ZNF202 is inversely regulated with its target genes ABCA1 and apoE during macrophage differentiation and foam cell formation

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Abstract The zinc finger protein ZNF202 is a transcriptional repressor that binds to promoter elements predominantly found in genes involved in lipid metabolism. Here we demonstrate that ZNF202 mRNA expression is inversely correlated with ATP binding cassette A1 (ABCA1), ABCG1, and apolipoprotein E (apoE) in human monocytes. Upregulation of ABCA1, ABCG1, and apoE expression during monocyte differentiation and foam cell formation was accompanied by a simultaneous downregulation of both ZNF202 mRNA isoforms m1 and m3. Conversely, deloading caused upregulation of ABCA1, ABCG1, and apoE expression during macrophage differentiation. Using site-directed mutagenesis, we show that two highly conserved transcription factor binding sites, a GC-box and an Ets-binding motif, are required for ZNF202 gene expression. Furthermore, electrophoretic mobility shift assays demonstrate in vitro binding of Pu.1 and GC-box binding proteins to the ZNF202 proximal promoter. We conclude that the inversely correlated transcriptional activity of ZNF202 and its target genes during macrophage differentiation may reflect a direct regulatory interdependence and thus provide further evidence for ZNF202 as an important gatekeeper of lipid efflux.

Supplementary key words transcriptional repressor • HDL metabolism • promoter analysis • Pu.1 • GC-box binding proteins

The recruitment of monocytes to the arterial wall and their subsequent differentiation into macrophages may initially serve a protective function by removing atherogenic modified LDL particles and apoptotic cells. However, progressive uptake of lipid-rich particles and the resulting development of macrophage foam cells are a hallmark of both early and late atherosclerotic lesions (1). Macrophages are able to release excess cholesterol by efflux transport mechanisms, with nascent pre-β HDL particles serving as the primary extracellular acceptor. In addition, macrophages may contribute directly to the availability of extracellular cholesterol acceptors through secretion of apolipoprotein E (apoE) (2), which is capable of contributing to the formation of HDL particles. Thus, mechanisms mediating cholesterol efflux are critical for maintenance of cholesterol homeostasis in the macrophage and the prevention of foam cell formation.

A low HDL cholesterol locus on chromosome 11q23 has been recently identified that is distinct from the apoA-I/C-III/A-IV/A-V gene cluster (3). This novel familial susceptibility locus for hypoalphalipoproteinemia contains the zinc finger protein ZNF202 that is functionally characterized by a SCAN (SRE-ZBP; CT-finS1; AW-1; Number 18) oligomerization domain, a Kruppel-associated box repression domain, and eight CysX2His3 DNA binding motifs (4). Two splice forms have been described for ZNF202, the m1 form encoding the full-length protein, and the m3 form encoding a truncated version containing only the SCAN domain and lacking the DNA-binding motif (4). Interestingly, the majority of ZNF202 target genes play a critical role in HDL homeostasis. This group of genes comprises the apoA-I/C-III/A-IV/A-V gene cluster on chromosome 11, the apoE/C-
I/C-IV/C-II gene cluster on chromosome 19, phospholipid transfer protein, and the enzymes lipoprotein lipase, hepatic triglyceride lipase, LCAT, and phosphatidylethanolamine \( \text{N-methyltransferase} \).

Two additional target genes for repression by ZNF202, ATP-binding cassette A1 (ABCA1) and ABCG1, have been recently identified (5). ABCA1 is a key regulator of plasma HDL levels, and mutations in this molecule cause familial HDL-deficiency syndromes (6). The half-size transporter ABCG1 is another member of the group of cholesterol-responsive ABC transporters, and also supports lipid efflux in human macrophages (7, 8). Expression of both ABCA1 and ABCG1 is induced in human monocytes during phagocytic differentiation and subsequent lipid loading (7, 9). ZNF202, which acts as a transcriptional repressor of both genes, is able to reduce phospholipid and cholesterol efflux in transiently transfected macrophage cells, demonstrating the functional relevance of this gene (5).

In view of the large number of ZNF202 targets that are all involved in lipid and, in particular, in HDL cholesterol metabolism, ZNF202 seems to orchestrate the expression of these gene clusters, and therefore regulate a functional network that controls reverse-cholesterol transport and the susceptibility to atherosclerosis.

