Insulin resistance triggers the developments of diabetes mellitus and insulin resistance. Tribbles homolog 3 (TRIB3) is involved in insulin resistance. We aimed to investigate whether TRIB3 is implicated in diabetic atherosclerosis. Sixty 3-week-old apolipoprotein E (ApoE−/−)/LDLR receptor (LDLR−/−) mice were randomly divided into chow and diabetic groups. Diabetes was induced by a high-fat and high-sugar diet combined with low-dose streptozotocin. Mice in both groups were randomly divided into vehicle and TRIB3-silencing groups. After transfection, all mice were killed to evaluate the effects of TRIB3 on atherosclerosis. Silence of TRIB3 markedly decreased insulin resistance (P = 0.039) and glucose (P = 0.019), regardless of diabetes. Ultrasonography-measured parameters were similar in both groups, with and without silence of TRIB3. However, silence of TRIB3 decreased the aortic atherosclerotic burden (P = 1 × 10−5). Further study showed that in brachiocephalic lesions, fibrous cap thickness, cap-to-core ratio, collagen content, and the number of smooth muscle cells were significantly increased (P < 0.01 for all) by silence of TRIB3, whereas lipid and macrophage contents remained unaltered, with the vulnerability index significantly reduced. Moreover, the numbers of apoptotic cells and macrophages in brachiocephalic lesions were both significantly decreased (P < 0.01 for both). Macrophage migration was decreased (P = 4 × 10−5) by knockdown TRIB3, whereas adhesion and phagocytosis were increased (P < 0.05 for both). Silence of TRIB3 would diminish atherosclerotic burden and increase the plaque stability in diabetic mice. *Diabetes* 61:463–473, 2012

**RESEARCH DESIGN AND METHODS**

All animal procedures were performed in accordance with the institutional guidelines of Qilu Hospital of Shandong University and were approved by Shandong University Institutional Animal Care and Use Committee. Mice were housed five per cage and allowed access to diet and autoclaved water. A schema of the protocol is provided in Supplementary Fig. S1.

**Diabetic model and in vivo experiments**

Three-week-old male apoE/LDLR DKO mice were fed a high-fat diet (HFD; 20% fat, 20% sugar, and 1.25% cholesterol). After 6 weeks, the DKO mice underwent an intraperitoneal glucose tolerance test (IPGTT). Any mouse exhibiting IR was injected once with low-dose streptozotocin (STZ, 75–80 mg/kg i.p.) to induce partial insulin deficiency. Two weeks after the STZ injection, most HFD/STZ-treated mice displayed hyperglycemia, IR, and glucose intolerance, as previously reported (14). At age 11 weeks, animals with similar degrees of hyperglycemia and body weight were randomly divided into vehicle (DM, n = 15) and TRIB3-silencing (DM+RNA interference [RNAi], n = 15) groups. The mice fed a normal diet were used as nondiabetic controls, divided into chow (n = 15) and TRIB3-silenced (chow+RNAi, n = 15) groups.

**IPGTT.** Mice fasted overnight were challenged intraperitoneally with glucose at 1.5 g/kg body weight. Blood glucose levels of every animal were measured from tail blood with the OneTouch SureStep glucometer (LifeScan, Inc., Milpitas, CA) at specified times after glucose administration.

Transfection of recombinant adenovirus full-length TRIB3 short hairpin RNA was subcloned from pGenesil-1.2 into the pShuttle vector. Then, recombinant pAdxsi adenovirus was constructed using the pAdxsi Adenoviral System.

**See accompanying commentary, p. 265.**
SILENCE OF TRIB3 SUPPRESSES ATHEROSCLEROSIS

(SinoGenoMax, Beijing, P.R. China). After amplification, viruses were purified, titered, and stored at −80°C until used. All mice were administered 5 × 10⁶ plaque-forming units of virus by tail vein injection at 20 weeks and another 5 × 10⁶ plaque-forming units of virus at 22 weeks. The chow group was infected with control virus (vehicle). All mice were killed for further study 2 weeks later. Serum sampling. At the end of the experiment, the DKO mice were fasted overnight and killed by an overdose of pentobarbital. FASTing serum insulin, glucose, total cholesterol, triglyceride, LDL cholesterol, and HDL cholesterol (HDL-C) were measured. The homeostasis model assessment (HOMA) method was used to calculate IR (15).

