RNA Interference for Discoidin Domain Receptor 2 Attenuates Neointimal Formation in Balloon Injured Rat Carotid Artery

Kou-Gi Shyu, Bao-Wei Wang, Peiliang Kuan, Hang Chang

Objectives—Discoid domain receptor 2 (DDR2) plays potential roles in the regulation of collagen turnover mediated by smooth muscle cells (SMCs) in atherosclerosis. Little is known about the function of DDR2 in vascular system. We investigated whether inhibition of DDR2 by small interfering RNA (siRNA) can reduce neointimal formation after arterial injury.

Methods and Results—SMCs from thoracic aorta of adult Wistar rats were cultured. The carotid artery from adult Wistar rats was injured by balloon catheter. DDR2 significantly increased migration and proliferation of SMCs. DDR2 siRNA inhibited 86% of DDR2 protein expression in cultured SMCs. DDR2 protein and mRNA expression significantly increased at 14 days after carotid injury. DDR2 siRNA significantly reduced DDR2 protein and mRNA expression induced by balloon injury. The immunohistochemical stain demonstrated that DDR2 siRNA decreased MMP2 protein labeling induced by balloon injury, a pattern similar to that of DDR2 protein labeling. Neointimal area was significantly increased after carotid injury and was significantly reduced by DDR2 siRNA.

Conclusions—DDR2 increases migration and proliferation of SMCs, and expression of DDR2 in carotid artery significantly increases after injury. DDR2 siRNA attenuates neointimal formation after carotid injury. DDR2 may play a pivotal role in the pathogenesis of neointimal thickening after mechanical injury. (Arterioscler Thromb Vasc Biol. 2008;28:1447-1453)

Key Words: discoidin domain receptor 2 — smooth muscle cell — RNA interference — vascular injury

Development of neointimal thickening is the major cause of atherosclerosis and restenosis. Smooth muscle cells (SMCs) are the predominant cell types in the media of blood vessel walls and contribute to neointimal formation after arterial injury. After arterial injury, SMCs synthesize collagens, which act as important signaling molecules regulating SMC responses to arterial repair. Discoid domain receptor 1 (DDR1) and DDR2 have recently emerged as nonintegrin-type receptors for collagen. DDR1 is mainly expressed in epithelial cells, whereas DDR2 is found in mesenchymal cells. DDR1 and DDR2 play potential roles in the regulation of collagen turnover mediated by vascular smooth muscle cells (VSMCs) in obstructive diseases of blood vessel. Both DDRs were found to be highly expressed by SMCs within the fibrous cap. DDR1 has been found to play an important role in mediating intimal thickening after arterial injury; however, little is known about the function of DDR2 in the vascular system. We have previously demonstrated that stretching of VSMCs in vitro resulted in upregulation of DDR2 expression. In this study we hypothesize that DDR2 plays an important role in vascular injury in vivo.

RNA interference mediated by small interfering RNA (siRNA) can knock down gene expression at a translational level through interactions with its target messenger RNA and shows great promise for therapeutic applications. Here, we applied RNA interference to study the role of DDR2 in atherosclerosis in vitro and in vivo.

Methods

siRNA Designed

The siRNA duplexes targeting the mouse DDR2 mRNA (GenBank accession no. NM_022563) were designed. For the initial screening of the most effective siRNA duplexes, the 21-nucleotide RNAs were synthesized by in vitro transcription (Dharmacon Inc). Further experiments were performed with the most efficient siRNA-duplex. The most efficient siRNA-duplex does have 1 mismatch to the rat sequence NM_031764. The mismatch 1 is located at the 6th base of the duplex, U for mouse and C for rat. No significant similarities to any genes other than DDR2 were found using BLAST against the rat reference sequence database.

Vascular Smooth Muscle Cell Culture

Primary cultures of VSMCs were grown by the explant technique from the thoracic aorta of 220- to 260-g male Wistar rats, as

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1447
described previously.10 The detail culture methods are described in the
org. VSMCs cultured on the flexible membrane base were subjected
to cyclic stretch produced by Flexcell FX-2000 strain unit (Flexcell
International Co) and by vacuum to 20% of maximum elongation, at
60 cycles/min as described previously.2 VSMCs were transfected
with 800 ng DDR2 siRNA oligonucleotide (Dharmacon Inc). VSMCs
were transfected with siRNA oligonucleotides using Effectene
Transfection Reagent according to the manufacturer’s instruction
(Qiagen Inc). After incubation at 37°C for 24 hour, cells were
stretched for 18 hours and subjected to analysis of Western blot.