To further define the role of ZNF202 in modulating macrophage gene expression, we analyzed the transcriptional regulation of ZNF202 during monocyteic differentiation and lipid loading and unloading. Our data show that ZNF202 is inversely regulated to its target genes ABCA1 and apoE. Furthermore, we cloned the human ZNF202 promoter and determined important regulatory regions. In monocytic cells, PU.1 and GC-box binding proteins play a major role for ZNF202 gene expression. Therefore, we conclude that ZNF202 expression itself may be an important factor for controlling the balanced expression of genes involved in lipid metabolism.

**EXPERIMENTAL PROCEDURES**

**Cell culture**

THP-1 cells were obtained from ATCC; cultured in RPMI 1640 medium (Sigma, St. Louis, MO); supplemented with 10% fetal calf serum (Gibco BRL), 100 units of penicillin per ml, and 100 \( \mu \text{g} \) of streptomycin per ml; and incubated in 10% \( \text{CO}_2 \) in air at 37°C. To induce macrophage differentiation, THP-1 cells were cultured in the presence of 160 nM PMA. To induce foam cell formation of differentiated cells acetylated LDL (acLDL, 100 \( \mu \text{g} \)/ml) was added for 24 h.

Human monocytes were obtained from healthy donors by leukapheresis and counterflow elutriation. The cells were cultured on plastic petri dishes in macrophage serum-free keratinocyte growth medium (SFK) (Gibco BRL, Karlsruhe) and allowed to differentiate for 4 days in the presence of 50 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF) (R & D Systems, Wiesbaden, Germany). Macrophages were incubated in the presence of enzymatically modified LDL (eLDL, 40 \( \mu \text{g} \)/ml) to induce sterol loading and foam cell formation for additional 24 h. LDLs (d = 1.006–1.063 g/ml) were purified from human plasma obtained from healthy volunteers according to published standard protocols (7). The preparation was performed in a Beckman L-70 ultracentrifuge (70 Ti rotor) at 4°C, and densities were adjusted with solid KBr. Lipoprotein fractions were dialyzed repeatedly in PBS containing 5 mM EDTA. After the final dialysis step (0.15 M NaCl), LDL lipoproteins were sterilized using a 0.45 \( \mu \text{m} \) sterile filter (Sartorius, Göttingen, Germany). eLDL was prepared according to the protocol by Bhakdi et al. (10).

### 5′-RACE-PCR

In order to identify the 5′ end of the ZNF202 cDNA and to map the transcriptional start site, 5′-RACE-Ready cDNA prepared from human testis poly(A) RNA (BD Biosciences Clontech, Palo Alto, CA) was amplified according to the instructions of the manufacturer. In brief, nested PCR reactions were conducted with the provided anchor primers and nested primers derived from the published ZNF202 cDNA sequence (Acc. Nr. B0113882): 5′-CCTTGGGCTTCGTCACACCATGAGAGC3′ and 5′-GCAATTGCCAGTGTCCACACATTGTG-3′. The longest PCR products were cloned, analyzed by DNA sequencing, and compared with the human ZNF202 genomic sequence.

**Reporter and expression plasmid constructs**

Primers for the amplification of the human ZNF202 promoter sequence were based on the genomic sequence provided by Myriad Pharmaceuticals Inc. (WO-09945112). Human genomic DNA isolated from leukocytes using the Qiamp blood kit (Qiagen, Hilden, Germany) served as a template for the amplification of the promoter sequence with the High Fidelity PCR System (Roche, Mannheim, Germany). Reporter constructs of the ZNF202 promoter sequence were cloned by ligation of PCR fragments into the Xhol and HindIII restriction sites of the pGL3 basic vector. The 5′-deletion constructs, as well as the constructs with mutated transcription factor binding sites, were prepared by