Liver glycogen content. Liver glycogen content was determined by acid hydrolysis and then glucose measurement (16). In brief, 50 mg liver tissue was boiled for 2 h in 0.5 mL of 2 mmol/L HCl and neutralized with an equal volume of 2 mmol/L NaOH. Glucose content was measured using the Glucose (HK) assay kit (Sigma-Aldrich, St. Louis, MO).

Liver triglyceride content. Liver triglycerides were extracted, as previously reported (16), and measured using a Triglyceride Quantiﬁcation Kit (Abcam, Cambridge, MA), following the manufacturer’s instructions for colorimetric assay.

Quantitation of atheroma in vivo and in vitro

Ultrasoundography. All animals were anesthetized and laid supine on a heated table, and warned ultrasound transmission gel was placed on the chest. Two-dimensional imaging was performed by using the Real-Time Micro Visualization Scanhead (RMV 704) with a central frequency of 40 MHz at the mechanical transducer (Vevo 770; VisualSonics, Toronto, ON, Canada). Intima-media thickness (IMT) measurements were performed according to previously validated protocols in humans. For each image, three sets of measurements were taken.

Atherosclerotic lesion analysis. Mice were anesthetized and killed. Mouse hearts were perfused with 10 mL phosphate-buffered saline (PBS) and then 10 mL 4% paraformaldehyde (PFA) for 30 min at physiological pressure through the descending aorta. After incubation in 4% PFA overnight, the aorta was cut open longitudinally. To calculate the lesion area, aortas were stained with Oil Red O (Sigma-Aldrich) before the analysis.

Histology. Brachiocephalic arteries were embedded in optimal cutting temperature compound (Sakura Finetek, Beijing, P.R. China). Sections were cut 30 μm along the brachiocephalic artery and stained with hematoxylin and eosin (H&E; Merck Millipore, Darmstadt, Germany) and Oil Red O (Sigma-Aldrich), followed by staining with rat anti-mouse MOMA-2 antibody (Abcam, Cambridge, U.K.) and horseradish peroxidase-conjugated goat anti-rat second-antibody (Abcam, Cambridge, U.K.). Visualization of sections was performed using a fluorescence microscope (Leica TSC SP2; Leica, Wetzlar, Germany).

RESULTS

Generation of diabetic atherosclerosis mouse model.

To generate a nongenetic rodent model closely resembling human diabetic atherosclerosis disease, HFD alone could induce IR after 6 weeks, conﬁrmed by IPGTT, whereas the combination of HFD and STZ treatment led to frank hyperglycemia and IR (Fig. 1B). At the end of the experiment, DM mice still showed signiﬁcantly elevated blood glucose (P = 0.026, Table 1). The mean body weight was signiﬁcantly higher for DM than normal-chow mice at the age of 9 weeks (Fig. 1A).

High-resolution ultrasoundography examination revealed atherosclerotic plaques in the arteries of DM mice (Fig. 1C), and DM mice showed signiﬁcantly greater aortic and brachiocephalic IMT than those of chow mice at the age of 20 weeks, with no signiﬁcant difference at the age of 24 weeks. However, carotid IMT was signiﬁcantly increased in DM mice at 24 weeks (Fig. 1D). The HFD/STZ mouse model showed typical T2DM features of hyperglycemia, IR, and obesity that could persist throughout the experiment. As expected, these DM mice showed signiﬁcantly more atherosclerosis, as indicated by ultrasoundography.

Silence of TRIB3 in aorta. The relative mRNA expression of TRIB3 in the aorta was signiﬁcantly increased in DM mice compared with that in chow mice (4.97 ± 1.30 vs. 1.69 ± 0.76, P = 1 × 10⁻⁵), whereas silence of TRIB3 signiﬁcantly reduced relative TRIB3 mRNA expression in DM (4.97 ± 1.30 vs. 1.59 ± 0.52, P = 1 × 10⁻⁷) by 68.01% compared with 35.50% in chow mice (1.69 ± 0.76 vs. 1.00 ± 0.32, P = 2 × 10⁻⁴).
Silence of TRIB3 improves metabolism. Knocking down TRIB3 in this mice model led to a significant decrease in blood glucose in DM mice ($P = 0.019$, Table 1) and in chow mice ($7.00 \pm 4.54$ vs. $3.80 \pm 1.71$, $P = 0.033$, Table 1). However, DM and RNAi silencing of TRIB3 showed no significant interactions ($P = 0.375$, Table 1). Furthermore, silence of TRIB3 had a similar effect on HOMA-IR (Table 1).

