NR2F1 and IRE1β Suppress Microsomal Triglyceride Transfer Protein Expression and Lipoprotein Assembly in Undifferentiated Intestinal Epithelial Cells

Kezhi Dai, Irani Khatun, M. Mahmood Hussain

Objective—Our aim was to elucidate mechanisms involved in the acquisition of lipid transport properties during enterocyte differentiation.

Methods and Results—We show that lipid mobilization via apolipoprotein B lipoproteins is dependent on the expression of microsomal triglyceride transfer protein (MTP) during differentiation of Caco-2 cells into enterocyte-like cells. Mechanistic studies showed that binding of the nuclear receptor family 2 group F member 1 (NR2F1) to the DR1 element in the MTP promoter suppresses MTTP expression in undifferentiated cells. During cellular differentiation, NR2F1 expression and its binding to MTTP promoter decline and MTP induction ensues. Moreover, undifferentiated cells express inositol-requiring enzyme 1β (IRE1β), a protein that posttranscriptionally degrades MTP mRNA, and its expression substantially decreases during differentiation, contributing to MTP induction. Immunohistochemical studies revealed a significant negative relationship between the expressions of MTP and NR2F1/IRE1β in undifferentiated and differentiated Caco-2 cells, as well as in crypt–villus and jejunum–colon axes of mouse intestine.

Conclusion—We propose that transcriptional and posttranscriptional mechanisms involving NR2F1 and IRE1β ensure low MTP expression in undifferentiated intestinal cells and avoid apolipoprotein B lipoprotein biosynthesis.

Key Words: apolipoprotein B □ differentiation □ enterocytes □ gene transcription □ intestine □ lipoprotein assembly □ microsomal triglyceride transfer protein

Intestinal crypts harbor stem/progenitor cells that divide, migrate, and differentiate into enterocytes and absorb dietary constituents such as carbohydrates and lipids. Expression of carbohydrate hydrolyzing enzymes during enterocyte differentiation is dependent on Cdx2. Lipid absorption depends on the biosynthesis of triglyceride-rich lipoproteins that requires apolipoprotein B (apoB), a structural protein, and microsomal triglyceride transfer protein (MTP), an endoplasmic reticulum resident chaperone. Mechanisms promoting induction of apoB lipoprotein biogenesis and lipid absorption during differentiation of enterocytes are unknown.

Human colon carcinoma Caco-2 cells are used extensively to study cellular differentiation. Undifferentiated Caco-2 cells do not synthesize or secrete apoB lipoproteins. However, during culture these cells spontaneously differentiate into enterocyte-like cells and produce chylomicon-size apoB containing lipoproteins when supplemented with oleic acid. We report that 2 proteins, nuclear receptor family 2 group F member 1 (NR2F1) and inositol-requiring enzyme 1β (IRE1β), prevent MTP expression at transcriptional and posttranscriptional levels in undifferentiated intestinal cells and restrict apoB lipoprotein biosynthesis.

Materials and Methods

Chemicals and reagents, primary antibodies, and secondary antibodies were purchased from Sigma, Santa Cruz Biotechnology, and Invitrogen, respectively. Caco-2 cells were allowed to differentiate on transwells and studied for the secretion of apoB lipoproteins. These cells were also infected with adenoviruses expressing human MTP or transfected with various plasmids or siRNA. For Supplemental Methods, please see http://atvb.ahajournals.org. Experiments were performed in triplicate and repeated at least twice. Data are presented as mean±SEM. Statistical significances (P<0.05) were determined using the Student t test or 1-way analysis of variance (ANOVA), followed with the Bonferroni test (GraphPad Prism).

Results

ApoB Lipoproteins Are Secreted After MTP Induction in Differentiated Caco-2 Cells

Caco-2 cells were plated at 30% to 40% confluence and growth media were collected on alternate days to measure apoB and apolipoprotein AI (apoAI) secretions. These cells reached confluence at approximately day 6 as observed under light microscope. ApoB was barely detectable before day 6 (Figure 1A); its secretion increased 5-fold from day 8 to 16. ApoB100, a major form secreted by these cells, was...
detectable on day 8, and amounts increased thereafter (Figure 1B). ApoB48, another form of apoB arising from posttranscriptional editing of apoB mRNA, was evident on day 16. In contrast, apoAI was present throughout the culture period, consistent with its mRNA profile (Figure 1C, apoAI) and other studies.16 These data are in agreement with previous observations that proliferative Caco-2 cells do not secrete apoB lipoproteins; the ability to synthesize lipoproteins is acquired during differentiation into enterocyte-like cells.8,17