Migration Assay
The migration activity of VSMCs was determined using the growth
factor–reduced Matrigel invasion system (Becton Dickinson) follow-
ing the protocol provided by the manufacturer. Migration assay was
performed as described previously.11 DDR2 recombinant protein
(Upstate) and collagen I (CALBIOCHEM) was added to the cultured
medium in the upper chamber. The recombinant DDR2 protein
contains the amino acid substitution S642A (native coordinates)
with respect to GenBank NM_006182. Cells were incubated overnight,
and the top layer of the membrane was scraped gently to remove any
cells. Migration was quantified by counting the number of cells in 5
random X400 fields per filter and expressed as the average of
number of cells per field.

Proliferation Assay
The proliferation of VSMCs was determined using [3H]thymidine
incorporation. VSMCs were seeded on ViewPlate (Packard Instru-
ment) for 60 minutes at a density of 5×10^4 cells per well in
serum-free medium incubated overnight. Recombinant DDR2 (15
nmol/L), DDR2 siRNA, and collagen I (20 μg/mL) was added to the
plate. Detailed methods for proliferation assay are described in the
online method supplement.

Balloon Injury of Rat Carotid Artery
Animal experiments were approved and carried out in accordance
with the Guide for the Care and Use of Laboratory Animals
published by the US National Institutes of Health (NIH Publication
No. 85-23, revised 1996). Adult Wistar rats were anesthetized with
isoflurane (3%) and subject to balloon catheter injury of the right
carotid artery as described previously.12 Briefly, a 2F Forgarty
balloon catheter (Biosensors International Inc) was inserted through
the right external carotid artery, inflated, and passed 3 times along
the length of the isolated segment (1.5 to 2 cm in length), then the
catheter was removed. DDR2 siRNA was injected to the segment
and electric pulses using PA-3000S Laboratory PulseAgile Electro-
poration System (PulseAgile, Cyto Pulse Sciences Inc) were admin-
istered with 2 pulses at 120 μCm, 0.1 ms duration, 0.5-second
interval using Tweezer’s electrode (5 mm×10 mm rectangular pad).
The injected siRNA was incubated for 10 minutes. After incubation,
unbound siRNA was aspirated. The carotid artery was then tied off,
and the neck was closed. The rats were euthanized at 7 or 14 days
after balloon injury and the carotids were perfused fixed at constant
physiological pressure with 4% paraformaldehyde. The vessels were
excised and embedded in paraffin blocks, and 2 cross-sections were
sliced at positions 1 and 2 cm upstream of the carotid bifurcation.
Intimal, medial, and adventitial cross-sectional areas were measured
by a image software (Image Pro Plus Olympus BX 51).

Western Blot Analysis
Arterial protein extracts were prepared by pulverizing the arteries
under liquid nitrogen (LN), then lysed in buffer containing 1% SDS,
1 mmol/L PMSF, and 10 mg/mL leupeptin in 50 mmol/L Tris (pH
7.6). Protein from cultured VSMCs and arterial extracts were used to
generate Western blots. Western blot was performed as previously
described.13 Rabbit polyclonal anti-DDR2, anti-DDR1, cyclin D1,
and PCNA antibodies (Santa Cruz Biotechnology) were used.

Real-Time Polymerase Chain Reaction
For analysis of DDR2 mRNA, real-time polymerase chain reaction
(PCR) was performed. The carotid arteries were harvested, stripped
of adventitia, and then pooled for RNA extraction. Total RNA was
isolated as described previously.13 Reverse transcription and real-
time PCR were performed as described previously.13 The cDNA
produced by reverse transcription was used to generate rat DDR2
cDNA probe by PCR. The PCR primer sequences are listed in the
online supplemental methods.

Immunohistochemical Analysis
The carotid artery was harvested and fixed in 10% formaldehyde and
sliced into 5-μm paraffin sections. The method for immunohisto-
chemical stain is described in the online method supplement.

Zymography
ECM-degrading activity was detected by zymography. The protein
was extracted from cultured VSMCs, and equal amounts of sample
protein were subjected to SDS-PAGE on gelatin-containing acryl-
amide gels (7.5% polyacrylamide and 2 mg/mL gelatin) under
nonreducing conditions. Zymogram was performed as previously
described.14

Measurement of Interferon γ Concentration
The level of interferon (INF-γ) was measured by a quantitative
sandwich enzyme immunoassay technique (R&D Systems). Blood
from the rat tail vein was collected for measurement. The lower limit
detection of rat INF-γ was 10 pg/mL. Both the intraobserver and
interobserver coefficient of variance were <10%.

