

# TIMP3 Is Reduced in Atherosclerotic Plaques From Subjects With Type 2 Diabetes and Increased by SirT1

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**OBJECTIVE**—Atherosclerosis is accelerated in subjects with type 2 diabetes by unknown mechanisms. We identified tissue inhibitor of metalloproteinase 3 (TIMP3), the endogenous inhibitor of A disintegrin and metalloprotease domain 17 (ADAM17) and other matrix metalloproteinases (MMPs), as a gene modifier for insulin resistance and vascular inflammation in mice. We tested its association with atherosclerosis in subjects with type 2 diabetes and identified Sirtuin 1 (SirT1) as a major regulator of TIMP3 expression.

**RESEARCH DESIGN AND METHODS**—We investigated *ADAM10*, *ADAM17*, *MMP9*, *TIMP1*, *TIMP2*, *TIMP3*, and *TIMP4* expression levels in human carotid atherosclerotic plaques ( $n = 60$ ) from subjects with and without diabetes. Human vascular smooth muscle cells exposed to several metabolic stimuli were used to identify regulators of *TIMP3* expression. SirT1 small interference RNA, cDNA, and *TIMP3* promoter gene reporter were used to study SirT1-dependent regulation of *TIMP3*.

**RESULTS**—Here, we show that in human carotid atherosclerotic plaques, TIMP3 was significantly reduced in subjects with type 2 diabetes, leading to ADAM17 and MMP9 overactivity. Reduced expression of TIMP3 was associated in vivo with SirT1 levels. In smooth muscle cells, inhibition of SirT1 activity and levels reduced TIMP3 expression, whereas SirT1 overexpression increased TIMP3 promoter activity.

**CONCLUSIONS**—In atherosclerotic plaques from subjects with type 2 diabetes, the deregulation of ADAM17 and MMP9 activities is related to inadequate expression of *TIMP3* via SirT1. Studies in vascular cells confirmed the role of SirT1 in tuning *TIMP3* expression. *Diabetes* 58:2396–2401, 2009

**D** diabetes is characterized by accelerated atherosclerosis, although molecular mechanisms explaining this phenomenon are still undefined (1,2). We and others have shown that chronic hyperglycemia increases matrix metalloproteinase (MMP) and A disintegrin and metalloprotease domain (ADAM) activities providing a potential clue to atherosclerotic

plaque progression, as confirmed by studies using vasculature from subjects with diabetes (3–5). Increased MMP and ADAM activities may be linked also to unbalanced expression of endogenous inhibitors called tissue inhibitor of metalloproteinases (TIMPs) 1–4 (4). We identified the deficiency of TIMP3 as a link between insulin resistance and vascular inflammation (6–8). Recently, Paigen and colleagues (9) found *Timp3* gene among quantitative trait loci associated with diabetes and dyslipidemia, identifying a mutation causing lower gene expression in diabetic mice. Moreover, *Timp3* is among the few genes downregulated in a microarray analysis of pericytes treated with glycated oxidized LDLs (10). Because TIMP3 uniquely among TIMPs retains the ability to inhibit shedding enzymes such as ADAM17, which are involved in inflammatory processes (11), we hypothesized downregulation of TIMP3 as a hallmark for atherosclerosis in diabetic subjects. We tested this hypothesis in atherosclerotic plaques from subjects with different degrees of glucose tolerance, linking *TIMP3* expression to activity of deacetylase Sirtuin 1 (SirT1). SirT1 is a deacetylase localized at nuclear levels acting as transcriptional regulator either on histones or on transcription factors such as forkhead box class O1 (FoxO1), liver X receptor (LXR), p53, and transcriptional cofactors such as peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (12). Recently, it has been suggested that loss of SirT1 activity may be associated with metabolic diseases such as type 2 diabetes and atherosclerosis (13). Several laboratories have shown that SirT1 gain of function either by genetic manipulation or through ligand activation may protect from insulin resistance associated with obesity and from atherosclerosis in experimental disease models (14,15).

However, little is known about SirT1 activity in human subjects affected by atherosclerosis and diabetes. Our data reveal a new potential role for TIMP3 and SirT1 in the atherosclerosis process in subjects with diabetes.