We reasoned that induction of apoB lipoprotein assembly might be linked to APOB gene transcription. However, apoB mRNA levels (Figure 1C, apoB) did not correlate with apoB secretion (Figure 1A) during culture. Hence, we hypothesized that APOB gene transcription might not be sufficient for its enhanced secretion after differentiation and other proteins are, perhaps, required. In addition to apoB, MTP is required for apoB lipoprotein secretion. MTP mRNA, activity, and protein increased slowly until day 8 and then rapidly until day 12; thereafter, triglyceride transfer activity either plateaus or declines modestly (Figure 1D, E). Therefore, MTP might be important for increases in apoB secretion during the early phase of cellular differentiation. Despite no further increase in MTP, apoB secretion continues to increase after day 12, indicating another protein/factor/mechanism that augments apoB secretion that was not studied here.

To determine the need for MTP in apoB secretion, undifferentiated cells were infected with adenoviruses expressing human MTP (AdMTP) or adenoviruses expressing human green fluorescent protein. Cells infected with AdMTP secreted more apoB than did adenoviruses expressing human green fluorescent protein-infected cells without altering expression of protein disulfide isomerase or tubulin (Figure 1F). Cells infected with multiplicity of infection of 1 and 10 AdMTP secreted 2-fold and 6-fold higher amounts of apoB, respectively, compared with adenoviruses expressing human green fluorescent protein-transduced cells (Figure 1G). Density gradient ultracentrifugation indicated that secreted particles had flotation density <1.024 g/mL (Figure 1H). Because MTP expression was very low in undifferentiated cells, no attempts were made to decrease MTP using siRNA. These data suggest that MTP transcription might be a limiting factor for apoB lipoprotein secretion in undifferentiated Caco-2 cells.

### HNF1, HNF4, and DR1 cis Elements Are Critical for MTP Induction During Differentiation

To investigate mechanisms of MTP induction, we performed run-on assays using nuclei isolated from undifferentiated (day 4) and differentiated (day 14) Caco-2 cells. Amounts of apoB mRNA synthesized on day 14 were higher than those seen on day 4 (Figure 2A, apoB). However, amounts of newly transcribed MTP mRNA from day 14 nuclei were 3-times higher after correction for increases in apoB mRNA than those from day 4 nuclei (Figure 2A), indicating that MTP gene transcription was induced significantly more than apoB gene transcription during differentiation.

To identify promoter sequences necessary for MTP induction, we performed transient expression using Renilla luciferase as transfection
control. The MTP promoter activity, as measured by increases in luciferase activity, was enhanced with increasing promoter length (Figure 2C, open bars). All constructs showed $\sim$2-fold higher promoter activity on day 10 compared with day 4 (Figure 2C, solid bars), indicating that the 204-bp promoter was as efficient as the longest in inducing luciferase expression during differentiation. Second, pMTP-204 and pMTP-1483 were stably expressed in Caco-2 cells and luciferase activity and apoB secretion were monitored over time. ApoB secretion increased during culture (Figure 2D) as seen in nontransgenic cells (Figure 1A). Moreover, luciferase expression in both cell lines increased by $\geq$4-fold (Figure 2D). This increase was similar to that observed in the run-off assay (Figure 2A), suggesting that the 204-bp promoter might be sufficient for MTP expression during Caco-2 cell differentiation.