Statistical Analysis
All results were expressed as mean±SEM. Statistical significance
was evaluated by analysis of variance followed by Tukey-Kramer
comparison test. A value of P<0.05 was considered to denote
statistical significance.

Results
Selection and Characterization of Effective
Anti-DDR2 siRNAs
Four siRNAs were designed and generated by in vitro
transcription to target-defined positions within the coding
region of the mouse DDR2 mRNA (Table). The potencies of
the 4 siRNAs relative to a randomized control siRNA were
compared at the translational level by Western blot analysis.
To better discriminate the effect of different siRNAs on
DDR2 protein level, we performed cyclic mechanical stretch
of cultured VSMCs, which upregulated DDR2 expression. As
shown in Figure 1A and 1B, the DDR2 siRNA-2 was the
most potent DDR2 silencer capable of reducing DDR2
protein expression below its basal level, giving an overall
inhibition of 86% at 24 hours. Control with scramble siRNA
of DDR2 siRNA-2 did not affect the DDR2 protein expres-
sion induced by cyclic stretch. DDR2 siRNA-2 as DDR2
siRNA was used at the following experiments.

DDR2-Fc fusion protein, which contains only the extracel-
lular domain, has been shown to increase binding of DDR2
and collagen.15 We have demonstrated that the exogenous
addition of recombinant DDR2 protein increased DDR2
density by fluorescence microscope (supplemental Figure I,
available at http://atvb.ahajournals.org). The increased DDR2
density may increase the collagen-DDR2 binding activity.
We then used recombinant DDR2 for the in vitro study. The
Fc-DDR2 increased endogenous DDR2 protein expression by
about 4-fold in a dose-dependent manner as compared to control (data not shown). The DDR2 protein contains N-terminal His6-tagged, recombinant, human DDR2 amino-acids 476-end expressed by baculovirus in Sf21 insect cells. There is no contamination of lipopolysaccharide (LPS) in the recombinant DDR2 protein. As shown in Figure 1C and 1D, DDR2 recombinant protein increased DDR2 protein expression in cultured VSMCs, and DDR2 siRNA attenuated the DDR2 protein expression induced by recombinant DDR2 protein. Addition of collagen I increased DDR2 protein expression whereas DDR2 siRNA also attenuated DDR2 protein expression induced by collagen I. The dose for each siRNA used in the experiment was 10^8 mol/L. The effect of DDR2 recombinant protein and siRNA on mRNA expression of VSMCs was similar to that on protein expression (supplemental Figure II).

**DDR2 Increases Migration of VSMC**

When the same numbers of VSMCs were cultured, rat VSMCs migrated significantly through the filter membrane after exogenous addition of DDR2 from 10 to 15 nmol/L as compared with the control group without treatment. The increased migration activity of DDR2 was dose-dependent (supplemental Figure III). Addition of DDR2 siRNA significantly inhibited migration of VSMCs induced by DDR2 or collagen I (Figure 2A and 2B). The migration activity of VSMCs was similar in control and scramble siRNA treated group.

**DDR2 Increases Proliferation of VSMCs**

The proliferation of VSMCs significantly increased from 6 hours to 24 hours in the control, DDR2-treated, and collagen I–treated group (supplemental Figure IV). The [3H]thymidine incorporation was significantly higher in the DDR2-treated group than in the control group. DDR2 siRNA significantly decreased the [3H]thymidine incorporation induced by DDR2 or collagen I. As shown in supplemental Figure V, recombinant DDR2 protein significantly increased and DDR2 siRNA decreased the cell number of VSMCs. Scramble siRNA did not affect the cell number of cultured VSMCs.

**Evidence of siRNA Transfection Into Carotid Artery**

To demonstrate the efficiency of siRNA delivery into the carotid artery by electric pulses, control siRNA was labeled with cyanine 5 (Cy5) at 5′/3′-side (Dharmacon Inc). In the carotid artery transfected with siRNA by electric pulses, successful transfection was evidenced by red stains under fluorescence microscope that were observed at the area of intimal thickening 14 days after balloon injury (Figure 3A).