Body weight, insulin, cholesterol, and LDL levels were not significantly altered by TRIB3 silence. Further studies showed that liver glycogen content was significantly increased ($2.59 \pm 0.30$ vs. $4.79 \pm 0.57$, $P = 2 \times 10^{-7}$, Table 1) with silence of TRIB3, but not the hepatic triglyceride level (Table 1).

Silence of TRIB3 decreases aortic atherosclerotic burden. Silencing TRIB3 decreased the number and size of aortic plaques, regardless of diabetes status. Face-to-face
Silencing TRIB3 stabilized lesions in the brachiocephalic artery. Spontaneous plaque rupture has been demonstrated in the brachiocephalic artery of mice (18). Fibrous cap thickness was significantly reduced in DM mice compared with chow mice \((P = 2 \times 10^{-6}, \text{Table 2 and Fig. 3, upper panel})\) but was significantly increased in DM+RNAi mice compared with DM mice \((P = 0.021, \text{Table 2 and Fig. 3, upper panel})\), with no change in chow mice. Further factorial analyses showed significant interactions between DM and RNAi for fibrous cap thickness \((P = 1 \times 10^{-9}, \text{Table 2 and Fig. 3, upper panel})\), suggesting the benefit of silencing TRIB3 in DM. Because silencing of TRIB3 would improve metabolism, even after adjusting for blood pressure, glucose, and HDL-C, it would significantly increase the cap thickness in DM mice \((P = 0.021, \text{Table 2})\).

In the current study, the cap-to-core ratio was significantly lower in DM mice than in chow mice \((P = 0.033)\), while silencing TRIB3 significantly increased the ratio in DM \((P = 0.025)\) and chow mice \((P = 0.043)\). Further factorial analyses showed no significant interactions between DM and RNAi \((P = 0.625)\), indicating that silenced TRIB3 and DM independently exerted effects on the ratio. However, after adjusting for blood pressure, glucose, and HDL-C, silence of TRIB3 had no effect on the ratio \((P = 0.914, \text{Table 2})\).

After adjusting for blood pressure, glucose, and HDL-C, collagen content was significantly lower in DM mice than in chow mice \((P = 0.004, \text{Table 2 and Fig. 3, middle panel})\), whereas it was significantly increased in DM+RNAi mice compared with DM mice \((P = 0.02, \text{Table 2 and Fig. 3, middle panel})\), with no difference in chow mice. Subsequent factorial analyses showed significant interactions between DM and RNAi \((P = 0.013, \text{Fig. 3, middle panel})\). After adjusting for blood pressure, glucose, and HDL-C, no significant interactions between DM and RNAi were revealed \((P = 0.215, \text{Table 2})\). Further studies demonstrated that silencing TRIB3 could significantly augment the collagen I-to-III ratio in DM \((P = 0.034)\) and chow mice \((P = 0.038, \text{Fig. 3, middle panel})\), for an increased proportion of collagen I to stabilize the plaques. However, after adjusting for blood pressure, glucose, and HDL-C, silence of TRIB3 had no effect on the collagen I-to-III ratio (Table 2).

Lipid, the other extracellular component, was significantly enhanced in DM mice compared with chow mice \((P = 0.032, \text{Table 2 and Fig. 3, bottom panel})\) after adjusting for blood pressure, glucose, and HDL-C, and not substantially altered by silencing TRIB3.

The cellular component, smooth muscle cells, was not significantly altered by DM but was significantly increased with TRIB3 silencing. However, macrophage content was significantly higher in DM mice than in chow mice \((P = 0.0005, \text{Table 2 and Fig. 3, bottom panel})\) after adjusting for blood pressure, glucose, and HDL-C, and not significantly altered by silencing of TRIB3.