## RESEARCH DESIGN AND METHODS

This study included 60 atherosclerotic plaques from normal glucose tolerant (NGT;  $n = 37$ ) or type 2 diabetic ( $n = 23$ ; according to medical records or oral glucose tolerance test) subjects in whom carotid endarterectomy for symptomatic disease was performed at Policlinico Tor Vergata University Hospital, Rome, Italy. Subject characteristics and treatments are described in Table 1. The study was approved by the ethics committee, and subjects provided informed written consent for the use of atherosclerotic material for research use. All procedures were performed according to the Declaration of Helsinki.

**Histological analysis.** Carotid plaques were removed en bloc during surgery to preserve plaque structure entirely. For histology, surgical samples were fixed for 24 h in 10% buffered formalin immediately upon removal. After decalcification, specimens were sectioned transversely every 5 mm and paraffin embedded. Hematoxylin-eosin was performed for morphologic study (4).

Immunohistochemistry was performed on serial 3- $\mu$ m thick sections cut from paraffin blocks of carotid plaques using the following antibodies: 1)

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TABLE 1  
Clinical data of patients subjected to carotid endarterectomy

	NGT	Type 2 diabetes
<i>n</i>	37	23
Sex (men/women)	25/12	14/9
Age (years)	71.0 ± 6.6	70.6 ± 8.9
BMI (kg/m <sup>2</sup> )	25.3 ± 3.9	24.1 ± 4.8
Hypertension (yes/no)	29/8	20/3
Antihypertensive drugs (yes/no)	23/14	15/8
Antiaggregant drugs (yes/no)	35/2	22/1
Statins (yes/no)	25/12	15/8
Total cholesterol (mg/dl)	188.9 ± 31.0	192.35 ± 36.86
HDL cholesterol (mg/dl)	45.32 ± 10.2	43.3 ± 9.6
LDL cholesterol (mg/dl)	115.7 ± 24.6	122.18 ± 28.1
Triglycerides (mg/dl)	125.69 ± 62.26	139.13 ± 72.04
A1C (%)	5.6 ± 0.2	7.2 ± 1.6*
Fasting plasma glucose (mg/dl)	89.7 ± 12.0	126.9 ± 51†
Fasting plasma insulin (μU/ml)	7.38 ± 5.59	12.96 ± 3.51
Oral agents/insulin treatment	—	15/8

Data are means ± SD and *n*. \**P* < 0.001 by Student's *t* test. †*P* < 0.001 by Student's *t* test.

polyclonal rabbit anti-human TIMP3 antibody (Calbiochem, San Diego, CA), 2) monoclonal anti-CD68 (human macrophage) antibody (DAKO, Glostrup, Denmark), and 3) monoclonal anti-α smooth muscle actin antibody (Europa Ventana Medical System, Illkirch, France). The primary antibodies were detected with avidin-biotin-peroxidase complexes (DAKO). All antibodies were used with positive and negative controls.

**Real-time quantitative RT-PCR analysis.** Frozen plaque samples were homogenized using a polytron homogenizer. Total RNA and single-strand cDNA were obtained as described (7). Real-time PCR RNA expression analysis of *ADAM17*, *ADAM10*, *TIMP3*, *TIMP1*, *TIMP2*, *TIMP4*, *MMP9*, and *Sirt1* (primers available upon request) was performed with ABI PRISM 7000 System (Applied Biosystems, Foster City, CA) and normalized to 18S rRNA. Each reaction was carried out in duplicate and analysis performed by 2<sup>ΔΔCt</sup> method as described previously (8).

**ADAM17 and MMP9 activities.** Proteins were extracted as previously described (4,6–8). ADAM17 activity was determined by the Sensolyte 520 ADAM17 Activity Assay Kit Fluorimetric (AnaSpec, San Jose, CA). MMP9 activity was measured by the Amersham MMP-9 Biotrack Activity Assay System (GE Healthcare U.K.) according to manufacturer's instructions. Active MMP9 was detected through activation of the modified prodetection enzyme and subsequent cleavage of its chromogenic peptide substrate. The resultant color was read at 450 nm in a microplate spectrophotometer (Victor 1420).