**TABLE 1. Oligonucleotides used to generate luciferase constructs and in EMSA**

| Oligonucleotides used to generate luciferase constructs | Oligonucleotides used in EMSA (sense strand) |
|--------------------------------------------------------|---------------------------------------------|
| ZNF202pro-144f 5′-gcccagactactctgcgtgtagcaagcgtc-3′   | ZNF202 GC-box 5′-ctactgacagagggctggaggtg-3′ |
| ZNF202pro-94f 5′-gcccagactaccccttcgtgtagcaagcgtc-3′ | ZNF202 GC-box_mut 5′-ctactgacagagggctggaggtg-3′ |
| ZNF202pro-44f 5′-gcccagactaccccttcgtgtagcaagcgtc-3′ | ZNF202 GC-box_mut 5′-ctactgacagagggctggaggtg-3′ |
| ZNF202pro-94f 5′-gcccagactaccccttcgtgtagcaagcgtc-3′ | ZNF202 GC-box_mut 5′-ctactgacagagggctggaggtg-3′ |
| ZNF202pro-44f 5′-gcccagactaccccttcgtgtagcaagcgtc-3′ | ZNF202 GC-box_mut 5′-ctactgacagagggctggaggtg-3′ |
| ZNF202pro-94f 5′-gcccagactaccccttcgtgtagcaagcgtc-3′ | ZNF202 GC-box_mut 5′-ctactgacagagggctggaggtg-3′ |

EMSA, electrophoretic mobility shift assay; Inr, pyrimidine-rich initiator; ZNF202, zinc finger protein 202.

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PCR. The sequences of the oligonucleotides used for generating the various constructs are listed in Table 1. The identity of the subcloned DNA fragments was confirmed by DNA sequencing. A promoterless pG3 basic vector served as negative control, while a pG3 control vector that contains the cytomagavirus promoter was used as positive control. THP-1 cells were transfected by the diethylaminoethyl-dextran procedure as described previously (11, 12), and cultured in the presence or absence of PMA. In all experiments, THP-1 cells were cotransfected with the pSV β-galactosidase vector in order to normalize differences in transfection efficiency. Cells were harvested 24 h after transfection and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase assay reagent containing lucryl-CoA was added after centrifugation. Luciferase activity was determined in a LUMAT LB9501 (Berthold). The β-galactosidase enzyme assay (Promega) was used for the determination of β-galactosidase activity. Each experiment was repeated three times with two distinct plasmid preparations, and measurements were done in triplicate.

**Northern blot analysis**

The isolation of total RNA was achieved using the TRI system according to the manufacturer’s protocol (Molecular Research Center, Cincinnati, OH). Briefly, monolayer cell cultures from THP-1 or human monocytes were lysed directly in 100 mM culture dishes by the addition of 0.8 ml of TRI reagent supplemented with 8 μl of microcarrier gel. The lysate was mixed with 0.1 ml of 1-bromo-3-chloropropane and centrifuged at 12,000 g for 15 min at 4°C. Total RNA was precipitated from the separated aqueous phase by the addition of isopropanol, and the pellet was dissolved in stabilized formamide. RNA samples were quantified spectrophotometrically. Total RNA (6.0 μg) was fractionated by agarose-formaldehyde denaturing gel electrophoresis and transferred to nylon membranes (Hybond-N; Amersham). In order to hybridize multiple probes simultaneously, the nylon membrane was cut according to the expected mRNA molecular weights. Labeled ABCA1, apoE, ZNF202, and β-actin cDNAs were synthesized in vitro in the presence of [α-32P]dCTP (Amersham) from a human cDNA template using random primers and Klenow enzyme (Prime-a-Gene; Promega), and were used to probe the Northern blots. The cDNA templates were generated by PCR amplification from a human liver cDNA (Clontech, Palo Alto, CA) using the primer pairs 5'-GGTGTGGTCGTGTGGAAAGACCTC-3' and 5'-AGATAATCCCCGTAAACCAAGG-3' for ABCA1, 5'-ATGAGGTCTGGTGGCTGTTG-3' and 5'-CAGTTCTGGGTGACTTGGGAAG-3' for apoE, and 5'-ACTGCCGATGCTTTCACAG-3' and 5'-CCTGGACGTGCTTATTCACACAG-3' for ZNF202. The cDNA template for β-actin was obtained from Clontech. Hybridization analysis was carried out in Quickhyb solution (Stratagene) at 65°C and visualized by autoradiography. The mRNAs were quantified and normalized to β-actin mRNA abundance using densitometric scanner analysis (Molecular Dynamics) of autoradiograms exposed in the linear range of film density.