The vulnerability index, describing the plaque stability, was significantly higher in DM mice than in chow mice.
FIG. 2. Images and quantifications of aortic atherosclerotic lesions in ApoE<sup>−/−</sup>/LDLR<sup>−/−</sup> mice with and without TRIB3 silencing. A: Images of aortic atherosclerotic lesions from mice. Experimental groups are indicated as follows: 1, chow diet (Chow); 2, chow diet with silence of TRIB3 (chow +RNAi); 3, DM; 4, DM with silence of TRIB3 (DM+RNAi). B: The degree of atherosclerosis determined by Oil Red O staining of face-to-face lesion areas in ApoE<sup>−/−</sup>/LDLR<sup>−/−</sup> mice. C: Quantification of atherosclerotic lesion areas. One-way ANOVA was performed. (A high-quality digital representation of this figure is available in the online issue.)
A 2 × 2 factorial ANOVA was used adjusting for blood pressure, glucose, and HDL-C. P was adjusted for blood pressure, glucose, and HDL-C. Fibrous cap was measured at the thinnest part of each plaque (24). SMC, smooth muscle cell. *The P value for DM means comparison of DM with chow. The P value of RNAi means comparison of DM with DM+RNAi.

(P = 0.004, Table 2) after adjusting for blood pressure, glucose, and HDL-C, whereas silence of TRIB3 significantly decreased the vulnerability index in DM (P = 0.015, Table 2) but not chow mice. Further factorial analyses showed no significant interactions between DM and RNAi for the vulnerability index (P = 0.245, Table 2), indicating that silencing TRIB3 and DM independently exert effects on the vulnerability index. Even with no adjustment for blood pressure, glucose, and HDL-C, silence of TRIB3 significantly decreased the vulnerability index (Table 2).

![Image of histological analyses of brachiocephalic artery lesions of ApoE−/−/LDLR−/− mice with and without TRIB3 silencing.](FIG. 3. Histological analyses of brachiocephalic artery lesions of ApoE−/−/LDLR−/− mice with and without TRIB3 silencing. Top panel shows sample cross-sections of brachiocephalic artery stained with hematoxylin and eosin. Middle panel shows atherosclerotic lesions in brachiocephalic artery stained with Picrosirius red for collagen. Bottom panel shows atherosclerotic lesions in brachiocephalic artery stained with the macrophage marker MOMA-2 (green), lipid marker Nile red (yellow), and nuclei marker DAPI (blue). Experimental groups are indicated as follows: 1, chow diet (Chow); 2, chow diet with silence of TRIB3 (Chow+RNAi); 3, DM; 4, DM with silence of TRIB3 (DM+RNAi). Scale bar, 100 μm. (A high-quality digital representation of this figure is available in the online issue.)]
Silence of TRIB3 decreases macrophage apoptosis in the brachiocephalic artery. Cell apoptosis was significantly greater in DM mice than in chow mice (201.00 ± 61.01 vs. 85.63 ± 38.46, \( P = 0.00003 \), Fig. 4), whereas silence of TRIB3 significantly reduced cell apoptosis in DM+RNAi mice compared with DM mice (201.00 ± 61.01 vs. 95.86 ± 39.80, \( P = 0.0001 \), Fig. 4), with no effect on chow mice. Further factorial analyses showed significant interactions between DM and RNAi for apoptosis (\( P = 0.007 \), Fig. 4).

To evaluate the role of macrophages in plaque stability, macrophage apoptosis was analyzed. We found a significantly higher number of TUNEL-positive, MOMA-2–positive macrophages in lesions from DM mice than in chow mice (108.80 ± 33.44 vs. 48.31 ± 28.39, \( P = 0.0001 \), Fig. 4). Silence of TRIB3 significantly decreased macrophage apoptosis in lesions from DM+RNAi mice compared with DM mice (46.20 ± 15.21 vs. 108.80 ± 33.44, \( P = 0.00006 \), Fig. 4), with no effect on chow mice. Further factorial analyses showed
significant interactions between DM and RNAi for macrophage apoptosis ($P = 0.013$, Fig. 4), indicating a benefit of silencing TRIB3 in DM.