**LDL preparation, cell culture, and Western blots.** LDL preparation (16), cell culture, and Western blots are described in detail in the online appendix at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-0280/DC1>.

**Timp3 promoter regulation assay.** For *Timp3* promoter regulation assay, coronary artery smooth muscle cells (CASMCs) were transfected with 2 μg Human SirT1 cDNA Clone (RG218134; Origene), 10 ng renilla, and *Timp3* Gene Promoter Reporter Vector (LR1034; Panomics) or 2 μg TransLucent Control Vector using primary smooth muscle cells Nucleofector solution with program A-033 (AMAXA). The luciferase assay was performed with Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions.

**Statistical analysis.** Statistical analysis was performed using Student's *t* test, one-way ANOVA, and Pearson's correlation coefficient (*r*) on SPSS software program v. 13.0 for Windows. Data are expressed as means ± SD. *P* < 0.05 was considered statistically significant.

## RESULTS

Our data show that in atherosclerotic plaques, among *ADAM10*, *ADAM17*, *MMP9*, *TIMP1*, *TIMP2*, *TIMP3*, and *TIMP4*, only *TIMP3* expression was lower in those with type 2 diabetes than those with NGT (Fig. 1A and B). Western blot assay confirmed the significant decrease of

*TIMP3* in subjects with type 2 diabetes (Fig. 1C). Immunohistochemistry completed the link between *TIMP3* downregulation and diabetes (Fig. 1D and G, for non-type 2 diabetic and type 2 diabetic subjects, respectively). Analysis of consecutive sections with anti-CD68 for macrophages and anti-α smooth muscle actin for smooth muscle cells suggested that both cell types are associated with *TIMP3* expression in NGT subjects (Fig. 1E and F for NGT subjects and Fig. 1H and J for type 2 diabetic subjects; negative control for antibodies in supplemental Fig. 1). To verify that the reduction of *TIMP3* in atherosclerotic plaques resulted in increased ADAM17 and MMP9 activity, we used a fluorimetric assay. We found that both ADAM17 and MMP9 activities were higher in those with type 2 diabetes than in those with NGT (Fig. 1K and L).