To identify cis elements within the 204-bp critical for MTP transcription during differentiation, we mutated different cis elements as shown in Figure 2E. Mutation-bearing and wild-type promoter constructs were transiently expressed in undifferentiated cells, and luciferase activities were measured (Figure 2F). Promoter activity was reduced by 89%, 78%, and 35%, respectively, when HNF1, DR1, and HNF4 sites contained mutations (Figure 2F). Double mutations of DR1 and HNF4 decreased activity by 98% (Figure 2F, 2mut), indicating their importance in MTP induction during differentiation. A, Nuclei (5 $\times$ 10$^6$) from day 4 and 14 cultures were labeled with digoxigenin-UTP at 37°C for 30 minutes. The nascent RNA was hybridized to MTP and apoB48 cDNA cross-linked to nylon membranes. The RNA–DNA complexes were visualized using monoclonal antibodies against digoxigenin (Roche) and antimouse Alexa Fluor 633 antibody (top panel). Data from 3 experiments are presented as fold change compared to day 4 (bottom). B, A scheme showing the MTP promoter and 4 constructs with different 5’ ends cloned in a pGL2-Basic background so that expression of firefly luciferase was now under the control of MTP promoter sequences. ADH, alcohol dehydrogenase; DAPP1, dual adaptor of phosphotyrosine and 3-phosphoinositides 1. C, Each promoter construct was transiently transfected along with pCMV-RL that served as a transfection control. Cis-acting promoter activities were measured after 4 or 14 days and ratios of firefly:Renilla luciferase were obtained. Units are presented as fold change of pMTP-204 ratio on day 4. D, Cells stably expressing pMTP-204 or pMTP-1483 were cultured for 14 days. The specific luciferase activities (light unit/mg/sec) were presented as fold change from day 4. In addition, apoB secretion was determined using enzyme-linked immunosorbent assay. E–G, The cis elements SRE, HNF1, HNF4, and DR1 in the 204-bp promoter were subjected to site-directed mutagenesis. Mutant sequences are shown above the original (E). Activities of wild-type and mutant promoters were evaluated in Caco-2 cells by transient cotransfection with pCMV-RL, a transfection control (F). Caco-2 cells stably expressing wild-type pMTP-204 and different mutant sequences were cultured for 4 or 10 days (F). Specific luciferase activities (light unit/mg/sec) on day 4 were normalized to 1. Mean ± SEM; * P < 0.05; ** P < 0.01; *** P < 0.001, Student t test.
nation (Figure 2G) completely abolished differentiation-induced activation. These data indicate that cis elements HNF1, HNF4, and DR1 in the minimal promoter are critical for differentiation-dependent MTP induction.

Increased MTTP Transcription Is Attributable to Reduced Expression of NR2F1 During Caco-2 Cell Differentiation

Attempts were then made to identify transcription factors that bind to these cis elements and reduce MTP expression in undifferentiated Caco-2 cells. The enhanced binding of transcription factors HNF-4α, HNF-1α, and HNF-1β (Figure 3A, activators) to the 3 identified cis elements could induce MTTP expression during differentiation. To determine whether these proteins exhibit differential binding to the MTTP promoter before and after differentiation, we used chromatin immunoprecipitation followed by standard polymerase chain reaction (Figure 3B). Binding of HNF-4α and HNF-1α to the MTTP or APOB promoter did not change during differentiation. However, binding of RNA polymerase II to the MTTP promoter was enhanced after differentiation. Using chromatin immunoprecipitation and quantitative polymerase chain reaction, we noted 2-fold and 5-fold elevations in the binding of polymerase II and acetylated histone H3 to the MTTP promoter in day 14 cultures compared with day 4 cultures (Figure 3C), respectively, indicating transcriptionally active MTTP promoter after differentiation. Again, the bindings of HNF-4α and HNF-1 proteins to the MTTP promoter were not significantly changed before and after differentiation (Figure 3C). These studies indicate that differential binding of HNF-4α and HNF-1α to the MTTP promoter does not determine differentiation-dependent induction of MTP.

We then hypothesized that the induction of MTTP transcription might be attributable to reduced bindings of putative repressors (Figure 3A) such as NR2F1 (COUP-TF1 or Ear3), NR2F2 (COUP-TFI or Arp-1), and NR2F6 (Ear2). To test this hypothesis, we first determined their mRNA levels during Caco-2 cell differentiation. Expression of these repressors was high in undifferentiated cells but low after differentiation (Figure 3D). To identify their role in MTTP gene regulation, we reduced their expression using siRNA; siNR2F1, siNR2F2, and siNR2F6 specifically reduced expression of their respective target genes (Figure 3E). We then evaluated the effect of these siRNA on MTP (Figure 3F). Whereas siNR2F1 increased MTP mRNA, siNR2F2 and siNR2F6 had no effect, suggesting that NR2F1 might act as a repressor. Consistent with this impression, NR2F1 overexpression in undifferentiated Caco-2 cells significantly inhibited MTTP promoter activity (Figure 3G). Moreover, chromatin immunoprecipitation showed >60% reduction in NR2F1 binding to the MTTP promoter after differentiation (Figure 3H), as well as increased acetylation of the MTTP promoter in siNR2F1-treated Caco-2 cells (Figure 3I). Furthermore, NR2F1 protein levels were diminished after differentiation (Figure 3J). These observations indicate that NR2F1 acts as a repressor in undifferentiated cells. Decreases in NR2F1 during differentiation might be linked to MTTP promoter activation.