**Real time quantitative PCR analysis**

Real time quantitative RT-PCR (TaqMan™) analysis of ZNF202 expression was performed with an ABI7700 machine (PE Biosystems). All reagents necessary for running a TaqMan™ RT-PCR assay, including primers and probes, were purchased from PE Biosystems and used according to the manufacturer’s instructions. The sequences of forward and reverse primers as designed by Primer Express (PE Biosystems) and specific for isoform m1 of ZNF202 were 5'-GCCCTCCCCACCAGCATCA-3' and 5'-CTTCTGTACTCCGCCTCTTGCTC-3', and for isoforms m1-m3 were 5'-AGAAGAGGAGGAATTCCTCAGGTGAA-3' and 5'-CCGGCTCTCCTCTGTAAAG-3'. The sequence of the TaqMan™ fluorogenic probes for ZNF202 m1 was 5'-TGTTATACCGTGCCCGTTGGA-3' and for m2 ZNF202 m1+m3 5'-AGATGATTTTACCCTGCGGCCAGAGTCTG-3'. The reaction parameters were: 2 min, 50°C hold; 30 min, 60°C hold; 5 min, 95°C hold, followed by 35 cycles of 20 sec 94°C melt and 1 min 60°C anneal/extend. Measurements were carried out in triplicate. Results were analyzed with an ABI sequence detector software version 1.6.3 (PE Biosystems). Quantitation was performed relative to a standard curve for GAPDH. For GAPDH quantitation, the detection kit was purchased (PE Biosystems) and used according to the manufacturer’s instructions.

**Electrophoretic mobility shift assay**

An equivalent of 40,000 cpm of double-stranded oligonucleotide probe containing the desired promoter sequence was incubated with 10 μg of nuclear extract from THP-1 cells in a buffer containing 50 mM Hepes/Cl, pH 7.9, 6 mM MgCl2, 50 mM dithiothreitol, 100 μg/ml BSA, 0.01% NP40, and 2 μg poly(dI-dC) (Pharmacia) at room temperature for 20 min. Supershift analysis was carried out using 1 μl antisera (Santa Cruz) against the indicated transcription factors. In competition experiments, nuclear extracts were preincubated with a 100-fold molar excess of competitor for 10 min prior to addition of the radiolabeled probe. The oligonucleotides used in electrophoretic mobility shift assay (EMSA) analysis are listed in Table 1. DNA-protein complexes were resolved on a native 8% polyacrylamide gel and autoradiographed with Kodak BioMax MR films at −80°C.

**DNA sequence analysis**

Alignments of human and mouse promoter sequences were generated using by BLAST2 (13). Analysis of promoter regulatory elements was performed using MATINSPECTOR (14) and MOTIF (15, 16) search analysis. The transcription start site was predicted with TSSW and TSSC.

**RESULTS**

Phagocytic differentiation of human monocytes and subsequent lipid loading using modified LDL has been shown to induce the expression of ABCA1, ABCG1, and apoE (7, 9, 17, 18). In contrast, overexpression of the transcriptional repressor ZNF202 in macrophages has been shown to result in a significant reduction of apoA1-mediated, and particularly HDL3-mediated, lipid efflux (5). Here we have analyzed the mRNA expression of ZNF202 during monocytic differentiation and foam cell formation. In the first model, THP-1 cells were in vitro differentiated into macrophages by culturing in the presence of 160 nM PMA and subsequently into foam cells by incubation with modified LDL (acLDL). The expression patterns of ABCA1, apoE, and ZNF202 were analyzed by Northern blotting. A representative experiment is shown in Fig. 1. We observed an induced expression of ABCA1 (Fig. 1A) and apoE (Fig. 1B), whereas the mRNA level of ZNF202 was downregulated during differentiation, and further repressed by cholesterol loading (Fig. 1C). These data clearly indicate for the first time an inverse mRNA regulation of ZNF202 and its target genes ABCA1 and apoE.