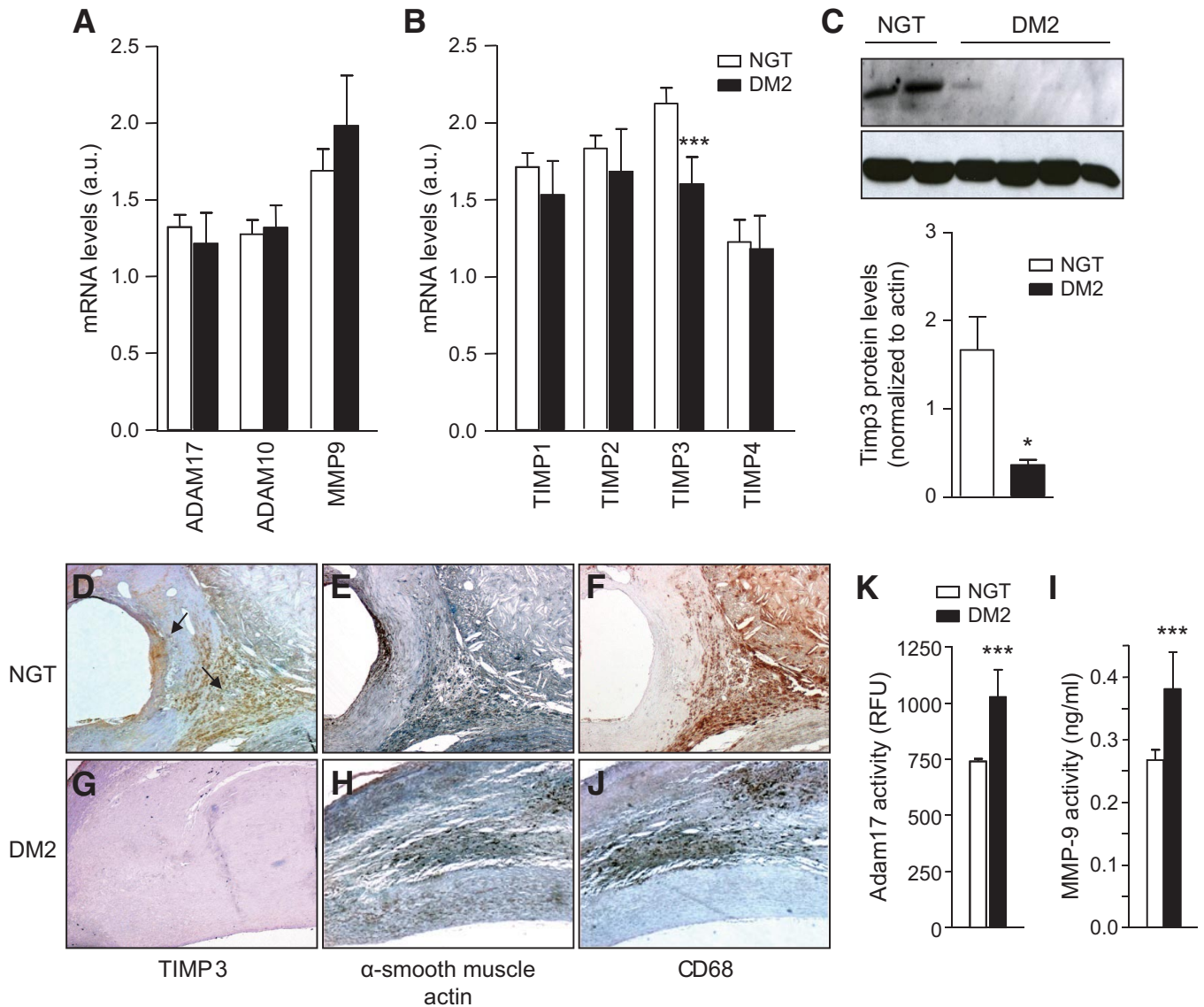
Analysis of clinical characteristics (Table 1) showed that *TIMP3* expression negatively correlates with LDL cholesterol (*r* = -0.29; *P* < 0.03) and A1C (*r* = -0.31; *P* < 0.02) but not with sex, age, or pharmacological treatment.

To identify metabolic factors reducing *TIMP3* expression, CASMCs were treated with high glucose (20 mmol/l), mannitol (osmotic control, 20 mmol/l), hyperinsulinemia (10<sup>-7</sup> M), LDL, oxidized LDL, and glycated LDL (100 μg/ml). Because previous data suggested that LXR regulates *TIMP3* expression (17), we used LXR agonists such as T0901317, 22-s, and 22-r hydroxycholesterol as well as GW3965. Because LXR is regulated by SirT1 deacetylase (12), we also used SirT1 inhibitor Sirtinol. Interestingly, we found that among the various treatments, only high glucose and Sirtinol significantly reduced *TIMP3* expression in CASMC (Fig. 2A).

Treatment of CASMC with Sirtinol determined an increased metalloprotease activity as measured by ADAM17 activity assay (Fig. 2B). Sirtinol and high glucose significantly reduced *TIMP3* expression also in human umbilical vein endothelial cells (HUVECs) and monocytic THP1 cells (Fig. 2C). Analysis of CASMC, HUVEC, and THP1 revealed that high glucose significantly reduced *Sirt1* expression (Fig. 2D) in CASMC and THP1 with a trend also in HUVEC. *Sirt1* expression was significantly reduced in atherosclerotic plaques from subjects with type 2 diabetes compared with those from NGT subjects (Fig. 2E), and we observed that *TIMP3* expression positively correlated with *Sirt1* expression (*r* = 0.4, *P* < 0.03; Fig. 2F), although NGT and type 2 diabetic subjects were mixed and therefore diabetes may represent a confounding factor. To confirm a direct role of SirT1 in regulating *TIMP3* expression, we used a small interference RNA approach. Knockdown of SirT1 in CASMC resulted in marked reduction of *TIMP3* expression but not *TIMP1*, *TIMP2*, *TIMP4*, *ADAM10*, *ADAM17*, or *MMP9* (Fig. 3A); a similar effect was observed in HUVEC and THP1 cells (Fig. 3B). To substantiate SirT1 effects on *TIMP3*, CASMC were co-transfected with human *Sirt1* cDNA and *Timp3* promoter luciferase reporter vector, confirming that SirT1 positively modulates *TIMP3* expression (Fig. 3C). Finally, in CASMC, HUVEC, and THP1 we found that *Sirt1* overexpression prevented the reduction of *TIMP3* expression determined by high glucose (Fig. 3D).