To confirm whether the MTTP promoter is repressed by NR2F1 and to identify cis elements required for this repression, Caco-2 cells stably expressing pMTP-204-luciferase and DR1m-
luciferase constructs were treated with various siRNA. Compared with siControl, siGL2 reduced luciferase activity by \( \approx 50\% \) indicating transfection efficiency. Knockdown of NR2F1 increased MTP promoter activity (Figure 3K, pMTP-204), whereas siNR2F2 and siNR2F6 had no effect. Caco-2 cells stably expressing a DR1m promoter construct did not respond to siNR2F1 (Figure 3K, DR1m). These observations indicate that the DR1 element is required for NR2F1 to repress MTP transcription in undifferentiated Caco-2 cells.

**Reduction in IRE1\( \beta \) Expression Contributes to Increased MTP mRNA During Differentiation**

MTP mRNA levels were enhanced by 15-fold after differentiation (Figure 1D). This was higher than the increases seen in MTP promoter activities (4-fold–5-fold; Figure 2D, G), suggesting that mechanisms other than transcriptional induction are also in play. Therefore, we looked for posttranscriptional mechanisms. Recently, IRE1\( \beta \) has been shown to regulate MTP mRNA posttranscriptionally.\(^1\) We found that undifferentiated Caco-2 cells highly express IRE1\( \beta \) mRNA; its amounts decline significantly \((\approx 4\)-fold decline\) after day 6, indicating differentiation-dependent reduction (Figure 4A). Next, we examined IRE1\( \beta \) protein by immunostaining (Figure 4B). Proliferating cultures at day 4 exhibited abundant IRE1\( \beta \) protein that colocalized with an ER marker, calnexin, as shown in yellow on the merge image. On day 14, Caco-2 cells showed a polarized morphology with the staining of calnexin suggestive of differentiated phenotype (Figure 4B, asterisk). IRE1\( \beta \) was significantly reduced in these differentiated cells. To understand the relationship between IRE1\( \beta \) reduction and MTP induction, we studied MTP expression in undifferentiated cells treated with siIRE1\( \beta \). As a control, we used siRNA against its homologue, IRE1\( \alpha \) (Figure 4C). IRE1\( \beta \) knockdown increased MTP mRNA without altering apob mRNA when compared with siGL2, whereas siIRE1\( \alpha \) had no effect, indicating that suppression of IRE1\( \beta \) increases MTP mRNA.

The studies described identified transcriptional and posttranscriptional mechanisms involving NR2F1 and IRE1\( \beta \) that control MTP expression during Caco-2 cell differentiation. Next, we determined individual and combined effects of these mechanisms on MTP expression and apoB secretion (Figure 4D). Knockdown of NR2F1 or IRE1\( \beta \) had no effect, whereas siNR2F2 and siNR2F6 had no effect. Caco-2 cells stably expressing a DR1m promoter construct did not respond to siNR2F1 (Figure 3K, DR1m). These observations indicate that the DR1 element is required for NR2F1 to repress MTP transcription in undifferentiated Caco-2 cells.

**Expression of NR2F1, IRE1\( \beta \), and MTP Along the Crypt–Villus and Jejunum–Colon Axes**

To obtain in vivo corollary to the cell culture experiments, we studied MTP, NR2F1, and IRE1\( \beta \) expression in mouse intestine by immunohistochemistry (Figure 5A). MTP was more abundant in jejunum than in ileum and was low in colon. In the jejunum and ileum, it was present mainly in the villi consistent with previous findings.\(^{20–23}\) IRE1\( \beta \) protein (Figure 5B) expression followed the order of: jejunum < ileum < colon. More IRE1\( \beta \) was present in crypts than in villi of the jejunum (Figure 5B). IRE1\( \beta \) expression gradient from jejunum to colon and from villus to crypt in the jejunum was reciprocal to intestinal MTP expression. Similarly, the expression patterns of NR2F1 (Figure 5C) were also reciprocal to those of MTP. Its expression was high in cells that express Ki67, a marker for dividing cells, and low in villi. These data indicate an inverse relationship in the expression of MTP with NR2F1 and IRE1\( \beta \) in the intestine.