In order to analyze ZNF202 expression in primary cells, human monocytes were cultured in the presence of M-CSF to induce differentiation, incubated with eLDL to achieve
foam cell formation, and subsequently exposed to HDL₃ for cholesterol depletion. To distinguish the expression pattern of the two common ZNF202 mRNA splice variants (the full-length m1 form and the truncated m3, which lacks the eight DNA binding zinc fingers and contains only the SCAN domain), we established a selective quantitative TaqMan™ RT-PCR assay. As shown in Fig. 2, both ZNF202 isoforms are highly expressed in freshly isolated human monocytes and are significantly downregulated during differentiation and foam cell formation (56% ± 1.1%, P < 0.001 and 50% ± 1.4%, P < 0.001, respectively), confirming our results obtained with the THP-1 cell model. Interestingly, deloading of macrophages with HDL₃ restores ZNF202 mRNA levels (71% ± 1.6%, P < 0.001). This induction seems to depend on the cholesterol content of the cells and may not be due to longer differentiation, since foam cells cultured in the absence of HDL₃ for 6 days do not upregulate ZNF202 expression to the same extent (53% ± 0.4%, P < 0.001) (Fig. 2). As seen in Fig. 2, no significant difference between the m1 and m3 splice forms could be observed. Therefore, the function of ZNF202 may not be modulated by differential expression of the two splice forms. Again, the expression pattern of ZNF202 reflects a regulatory interdependence with ABCA1, whose expression has been previously shown to be regulated inversely (9).

The intriguing expression pattern of ZNF202 in monocytic cells prompted us to clone and characterize the human ZNF202 promoter. As a first step, we mapped the transcriptional start site by 5′-RACE-PCR with primers derived from the 5′ end of the published human ZNF202 cDNA sequence (Acc. Nr. BC013382). Following cloning and sequence analysis, the longest 5′-RACE-PCR products were compared with the human and murine genomic regions. This experimentally determined transcription initiation site, which is indicated by an arrow in Fig. 3A, is in good accordance with the position predicted with both TSSW and TSSG computer algorithm programs. The ZNF202 proximal promoter sequence is GC rich and lacks a canonical TATA-box within an appropriate distance to the transcription initiation site. However, a pyrimidine-rich initiator sequence that overlaps a consensus E-box motif (19) is located directly at the transcription start site (Fig. 3A).

The BLAST2 algorithm was applied to search for conserved sequences in the proximal 1,000 bp between the human and murine 5′ flanking regions. As depicted in Fig. 3B, the sequence alignment reveals more than 70% similarity and displays (particularly in the very proximal upstream region) islands of high conservation that indicate the presence of a functional ZNF202 promoter. The potential DNA binding sites for transcription factors in the human ZNF202 promoter sequence predicted by the MatInspector software are shown in Fig. 3A and B. The first 500 bp of the proximal promoter region feature binding sites for Ets proteins (pos. −23), a GC-box (pos. −40), MZF1 (pos. −127), HSF2 (pos. −148), E47/AP4 (pos. −165), CCAAT/enhancer binding protein β (c/EBPβ) (pos. −241), SR-Y (pos. −348, −275, −265, −257), CP2 (pos. −391), AP1 (pos. −399), GATA1 (pos. −409), and YY1 (pos. −447). Among these putative binding sites, the sequence motif for E47/AP4, the GC-box, and the Ets binding site are highly conserved in the promoter regions of ZNF202.
of both human and murine ZNF202 genes (Fig. 3B), implying a functional relevance for these promoter regions. The four binding sites for SR-Y in the proximal promoter may imply an important role for this transcription factor in the high testis tissue-specific expression of ZNF202 (20). c/EBPβ and MZF1 have been characterized to mediate myeloid-specific gene expression, whereas YY1 has been established as a critical transcription factor for sterol homeostasis (21–23).

In order to locate the core promoter region of the ZNF202 gene and to analyze its activity in monocytic cells, we cloned a series of 5′ deletions of the ZNF202 upstream region in front of a luciferase reporter gene. Since primary human monocytes/macrophages are not transfectable, we used the monocytic THP-1 cell line for our promoter assays. The differentiation of THP-1 cells into macrophages was accomplished by incubation with the phorbol ester PMA (11). A reporter construct encompassing 1,061 bp of the ZNF202 5′ flanking sequence reflected low promoter activity in THP-1 cells (Fig. 4A). Reporter constructs with shortened proximal regulatory sequences of only 511 bp and 247 bp, however, revealed significant increases in promoter activity. In contrast, a reporter construct with only 85 bp of proximal regulatory sequence reflected again low promoter activity in the range of the 1,061 bp construct. Exposure of the transfected THP-1 cells to PMA led to a strong activity repression of all investigated reporter constructs (Fig. 4A). Particularly, the activity of the reporter construct for the 247 bp proximal promoter region was reduced 6-fold. These deletion analyses revealed that i) the ZNF202 promoter region between 1,004 bp and 511 bp contains a silencing element, ii) the 247 bp proximal region contains the core promoter sequence required for basal activity, and iii) the −247 bp