## DISCUSSION

We recently observed that *TIMP3* deficiency is necessary to develop fatty streaks characterized by macrophage infiltrate using the insulin receptor heterozygous mouse

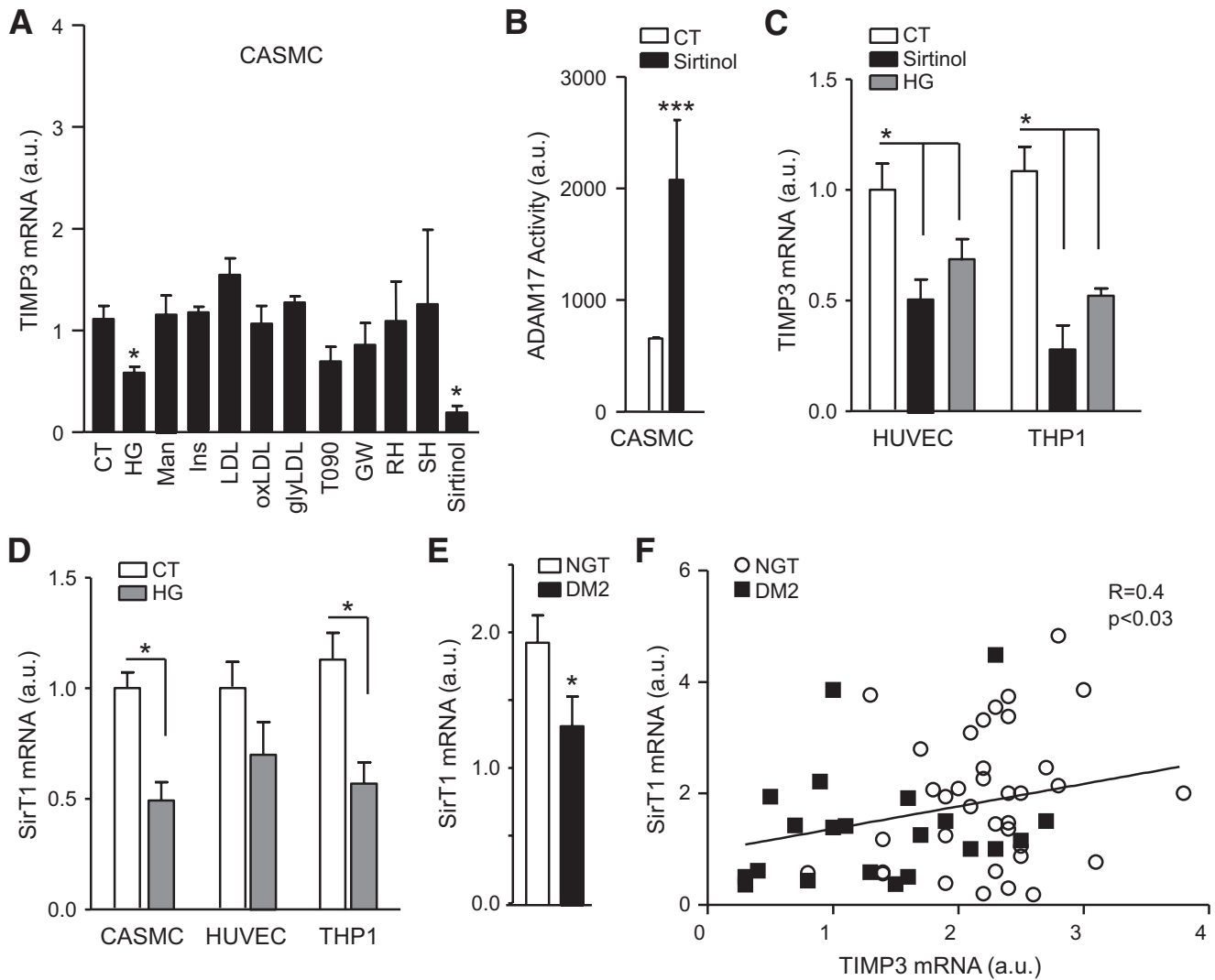


**FIG. 1.** TIMP3 is reduced in atherosclerotic plaques of subjects with type 2 diabetes (DM2). ADAM10, ADAM17, and MMP9 (A) as well as TIMPs (B) expression in NGT ( $n = 37$ ) and type 2 diabetes ( $n = 23$ ) subjects;  $***P < 0.001$  by one-way ANOVA. C: Western blot using extracellular matrix extracts from representative NGT ( $n = 2$ ) and type 2 diabetic ( $n = 4$ ) subjects.  $*P < 0.05$  NGT vs. type 2 diabetes by Student's  $t$  test. D–J: Immunohistochemistry confirmed that TIMP3 is reduced in type 2 diabetic ( $n = 8$ ) versus NGT ( $n = 8$ ) subjects; one representative image is shown for TIMP3, anti- $\alpha$  smooth muscle actin, and CD68 for NGT (D–F; 4 $\times$  magnification) and type 2 diabetic (G–I; 4 $\times$  magnification) subjects. K and J: ADAM17 activity measured by a fluorimetric assay (K) and MMP9 activity measured by a fluorimetric assay (J) are increased in type 2 diabetic ( $n = 23$ ) compared with NGT ( $n = 37$ ) subjects;  $***P < 0.001$  by Student's  $t$  test for both. (A high-quality digital representation of this figure is available in the online issue.)

model fed a diet rich in lipids (8). The relevance of TIMP3 is demonstrated by the reverse phenotype caused by TACE deficiency in the same mouse model, suggesting that loss of TIMP3 may favor the development of atherosclerotic lesions (8). Whereas other studies on models such