**Table. Sequence Composition and Target Localization Within the Mouse DDR2 mRNA of the siRNAs (siRNA-1 Through siRNA-4) Designed to Screen for Effective DDR2 Gene Silencing**

<table>
<thead>
<tr>
<th>siRNA</th>
<th>mDDR2 mRNA</th>
<th>Targeted Region*</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA-1</td>
<td>1564–1582</td>
<td>5'-CGAGAUCUAIUUCGAIUU-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-UUGCUCAUAGUGAGGCUAG-5′</td>
<td></td>
</tr>
<tr>
<td>siRNA-2</td>
<td>1664–1682</td>
<td>5'-GAUGAUAGCAACACUGGAGA-3′</td>
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<tr>
<td></td>
<td></td>
<td>3'-UUCUACUUGAGUCAGCUA-5′</td>
<td></td>
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<tr>
<td>siRNA-3</td>
<td>1885–1903</td>
<td>5'-CAACUCAUCAUGAUAGCAU-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-UUGUUGAGAUAUGAUCUG-5′</td>
<td></td>
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<tr>
<td>siRNA-4</td>
<td>1267–1285</td>
<td>5'-CGAAAGUCUAGGCGAACC-3′</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3'-UUGCUCUAGCGGACGCUU-5′</td>
<td></td>
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<td>1664–1682</td>
<td>5'-GAUAGUUCAGUUGGCUAGA-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-UUCUACUUGCAUAGUUCCU-5′</td>
<td></td>
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</tbody>
</table>

The 21-nucleotide RNA sequences displayed in each row are arranged complementary homology yielding the corresponding siRNA duplexes, with 2 unpaired uridine nucleotides at the 3′-end of each strand. siRNA-C indicates control siRNA. scsiRNA-2- indicates absence of homology with respect to the DDR2 mRNA sequence. siRNA-C indicates control. Numbers indicate the position of the targeted region within the mDDR2 mRNA sequence from ATG initiation codon.

Figure 1. DDR2 siRNA-mediated silencing of DDR2 protein expression in cultured VSMCs. A and C, Representative Western blots for DDR2 in VSMCs subjected to cyclic stretch by 20% for 24 hours. B and D, Quantitative analysis of DDR2 protein levels (n=3 per group). *P<0.001 vs stretch 24 hours. **P<0.05 vs control.
The efficiency of siRNA delivery using control siRNA was dose-dependent. No red stain in the injured carotid artery was observed when no siRNA was applied in the control group.

**DDR2 siRNA Reduces DDR2 Protein and mRNA Expression After Carotid Injury**

After 7 and 14 days of balloon injury, DDR2 protein expression was evident. Delivery of siRNA into the carotid artery after balloon injury decreased DDR2 protein expression at a dose-dependent manner. The most potent dose of DDR2 siRNA to reduce DDR2 protein expression after balloon injury was 10 μmol/L (Figure 3B and 3C). There was no inhibitory effect of different concentrations of scramble DDR2 siRNA on DDR2 protein expression. The DDR2 protein and mRNA expression after balloon injury was time-dependent. As shown in supplemental Figure VI, DDR2 protein significantly increased at 14 days after balloon injury as compared to control group without injury. DDR2 mRNA expression was similar to DDR2 protein expression (supplemental Figure VII). DDR2 siRNA significantly reduced DDR2 protein and mRNA expression induced by balloon injury. Cyclic D1 and PCNA protein expression was significantly induced at 14 days after balloon injury, and DDR2 siRNA significantly attenuated the increased cyclic D1 and PCNA expression (supplemental Figure VI).

**DDR2 siRNA Decreases the DDR2 and MMP2 Protein Labeling**

The immunohistochemical stain demonstrated that there were strong DDR2-positive labeling cells at 14 days after carotid injury (Figure 4A). MMP2-positive labeling cell were strongly positive, similar to that of DDR2. siRNA decreased MMP2 protein labeling induced after balloon injury, a pattern similar to that of DDR2 protein labeling. Control siRNA did not decrease the MMP2 and DDR2 protein labeling after balloon injury. Direct red stain also demonstrated that DDR2 siRNA decreased the content of red stain induced by carotid injury after 14 days. This indicates that carotid injury increased collagen content in the arterial wall, and DDR2 siRNA decreased the collagen content. As shown in Figure 4B and 4C, matrix metalloproteinase (MMP)-2 activity increased after treatment with DDR2 or collagen I, and DDR2 siRNA decreased the MMP2 activity induced by DDR2 and collagen I. The macrophage marker, anti-CD68, was similar between the injury and DDR2 siRNA group (data not shown).