To document further the reciprocal relationship between MTP and NR2F1 at functional level, we separated villus and crypt cells from mouse intestine. MTP mRNA and c-Myc, a marker for diving cells, protein levels were high in villus and crypt cells, respectively, indicating separation of these cells (Figure 5D, E). To determine whether NR2F2 interacts with

---

**Figure 4.** IRE1\( \beta \) expression is reduced during differentiation. A, IRE1\( \beta \) and APP\( \alpha \) mRNA were quantified and ratios on day 2 were normalized to 1. Fold change in IRE1\( \beta \) mRNA during Caco-2 cell differentiation is shown. B, Caco-2 cells were fixed (1% formaldehyde, 30 minutes), treated with methanol (5 minutes), blocked with phosphate-buffered saline containing 1% horse serum and 3% bovine serum albumin (30 minutes), and incubated (1 hour) first with antibody against IRE1\( \beta \) (sc-20575) in blocking buffer and then with anti-calnexin (sc-11397). Two secondary antibodies, antirabbit Alexa Fluor 555 and antigoat Alexa Fluor 488, were added and incubated (45 minutes). nDNA was stained with Topro3 blue (Invitrogen). Scale bar, 10 \( \mu \)m. C, Cells were treated with different siRNA and mRNA levels of IRE1\( \alpha \), IRE1\( \beta \), MTP, and apob were determined. Data were normalized to siGL2 and presented as means ±SEM. D, Caco-2 cells (100-mm dishes) were transfected with siGL2, siNR2F1, and siIRE1\( \beta \), alone or in combination. Intracellular MTP and GAPDH were determined with Western blotting. Cells were labeled with \( ^{35} \)S-methionine/cysteine and secreted apoB and apoAI were immunoprecipitated with 1D1 or 4H1 antibodies, respectively. Bands were scanned and quantified using ImageQuant (lower panel). Representative bands from 2 separate experiments are shown in the upper panel. E, Hypothetical model of intestinal MTP regulation. NR2F1 and IRE1\( \beta \) suppress MTP expression via transcriptional and posttranscriptional mechanisms. These 2 regulatory proteins might restrict MTP expression and chylomicron production in undifferentiated/dividing intestinal cells.
the MTTP promoter, we performed chromatin immunoprecipitation in these 2 fractions (Figure 5F). Binding of NR2F1 to the MTTP promoter was high in the crypt but low in the villi, similar to that observed in undifferentiated and differentiated Caco-2 cells, respectively. Therefore, we suggest that the binding of NR2F1 to MTTP promoter in crypt cells might suppress MTP expression in mouse intestine.

Discussion

MTP Expression Is Limiting for Lipoprotein Biosynthesis in Undifferentiated Intestinal Cells

We show that undifferentiated Caco-2 cells neither express MTP nor synthesize lipoproteins. In contrast, both undifferentiated and differentiated cells contain apoB mRNA. Adenoviral-mediated expression of human MTP induces apoB lipoprotein secretion in undifferentiated cells. Therefore, we propose that MTP is the limiting factor for the biosynthesis of apoB lipoproteins in undifferentiated cells. It is unclear why the regulation of MTP is related to lipid mobilization in enterocytes. Possibly, regulation of the synthesis of a shorter MTTP transcript than that of APOB might be more efficient.

NR2F1 Suppresses MTTP Expression in Undifferentiated Cells

Our studies indicate that HNF-4α and HNF-1α required for the synergistic activation of MTP in the liver bind to the MTTP promoter in both undifferentiated and differentiated Caco-2 cells. Despite their binding to the MTTP promoter, MTTP gene is not expressed in undifferentiated intestinal cells. Mechanistic studies revealed that NR2F1 suppresses MTP expression in undifferentiated cells by binding to the DR1 element in its promoter. During differentiation, NR2F1 expression is reduced and its binding to the MTTP promoter is decreased. Therefore, MTTP induction during differentiation depends on decreased binding of NR2F1 to its promoter.

NR2F1 and NR2F2 behave similarly during differentiation of neuronal cells into glial cells in mice. Consequently, we were surprised to observe that NR2F1, but not its homologues NR2F2 and NR2F6, modulates MTP expression during differentiation of Caco-2 cells. It has been shown that NR2F2 suppresses MTP expression in hepatoma cells, but very little is known about the role of NR2F1 in hepatic MTTP gene regulation. Our preliminary studies (not shown) indicate that NR2F1 is not expressed in hepatocytes. These observations indicate that NR2F1 might play a specific role in the suppression of MTTP gene in enterocytes.