as ApoE and LDL receptor knockout are necessary to fully characterize the role of TIMP3 in the progression of atherosclerosis under diabetic conditions, here we analyzed its role in human atherosclerosis accompanied by diabetes. Our results suggest that subjects with diabetes exhibit reduction of TIMP3, as well as increased activity of ADAM17 and MMP9, possibly because of the more intense oxidative stress caused by hyperglycemia, a known activator of both the enzymes in a protein kinase C-dependent manner (3,4,18,19). Therefore, our data suggest that metabolic-dependent reduction in TIMP3 expression may increase the activity of inflammatory and proteolytic en-

zymes, which play a role in atherothrombosis (20,21). Previous studies showed that TIMP3 expression was increased in extracts from atheroma compared with non-atherosclerotic tissue in nondiabetic subjects (22). In view of our results, TIMP3 reduction is emerging as a specific factor in the atherosclerosis process of subjects with diabetes. Loss of TIMP3 may lead to increased tumor necrosis factor- $\alpha$  and epidermal growth factor receptor signaling, potentially increasing the inflammatory burden inside the atherosclerotic plaque (6–8). Moreover, loss of TIMP3 increases MMP9 activity in atherosclerotic plaques, a feature known to be increased in vasculature from subjects with type 2 diabetes (5), and may potentially affect plaque stability in the long term.

The role of TIMPs in diabetic atherothrombosis is still undefined. Recent data in animal models supported a role for an imbalanced MMP-to-TIMP ratio favoring increased