![Fig. 3. Sequence of the upstream regulatory region of the ZNF202 gene. A: The genomic sequence of 1,061 nucleotides preceding exon 1 of the human ZNF202 gene is illustrated. Potential transcription factor binding sites identified by MATINSPECTOR and MOTIF search are boxed. The transcriptional start site determined by 5′-RACE-PCR and TSSW and TSSG computer algorithms is indicated by an arrow. The 5′ borders of luciferase reporter constructs are displayed by brackets. B: Comparison of the proximal mouse and human ZNF202 promoter sequences. Homologous sequences were identified using BLAST2 software, and potential transcription factor binding sites are indicated by boxes.](image-url)
Fig. 4. Identification of functional promoter elements within the human ZNF202 promoter. A: Putative regulatory elements and luciferase activity analysis of pGL3 reporter gene vectors containing successively truncated 5’ regions of the ZNF202 gene are shown. THP-1 cells and PMA-differentiated THP-1 cells were transfected with each reporter gene construct and a pSVβ-gal plasmid, and cultured for 24 h. Representative experiments are shown that were independently repeated three times. Luciferase activity was normalized for β-galactosidase activity. Results are expressed as relative promoter activity and given as mean ± SD of triplicate measurements. B: THP-1 cells were transfected with additional luciferase 5’ deletion constructs to locate the silencing element between the −511 and the −1,104 construct to the region −170/−511. Furthermore, the effect of the CCAAT/enhancer binding protein (c/EBP) binding site (pos. −241) was studied by the −237 deletion construct. C: Site-directed mutagenesis and promoter analysis of the six potential transcription factor binding sites within the −247 construct. The mutated constructs, along with the wild-type construct, were transfected in THP-1 cells and assayed for luciferase activity.
core promoter region is highly repressed during macrophage differentiation, indicating the important role of this segment in the transcriptional regulation of the ZNF202 gene in monocytes and macrophages. To further narrow the silencing element in the distal ZNF202 promoter region, additional 5′-deletion constructs were created. As obvious from Fig. 4B, the −910, −799, and −700 bp constructs still had very low luciferase activities, indicating that the silencer sequence is located in the region from −511 to −700 bp of the promoter.

Since the −247 bp construct displayed the strongest promoter activity in monocytic cells, and in order to analyze the role of the six potential transcription factor binding sites in this region, site-directed mutagenesis in these sequence motifs was performed. The influence of the c/EBP binding site at position −241 was analyzed by further shortening of the construct to −237 bp. Although a reduction of luciferase activity was observed, the −237 bp construct still showed significant promoter activity, which was in the range of the −511 bp construct (Fig. 4B). A similar observation was made when using constructs with mutations in the E-box (pos. +1), the MZF1 binding site (pos. −127), the HSF2 site (pos. −148), and the E47/AP4 binding motif (pos. −165), demonstrating that these transcription factor binding sites may be of minor importance for ZNF202 promoter activity, at least in monocytes. In contrast, as shown in Fig. 4C, mutation of either the Ets binding site (pos. −23) or the GC-box (pos. −40) resulted in a complete loss of promoter activity, implying a critical functional role for these sequence motifs in ZNF202 gene expression in monocytes.

To characterize transcription factors that interact with these critical regions in the ZNF202 regulatory region, EMSA analysis was performed with synthetic double-stranded oligonucleotides covering the conserved binding sequences for Ets factors (−34/−5) and the GC-box (−58/−29) (Fig. 5). Incubation of the GC-box probe with THP-1 nuclear extracts resulted in the formation of two shifted bands (Fig. 5A, lane 2). To further confirm the specificity of the binding reaction, unlabeled GC-box oligonucleotide was used in 100-fold molar excess as a competitor. This resulted in a clear reduction of the shifted DNA-protein complexes (Fig. 5A, lane 3), whereas the mutated −58/−29 competitor had no effect (Fig. 5A, lane 6). The computer-assisted search for transcription factor binding sites revealed a strong binding probability of Sp factors to the GC-box, and since monocyte differentiation is known to be regulated by these factors, supershift assays were performed with antibodies against Sp1 (Fig. 5A, lane 4) and Sp3 (Fig. 5A, lane 5), which have been tested previously using positive controls derived from the human ABCA1 promoter (12). However, no supershifted band or inhibition of complex formation could be observed in these reactions, implying that the two specific protein complexes are neither composed of Sp1, nor of Sp3, but may rather represent other GC-rich element binding factors.