Posttranscriptional Mechanisms Reduce MTTP mRNA Levels

In addition to the transcriptional suppression by NR2F1, we found that posttranscriptional mechanisms involving IRE1α might be operative in undifferentiated intestinal cells. IRE1α has been shown to posttranscriptionally degrade MTP mRNA and play a role in suppressing high-fat diet-induced MTP induction. This study shows that high expression of IRE1α in undifferentiated cells might ensure low MTP mRNA abundance. Knockdown of IRE1α alone or in combination with NR2F1 increases MTP expression and apoB secretion in undifferentiated cells. Therefore, reduced expression of proteins involved in MTTP gene repression and mRNA degradation may pave the way for MTP expression during differentiation.

MTTP Induction in the Intestine Involves Unique Mechanism

Mechanisms elucidated in this study for the induction of MTP during differentiation are different than those reported for the induction of sucrase-isomaltase and apoAI in intestinal...
epithelial cells. Induction of sucrase-isomaltase is dependent on the expression of Cdx2. ApoAI induction is high on day 8 and is preceded by significant induction in HNF-4α expression. Barrero and Malik have shown that, in addition to HNF-4α, PRMT1 acts as a coactivator of HNF-4α in the induction of apoAI during Caco-2 cell differentiation. Although MTP expression requires HNF-4α, we observed that MTP induction was not correlated with HNF-4α binding. Instead, MTP expression was dependent on the loss of NR2F1 and IRE1 expression during differentiation of intestinal cells. Thus, intestinal cells use different mechanisms to induce various genes during differentiation.

In addition to the intestine, MTP is also highly expressed in the liver. Very little is known about the induction of MTP during hepatic differentiation or regeneration. However, adenosine-mediated expression of PGC1β, Foxa2, and FoxO1 in hepatocytes increases MTP expression, and SHP reduces its expression. Surprisingly, NR2F2, which has been shown to repress MTP transcription in rat liver-derived cell lines, does not play a role in MTP induction in Caco-2 cells (Figure 2E, F). Because NR2F1 and IRE1β are not expressed in hepatocytes, the mechanisms identified in this report might be specific to intestinal MTP expression.

Acknowledgments

The authors thank Dr Titelman’s laboratory for help in confocal microscopy.

Sources of Funding

This study received funding from the National Institutes of Health (DK-46900) and the American Heart Association.

Disclosures

None.

References

NR2F1 and IRE1β Suppress Microsomal Triglyceride Transfer Protein Expression and Lipoprotein Assembly in Undifferentiated Intestinal Epithelial Cells
Kezhi Dai, Irani Khatun and M. Mahmood Hussain

*Arterioscler Thromb Vasc Biol.* 2010;30:568-574; originally published online December 10, 2009;
doi: 10.1161/ATVBAHA.109.198135

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/30/3/568

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/12/03/ATVBAHA.109.198135.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/
“Supplement Material”

Materials and Methods

Plasmids: Sequences 1483 bp upstream and 83 bp downstream from the transcription initiation site of human MTTP gene were cloned into pGL2-Basic (Promega) between Hind III and Kpn I sites to generate pMTP-1483. A similar strategy was used to clone pMTP-1181 and pMTP-204. For pMTP-775, a Hind III-Bgl II DNA fragment was excised from pMTP-1483 and inserted into pGL2-Basic. All insert sequences were identical to the human genomic sequence AC083902. QuickChange site-directed mutagenesis (Stratagene) was performed in pMTP-204 to yield SREm, HNF1m, HNF4m, DR1m, and 2mut constructs harboring specific mutations in different cis elements. The primers (Table 1) used for cloning were designed using Primer 3.0. Plasmid expressing NrF1 (pMT2-NR2F1) was kindly provided by Dr. Sotirios Karanthanasis.

Cell cultures: For differentiation, approximately 3 x 10^5 Caco-2 cells were placed in Transwells (Corning) and cultured in DMEM supplemented with 20% FBS \(^1\). The media were changed every other day until day 16. Conditioned media and cell cultures were collected and kept at -70°C until analysis. ApoB was quantified using ELISA \(^2,3\).