**Knocking down TRIB3 increased Akt activity.** The TRIB3 level was higher in DM mice than in chow mice (Fig. 5, lane 4 vs. lane 2). Correspondingly, phosphorylation of Akt was lower (Fig. 5, lane 4 vs. lane 2) in DM mice, and as a consequence, caspase-3 was upregulated in DM mice. However, silencing TRIB3 increased the phosphorylation of Akt in DM+RNAi mice (Fig. 5, lane 3 vs. lane 4). Meanwhile, downregulation of caspase-3 was detected in DM+RNAi mice. These phenomena were also observed in chow mice.

**Silence of TRIB3 and macrophage functions.** To test whether TRIB3 silence exerted an effect on macrophage adhesion, migration, and phagocytosis, peritoneal macrophages isolated from four different groups were examined. We first examined the effects of TRIB3 deficiency on chemotaxis by Transwell migration assay. DM mice showed significantly more migrated macrophages than did chow mice (14.00 ± 2.45 vs. 5.60 ± 2.37, $P = 1 \times 10^{-8}$, Fig. 6A and E). Silence of TRIB3 significantly decreased macrophage migration from DM+RNAi mice compared with DM mice (7.10 ± 3.28 vs. 14.00 ± 2.45, $P = 4 \times 10^{-4}$, Fig. 6A and E). Migration of macrophages was equivalent in cells isolated from both groups of chow mice ($P = 0.603$, Fig. 6A and E). Further factorial analyses showed significant interactions between DM and RNAi for macrophage migration ($P = 4 \times 10^{-4}$, Fig. 6E), indicating benefit of silencing TRIB3 in DM.

Peritoneal macrophages from DM mice showed significantly increased macrophage adherence to the surfaces coated with poly-lysine compared with those from chow mice (44.83 ± 8.81 vs. 25.71 ± 2.50%, $P = 3 \times 10^{-4}$, Fig. 6B and F). Silence of TRIB3 significantly increased macrophage adhesion from chow+RNAi mice compared with chow mice (34.07 ± 9.00 vs. 25.71 ± 2.50%, $P = 0.020$, Fig. 6B and F), with no effect on DM mice ($P = 0.515$, Fig. 6B and F). Further factorial analyses showed no significant interactions between DM and RNAi for macrophage adhesion ($P = 0.142$), which suggests that silencing TRIB3 and DM independently exert effects on macrophage adhesion.

Flow cytometry analysis revealed significantly higher phagocytosis ability of macrophages for DM mice than for chow mice (47.50 ± 15.92 vs. 27.67 ± 0.77, $P = 0.004$, Fig. 6G, C, and D). Silence of TRIB3 significantly increased macrophage phagocytosis, regardless of DM (Fig. 6G, C, and D). Further factorial analyses showed no significant interactions between DM and RNAi for macrophage phagocytosis ($P = 0.131$), which suggests that silencing TRIB3 and DM independently exert effects on macrophage phagocytosis.

**DISCUSSION**

Because IR triggers the development of DM and atherosclerosis and TRIB3 is involved in IR, we aimed to investigate whether TRIB3 is implicated in diabetic atherosclerosis in mice. We found that silence of TRIB3 in vivo improved metabolism, alleviated the atherosclerotic lesion burden, and stabilized atherosclerotic plaque in a mouse model of diabetic atherosclerosis. Mechanistically, this phenotype is attributed to a decrease in proatherogenic pathways, such as increased Akt phosphorylation, amelioration of IR, and diminished macrophage apoptosis. To our knowledge, these findings provide novel insights into the molecular processes underlying diabetic atherosclerosis.
mechanisms that directly link TRIB3 to atherosclerosis in DM. Amelioration of DM metabolisms with TRIB3 silencing. TRIB3 was found to inhibit insulin-stimulated Akt phosphorylation (3) and modulate gluconeogenesis in rodent liver (19). Several studies demonstrated TRIB3 induction as a novel molecular mechanism in human IR and diabetes (5,7,8). Previous research has described that a gain-of-function mutation of TRIB3 is associated with IR and related abnormalities (9–11). In the current study, TRIB3 expression was higher in the mouse models of diabetes. Moreover, silence of TRIB3 could ameliorate IR and lower blood glucose, which might be attributed to increased phosphorylation of Akt, crucial for insulin signaling (3).