Since we have shown the functional importance of the Ets binding site in luciferase reporter assays (Fig. 4C), we were interested in analyzing whether Ets proteins are able to bind to the GGAA core sequence in the ZNF202 proximal promoter. Therefore, the −34/−5 probe was subjected to EMSA analysis with THP-1 nuclear extracts. This resulted in the formation of a prominent, slowly migrating band. (Fig. 5B, lane 2). Again, to prove the specificity of the complex, wild-type (Fig. 5B, lane 3) or mutated (Fig. 5B, lane 4) unlabeled oligonucleotides were used for competition experiments. The wild-type −34/−5 competitor oligonucleotide clearly inhibited complex formation, whereas the mutated −34/−5 competitor had no effect. When an Ets1/PEA3 consensus wild-type and mutated oligonucleotide was used in EMSA, a similar competition pattern was observed, indicating that an Ets protein family member specifically binds to the −34/−5 promoter sequence of the ZNF202 gene. Up to now, more than 30 Ets domain-containing transcription factors have been identified. Therefore, we focused on such proteins that are important for myeloid gene expression. In the first series of experiments, supershift assays with THP-1 nuclear extracts and antibodies against Ets1, Etv1, Erg1, Erg2, NERF, and Tel were performed. None of these antibodies, although reactive with positive controls, influenced DNA-protein complex formation (data not shown). This suggests that these factors are either not expressed in THP-1 cells or do not interact with the −34/−5 region. Next, PU.1-, Spi-B-, Fli1-, and Ets1/Ets2-specific antibodies were tested in EMSA (Fig. 5B). Whereas no binding of Spi-B (Fig. 5B,
ABCA1 has been characterized as a key regulator of cellular cholesterol and choline-phospholipid homeostasis (24–26). In addition, ABC transporters of the ABCG (White) subfamily have been shown to critically control cellular lipid trafficking (8). Exposure of macrophages to oxysterols and to retinoic acid has been shown to induce ABCA1 and ABCG1 gene expression via activation of the nuclear receptors LXR and RXR.

Overexpression of ZNF202, however, prevented the differentiation-dependent and LXR-RXR-mediated induction of ABCA1, demonstrating the ability of ZNF202 to interfere with critical promoter elements of this ABC transporter gene (5). In this report, we observed an up-regulation of ABCA1 in human monocytes during differentiation and loading with modified lipoproteins. Interestingly, the up-regulation of ABCA1 correlated with the simultaneous downregulation of ZNF202. Moreover, incubation with HDL₃ restored ZNF202 expression, indicating that the gene is modulated by macrophage differentiation and cellular cholesterol levels. We hypothesize that this inversely correlated expression of ABCA1 and ZNF202 may indicate a direct regulatory interdependence. ZNF202 overexpression has also been shown to result in a reduction of apoA-I- and HDL₃-mediated lipid efflux (5). The particularly strong suppression of HDL₃-mediated cholesterol efflux suggests that ZNF202 modulates, in addition to ABCA1, other genes with selective cholesterol transport activities. Likewise, apoE and ABCG1 have been shown to be transcriptionally controlled by ZNF202 and inversely expressed during monocyte differentiation into foam cells. Thus, transcriptional regulation of ZNF202 in monocytic cells may represent an important mechanism to control the expression of its target genes involved in lipid efflux.