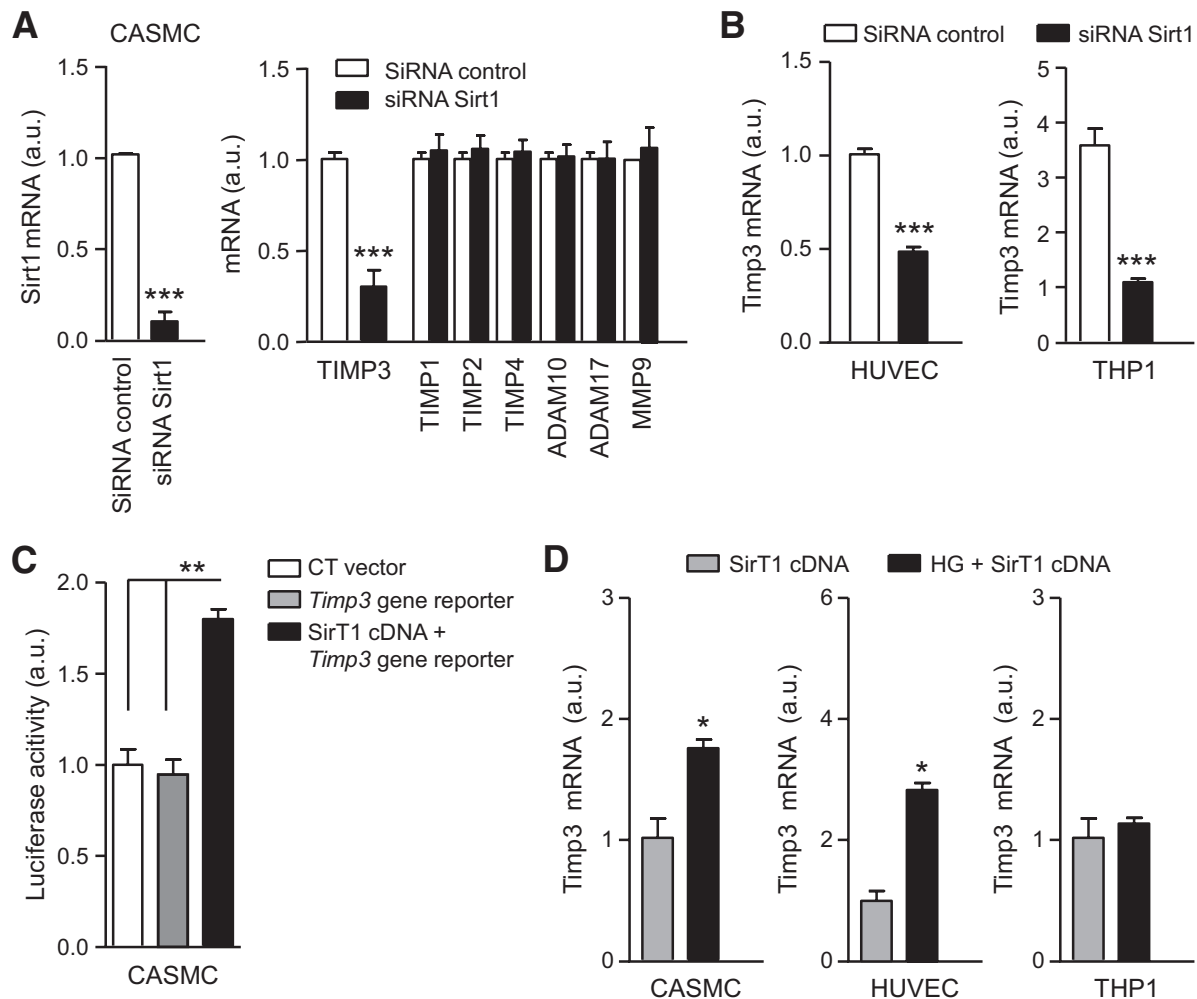


**FIG. 2.** Effects of diabetes on *TIMP3* expression in vascular cells. **A:** *TIMP3* expression in CASMC treated with various metabolic stimuli: high glucose (HG) 20 mmol/L; mannitol (Man) 20 mmol/L; insulin (Ins)  $10^{-7}$  M; LDL 100  $\mu$ g/ml; oxidized LDL (oxLDL) 100  $\mu$ g/ml; glycated LDL (glyLDL) 100  $\mu$ g/ml; LXR agonists (T0901317 [T090] 5  $\mu$ mol/L; GW3965 [GW] 3  $\mu$ mol/L; R-hydroxycholesterol [RH] 10  $\mu$ mol/L; 22-S-hydroxycholesterol [SH] 10  $\mu$ mol/L); SirT1 inhibitor (Sirtinol) 50  $\mu$ mol/L.  $n = 4$  for all experiments; \* $P < 0.05$  by Student's *t* test versus control (CT). **B:** Sirtinol increased ADAM17 activity in CASMC.  $n = 4$  for all experiments; \*\*\* $P < 0.001$  by Student's *t* test. **C:** *TIMP3* expression in HUVEC and THP1 treated with Sirtinol and high glucose (20 mmol/L).  $n = 4$  for all experiments; \* $P < 0.05$  by Student's *t* test versus control (CT). **D:** SirT1 expression is reduced in CASMC, HUVEC, and THP1 treated with high glucose (20 mmol/L) compared with control.  $n = 4$  for all experiments; \* $P < 0.05$  by Student's *t* test. **E:** SirT1 levels are decreased in type 2 diabetic compared with NGT subjects. \* $P < 0.05$  by Student's *t* test. **F:** *SirT1* correlates with *TIMP3* in atherosclerotic plaques from NGT ( $n = 37$ ) and type 2 diabetic (DM2) ( $n = 23$ ) subjects.