Infection of cells with adenoviruses: Caco-2 cells seeded at 30% confluency in 6-well plates. The next morning cells were washed once with DMEM containing 1%FBS and indicated MOI of adenovirus was added in 300 µl of DMEM containing 1%FBS. After 6 h incubation, 2 ml of DMEM supplemented with 10% FBS DMEM was added to each well and cells were allowed to grow overnight. Next day these infected Caco-2 cells were used for metabolic labeling or harvested for western blotting.

Transfection with plasmid DNA or siRNA: Cells in 12-well plates were 70% to 80% confluent on the day of transfection. Exgen 500 reagent (Fermentas) was used to deliver 500 ng of pMTP luciferase constructs with 20 ng of pCMV-RL (Promega) that expresses Renilla luciferase under the control of a cytomegalovirus promoter. After 48 h, cells were harvested for analyses. Variations in transfections were normalized to the expression of
Renilla luciferase. To stably transfect Caco-2 cells, we used 50 ng of pTRE2hyg (BD Bioscience) instead of pCMV-RL during transfection. After 2 days, cells were re-plated in 100-mm dishes and cultured in the presence of 300 µg/mL hygromycin. Colonies resistant to hygromycin were harvested after 2 weeks. Polyclonal cell lines were maintained in 100 µg/mL hygromycin. Lipofectamine 2000 reagent (Invitrogen) was employed to transfect 50 nM siRNA (Table 1) into Caco-2 cells at a ratio of 3.3 µl to 5 pmol siRNA. After overnight incubation, fresh media were added to dilute siRNA to 20 nM. Cells were collected for analysis after 2 days.

**Immunofluorescent Staining:** The cell staining procedure was described previously. Tissue samples from C57BL/6J mice were fixed in 4% paraformaldehyde, frozen in Tissue-Tek OCT compounds (Fisher Scientific), sectioned onto Tissue Tack slides (Polyscience), and incubated with 0.05% Tween 20 in 10 mM sodium citrate buffer (pH 6.0) at 90°C for 25 min. Slides were incubated in a blocking buffer, PBS containing 1% horse serum, 3% BSA and 1% Triton X-100, for 30 min before 1 hr incubation with primary antibodies followed by another hour incubation with secondary antibodies. All antibodies were diluted 1 to 300 in the blocking buffer and the whole immunostaining was carried out at room temperature. Images were obtained with a laser-scanning microscope (Model LSM 510, Carl Zeiss) and processed with Photoshop 6.0 before exporting to Illustrator CS2 (Adobe).

**Separation of mouse intestinal epithelial cells:** To isolate various intestinal cell types, jejunum (about 16 cm from the end of the duodenum) was removed from anesthetized mice, emptied, and rinsed with saline containing 1 mM dithiothreitol (DTT). The lumen was filled with 3 mL of 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH2PO4, 5.6 mM Na2HPO4 buffer pH 7.3, and incubated with continuous agitation in a 37°C saline bath for 15 minutes. The lumen was emptied and refilled with 3 mL of 1 mM EDTA and 0.5 mM DTT in PBS and incubated in the saline bath for 4, 2, 2, 3, 4, 5, 7, 10 or 15 minutes to collect 9 fractions. Rinsing the lumen with 2 mL of saline between incubations increased the yield.
RNA preparation and quantitative real-time PCR (qRT-PCR): RNA from Caco-2 or mouse intestinal cells was used to synthesize cDNA. The quantitative PCR \(^6\) was carried out with a SYBR Green I qPCR kit (Eurogenetic) using primers described in Table 1. Data were analyzed using the \(\Delta\Delta C_T\) method and are presented as fold change.

Chromatin immunoprecipitation (ChIP): ChIP was used to study the binding of transcription factors to the \(MTTP\) promoter \(^7\). Four 100-mm Transwell dishes (Corning) of 4-day Caco-2 cultures or 2 dishes of 14-day cultures were fixed with 1% formaldehyde. Isolated primary enterocytes were fixed with 1.5% formaldehyde. After 15 minutes at room temperature, cells were harvested. Nuclear fractions were isolated and sonicated (Sonic Dismembrator 550, 15 sec at setting 5, 16 bursts, 10-sec intervals). Approximately 25 \(\mu\)g of cross-linked genomic DNA in 1 mL 1%BSA/PBS was incubated with 50 \(\mu\)L of antibodies against NR2F1 (sc-6577), HNF-4\(\alpha\) (sc-8987) or HNF-1\(\alpha\) (sc-10791) overnight at 4\(^\circ\)C. Next day 50 \(\mu\)L of protein A/G beads (sc-2003) were added and centrifuged at 8,000 rpm for 5 min at room temperature. Precipitated DNA was extracted by phenol-chloroform after reversal of cross-linkage by incubating at 65\(^\circ\)C for 4 h. Real-time and regular PCR were carried out using primers (Supplementary Table 1) for \(MTTP\), \(HNF4A\), or \(APOB\) promoters to determine the binding of different transcription factors. For controls, non-immune serum (sc-2028) was used.
Supplementary Table 1. Oligonucleotide sequences of primers and siRNAs