Although two splice forms exist for ZNF202, the full-length m1 form and the m3 form lacking the DNA binding domain and containing solely the SCAN domain, no differential regulation of both isoforms could be observed in our monocytic cell model. A close relative to the m3 isoforms, the SCAN domain containing protein 1 (SDP1) has been shown to interact with ZNF202 via its SCAN domain, thereby preventing recruitment of the universal corepressor KAP1 and transcriptional silencing by ZNF202m1 (5). Very recently, it was demonstrated that SDP1 but not the ZNF202 m3 isoform is able to function as a coactivator of PPARγ2 by direct interaction via the SCAN domain and to enhance PPARγ2-dependent gene transcription (27). Thus, by activating PPARγ2 and blocking KAP1 binding to ZNF202, SDP1 could induce ABCA1 transcription. A similar mechanism could be envisioned for apoE, which is regulated by ZNF202 and indirectly regulated by PPAR ligands (28). Therefore, the balanced expression of ZNF202 and SDP1 is required to control transcriptional regulatory networks for the cellular lipid efflux machinery.

In this study, we have characterized crucial sequence motifs in the ZNF202 promoter and have started to elucidate the underlying mechanisms for the differentiation-dependent regulation of ZNF202 in monocytes/macrophages. Analysis of the human and mouse promoter sequence revealed stretches with strong sequence conservation that imply important biological functions. As for many other genes expressed in myeloid cells, the core promoter region is relatively short and lacks a classical TATA-box but contains a GC-box site in proximity to the transcriptional start site (29). We localized the core promoter region within the first 247 bp proximal sequence, and also observed the strongest luciferase reporter gene expression in undifferentiated THP-1 cells with this promoter region. A silencing promoter element resides within the region from −511 to −700 bp, and may also modulate ZNF202 gene expression.

Since the transcription factors Sp1, c-Ets, c/EBPβ, and MZF1 have been previously implicated in monocyte/macrophage gene expression (30), we especially focused on the binding sites for these factors in the ZNF202 promoter. MZF1 is a cell- and promoter-context-dependent bifunctional regulator of gene expression, which activates transcription in hematopoietic cells and represses transcription in nonhematopoietic cells (31, 32). In addition, MZF1 controls the proliferative potential of hematopoietic progenitor cells (33). Thus, MZF1 seems to stimulate cell proliferation in order to expand myeloid progenitors before going on to terminally differentiate (22, 34). Therefore, we speculated that the high expression of ZNF202 in undifferentiated monocytes and the concomitant downregulation during macrophage differentiation could be attributed to the loss of MZF1-dependent promoter activation. However, site-directed mutagenesis of the MZF1 binding site in the ZNF202 promoter region had no significant effect on promoter activity in monocytic cells. This indicated that at least in THP-1 cells, MZF1 is not a critical factor for ZNF202 gene expression.

In contrast to the MZF1 site, the GC-box (pos. −40) and the sequence motif for Sp1 (pos. −23) are highly conserved among the human and mouse promoters. Luciferase assays have clearly demonstrated a functional importance of both elements for ZNF202 promoter activity in THP-1 cells. Although two specific DNA-protein complexes have been identified at the GC-box, the nature of the transcription factors is still unknown and remains to be determined. We have shown that neither Sp1 nor Sp3, two important GC-box binding proteins (12), are able to bind to the proximal ZNF202 regulatory region.

In addition to the GC-rich element, the Ets factor-binding motif is of crucial importance for ZNF202 promoter activity. By competition with Ets1-PEA3 consensus oligo-
nucleotides, we could clearly demonstrate that an Ets factor binds to this sequence. Using antibodies against various Ets proteins, we could finally show that the PU.1 protein is the major component of the specific complex formed with THP-1 nuclear extracts and the ZNF202 Ets probe. Ets factors have several hematopoietic target genes and PU.1, Tel, Erg, Ets1, Ets2, and Fli1 are critically involved in hematopoiensis and leukemia (29). PU.1 is highly expressed in cells of the myeloid lineage, especially monocytes and neutrophils (35), and PU.1−/− mice develop multiple hematopoietic abnormalities (36–38). Therefore, it is conceivable that the high expression of ZNF202 in monocytes is controlled by PU.1.

In conclusion, our results suggest a direct transcriptional interdependence between ZNF202 and its target genes ABCA1, ABCG1, and apoE. Furthermore, we have characterized the ZNF202 core promoter region and demonstrated that a GC-box and an Ets consensus sequence binding PU.1 are absolutely necessary for ZNF202 promoter activity in monocytic cells. This study provides a useful basis for analysis of genetic variations in the ZNF202 regulatory regions, as well as potential pharmaceutical intervention to control the expression of ZNF202 target genes in lipid metabolism.

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