degradation of extracellular matrix that may promote progression of atherosclerosis (23).

Factors regulating *TIMP* expression in atherosclerotic plaques are undefined, although previous data suggested a role for growth factors such as transcription growth factor- $\beta$  and platelet-derived growth factor (23). However, the role and regulation of *TIMP3* in diabetic vascular disease has been thus far unexplored. Our data suggested that *TIMP3* is negatively associated with A1C and LDL cholesterol levels. Exposure of CASMC to different stimuli linked to gluco- and lipotoxicity revealed that both high glucose and inhibition of the deacetylase SirT1 led to reduced *TIMP3* expression and activity. SirT1 is emerging as a master of integrated metabolic response to nutrient availability (12–15,24). Data from Apolipoprotein E knockout mice suggest that overexpression of *SirT1* from endothelial cells may defend against atherosclerosis progression, although the basic mechanisms remained undefined. Our results, via knockdown of SirT1 or its

overexpression, confirmed a role for this deacetylase in the modulation of *TIMP3* expression in vascular smooth muscle cells, and especially monocyte/macrophage. SirT1 controls gene expression through deacetylation of histones and transcription factors; one or both of the two mechanisms may be involved in regulating *TIMP3* expression. Previous studies using T0901317 compound, an LXR agonist, suggested that LXR transcription factors regulate *TIMP3* expression (17). In our cell systems we did not observe an effect of T0901317. However, this may depend on several factors including different experimental models and culture conditions. Moreover, SirT1 is a positive regulator of LXR as well as other transcription factors potentially involved in *TIMP3* expression such as FoxO1 (25). Because *SirT1* overexpression is able to rescue *TIMP3* expression in the presence of glucotoxicity, it is possible that SirT1 affects events linked to de-repression of *TIMP3* promoter via transcription factors such as FoxO1 or histone deacetylation.



**FIG. 3.** Regulation of *TIMP3* expression in CASMC. **A:** SirT1 knockdown decreased *TIMP3* expression but not *TIMP1*, *TIMP2*, *TIMP4*, *ADAM10*, *ADAM17*, or *MMP9* in CASMC.  $n = 4$  for all experiments;  $***P < 0.001$  by Student's *t* test versus control. **B:** SirT1 knockdown decreased *TIMP3* expression in HUVEC and THP1.  $n = 4$  for all experiments;  $***P < 0.001$  by Student's *t* test. **C:** SirT1 cDNA overexpression increased *Timp3* promoter activity.  $n = 4$  for all experiments;  $**P < 0.01$  by one-way ANOVA. **D:** SirT1 overexpression increased and prevented loss of *TIMP3* expression caused by high glucose (HG; 20 mmol/l) in CASMC, HUVEC, and THP1.  $n = 4$  for all experiments;  $*P < 0.05$  by Student's *t* test.

To our knowledge, this is the first gene/mechanism linked to SirT1 identified in the context of diabetes and atherosclerosis diseases using human vascular specimens. Therefore, our results provide further support for the protective role played by SirT1 against metabolic diseases. In conclusion, we observed that atherosclerotic plaques from subjects with impaired glucose metabolism are characterized by *TIMP3* deficiency and increased metalloproteinase activity. In vitro studies and clinical correlations suggested that SirT1 regulates *TIMP3* expression, which is emerging as a specific factor for the atherosclerosis process in diabetes.

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No potential conflicts of interest relevant to this article were reported.

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