Silence of TRIB3 decreases atherosclerotic burden. TRIB3 has been revealed to be upregulated in atherosclerotic unstable plaque (20). Studies demonstrated that TRIB3 is upregulated by oxidized LDL (21) and mediates human monocyte-derived macrophage apoptosis (22). However, the impact of silence of TRIB3 on plaque progression remained to be specifically investigated. Here, we detected upregulated TRIB3 in the diabetic atherosclerotic mice, which was accompanied by increased macrophage apoptosis in the
atherosclerotic plaque. With the silence of TRIB3, the aortic
erosclerotic burden and lesion formation were mitigated.
Furthermore, data from nondiabetic mice showed the ather-
sclerotic burden was reduced by silence of TRIB3 even
if IR was not significantly improved.

Stabilization of atherosclerotic plaque by knocking
down TRIB3. Some phenotypic characteristics of athero-
sclerotic plaques, such as fibrous cap thickness, collagen
content, plaque cap-to-core ratio, and macrophage number
have been widely used as indicators of plaque stability.
We confirmed that DM mice had thinner fibrous caps than chow
mice and that silencing TRIB3 could lead to thicker fibrous
caps. The fibrous cap consists of collagen, and we found
that the collagen content of plaques was lower in DM mice
than in chow mice, whereas silence of TRIB3 increased the
collagen content of plaques to stabilize it.

Plaques with a thin fibrous cap and a large lipid core are
considered vulnerable (23). Hence, we analyzed the cap-to-
core ratio to determine the plaque vulnerability. The ratio
was lower in DM mice than in chow mice, and silence of
TRIB3 enhanced the ratio to stabilize the plaque. However,
silence of TRIB3 had no effect on the ratio when controlling
for the metabolic factors, which suggests that the ratio
might depend more on metabolism. Investigation of the vul-
erability index demonstrated that silence of TRIB3 in-
creased the stability of plaque when controlling for the
metabolic factors.

Macrophages with silence of TRIB3. The macrophages
were thought to be at the crossroad of IR and atherosclerosis
(13). IR in macrophages, more susceptible to apoptosis, may
promote the development of atherosclerosis, thus increasing
the vulnerability. Therefore, silence of TRIB3 should ame-
liorate IR and thereby reduce macrophage apoptosis in
diabetic atherosclerosis in vivo, in agreement with our
findings in vitro (12).

Further study showed that macrophages from DM mice
exhibited increased migration, enhanced adherence, and
augmented phagocytosis, which were altered by silence of
TRIB3. Increased migration suggested enhanced macrophage
recruitment, which has important roles in atherogenesis.
Knocking down TRIB3 would significantly decrease mac-
rophage migration to prohibit atherosclerotic plaque ex-
pansion. Enhanced adherence indicates an increased
ability of tissue infiltration under diabetic conditions.
Knocking down TRIB3 increased the adherence only in
chow mice, whereas this function maintained unchanged
in DM mice, which might be attributable to increased local
clearance. Phagocytosis was amplified by silence of TRIB3
in both mouse types, which indicates that more necrotic
lipid was engulfed by macrophages. Therefore, augmented
phagocytosis with silence of TRIB3 suggested regained
phagocytic clearance, which would restrain expansion of
the necrotic core.

Limitation. Adenoviral gene delivery by tail vein injection is
a simple yet effective in vivo gene-delivery method. Be-
cause of tail vein injection, the observed effect in macro-
phages could be derived from a direct action of TRIB3 small
interfering RNA in those macrophages and an indirect effect
of TRIB3 mRNA knockdown on whole-body insulin sensi-
tivity mice in the current study. Because intravenous de-
livery is minimally invasive and easily manipulated, it is
increasingly being used to elucidate the role of genes in the
pathogenesis of disease in animal models.

In conclusion, we collectively found that silence of TRIB3
diminishes the atherosclerotic burden and increases plaque
stability in diabetic atherosclerotic mice, which might
provide a therapeutic approach to decreasing atheroma
formation and promoting plaque stabilization in diabetes
state.

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