*. 1983; **80**:3734-3737.