### Primer pairs used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>5' primer</th>
<th>3' primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2F1</td>
<td>GCGTTCAGCGAGGAAGA</td>
<td>TGCCTACTGGCTGGATTG</td>
</tr>
<tr>
<td>NR2F2</td>
<td>ATCGCAACCAGTCCAGTACT</td>
<td>ACCGCTTTCCGTTCATG</td>
</tr>
<tr>
<td>NR2F6</td>
<td>ACCGAAACCAGTGCCAGTAC</td>
<td>TGACGCGCTCCTTCCT</td>
</tr>
</tbody>
</table>

### Primer pairs used in ChIP experiments

<table>
<thead>
<tr>
<th>Promoter ID</th>
<th>5' primer</th>
<th>3' primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOB</td>
<td>GAAGCCAGTGTAGAAAAAGCAAAAC</td>
<td>CAAGAGCAGCCAGATTGG</td>
</tr>
<tr>
<td>MTTP(^1)</td>
<td>CAGCCCACCTCAGTTAATATTT</td>
<td>GCAAACTCAGAAATACGAGCTA</td>
</tr>
<tr>
<td>MTTP(^2)</td>
<td>TGACCAGCAATCCTCAACT</td>
<td>CCAAACCTCAAGAATACGAGCTA</td>
</tr>
<tr>
<td>HNF4A(^3)</td>
<td>GGGTTCCCTAAGTGACTGGTT</td>
<td>ACCGCGCGTTTATTTATTT</td>
</tr>
<tr>
<td>mtp</td>
<td>TAGCCACCTCGCTTTAATCATT</td>
<td>CCAAACCAATACGCCAGACT</td>
</tr>
</tbody>
</table>

### Primers for cloning of WT or mutant MTTP promoter

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>5' primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Hind III) +83</td>
<td>CCGGCACTTTGGACAGCAATTCTCAAC</td>
</tr>
<tr>
<td>(Bgl II) -204</td>
<td>GGAAGATCTGTTAGCTCTCAAAAGTGA</td>
</tr>
<tr>
<td>(Kpn I) -1181</td>
<td>CGGGGTACCAGTTGAGAGTGACCAG</td>
</tr>
<tr>
<td>(Kpn I)-1483</td>
<td>CGGGGTACCCAAAGCACTTTACCATTC</td>
</tr>
<tr>
<td>Mut-SRE</td>
<td>GAGTGAGAGACTGAAAACTGCgaattCCTACGTCTAATC</td>
</tr>
<tr>
<td>Mut-HNF1</td>
<td>CCCACCTACGTTgAATCATgAATTAGTGAGCCC</td>
</tr>
<tr>
<td>Mut-HNF4</td>
<td>GGTCTGATTGTGGAGTGC CGGCCTTCCTCCCCAAG</td>
</tr>
<tr>
<td>Mut-DR1</td>
<td>GAGTTTTGGAGACTGACTgTTTCCCAC</td>
</tr>
</tbody>
</table>

### Sequences of siRNA forward strands

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Target sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2F1</td>
<td>rCrGrCrGrAUrAUrGUUrArGrCUrGUUrCU</td>
</tr>
<tr>
<td>NR2F2</td>
<td>rCrGrArGrArArUUUrUrGrCrArArCUrGUU</td>
</tr>
<tr>
<td>NR2F6</td>
<td>UrGrArCUrArArGrCrArArCUrArArUU</td>
</tr>
<tr>
<td>GL2-luciferase</td>
<td>rCrGuArCrGrGrArArCUrCUrCrGrATT</td>
</tr>
</tbody>
</table>

1. Primers for real-time PCR.
2. Primers for regular PCR.
3. β cell-specific P2 promoter, which is not accessible in intestinal cells.
Reference List