**DDR2 siRNA Reduces Intimal Thickening After Carotid Injury**

Balloon injury of carotid artery for 14 days significantly increased thickness of intimal area as compared to control group without injury.
group without injury. Immunohistochemical double stain demonstrated that Cy3-DDR2 siRNA was incorporated into DDR2 protein, affirming the effect of DDR2 siRNA (Figure 5A). The intimal layer in the sham group did not express DDR2 whereas in the 14 days after carotid injury, DDR2 was expressed in the intimal SMCs. DDR2 siRNA decreased the DDR2 labeling signal intensity. Delivery of DDR2 siRNA significantly reduced thickness of intimal area and lumen size (Figure 5B). The thickness of intimal area was reduced by 50% and the lumen size was increased by 45% by DDR2 siRNA. Control siRNA did not reduce intimal area induced by carotid injury.

**DDR2 siRNA Does Not Affect Serum Level of INF-γ**

To investigate whether delivery of DDR2 siRNA affects immune response of rats, serum levels of INF-γ were measured. The serum levels of INF-γ for sham group, 7 and 14 days after balloon injury, and DDR2 siRNA group were 27.3±0.5 pg/mL, 26.3±1.2 pg/mL, 25.2±1.8 pg/mL, and 26.9±0.3 pg/mL, respectively (n=4 for each group). There was no statistical difference among the 4 groups. This finding indicates that DDR2 siRNA does not affect serum level of INF-γ, implying that DDR2 siRNA does not affect the immune response.

**Discussion**

In this study, we found that DDR2 directly promoted the migration and proliferation of VSMCs, which contributes to the development of neointimal formation in restenosis and is an accelerated arteriopathy in response to vascular injury. We have demonstrated previously that DDR2 expression is regulated by cyclic stretch of VSMCs mediated by increases in transforming growth factor (TGF)-β 1 and angiotensin II production. Both TGF-β1 and angiotensin II play important roles in the atherosclerosis. These findings clearly indicate that hemodynamic forces can play a significant role in the modulation of DDR2 expression of VSMCs. In the present study, DDR2 was enhanced in the intimal area after injured carotid injury. Taken together, these data indicated that DDR2 might play an important role in the remodeling of vascular disease.

Prolonged stimulation of DDR2 has been associated with the upregulation of MMP-1 expression. Recently, Ferri et al have shown that DDR 1 and DDR2 play potential roles in the regulation of collagen turnover mediated by VSMCs in obstructive diseases of blood vessel. Overexpression of DDR2, but not DDR1, in human SMCs induced MMP1 as well as MMP2 expression. In contrast, Hou et al demonstrated that DDR1 deficiency inhibited neointimal lesion area after carotid balloon injury, an effect associated with reduced MMP-2 and MMP-9 activity. In this study, increased DDR2 expression after carotid injury was concurrent with increased MMP2 expression. DDR1 was not enhanced after carotid balloon injury. Furthermore, reduction of DDR2 by siRNA technique was also accompanied with reduction of MMP2 expression after carotid injury. MMP is necessary for SMC migration from media to intima. Reduction of DDR2 and MMP2 expression leads to attenuation of neointimal formation after carotid injury. These findings indicated that DDR2 plays an important role in the pathogenesis of intimal thickening after vascular injury and that DDR2 may potentially be the target for the management of atherosclerosis. After arterial injury, SMCs synthesize collagens. DDRs function as collagen receptors. In this study, we demonstrated that carotid injury increased collagen content, and DDR2 siRNA attenuated the increase of collagen induced by carotid injury.
proliferation. In addition to DDR2 expression, cyclin D1 and PCNA expression were also inhibited by DDR2 siRNA. This finding implicates that cyclin D1 and PCNA may be the DDR2-mediated signaling pathways. Our study demonstrated that recombinant DDR2 induced upregulation of endogenous DDR2 on VSMCs. The recombinant DDR2 may further oligomerize with endogenous DDR2, which increases the binding activity and therefore may induce endogenous DDR2 expression.

RNA has become the tool of choice for gene function study after the gene silencing effect of RNAi in mammalian cells has been confirmed. Synthetic siRNAs leverage the naturally occurring RNAi process in a manner that is consistent and predictable with regard to extent and duration of action. The safety and efficacy of an siRNA therapeutic agent against vascular growth factor in a validated nonhuman primate model of age-related macular degeneration. In this study, we used electric pulses to deliver DDR2 siRNA, and this therapeutic strategy decreased DDR2 protein and mRNA expression and attenuated neointimal formation induced by carotid injury. The electric pulses system used in the present study has been successfully applied to in vivo therapeutic delivery. We used fluorescence microscope to confirm that the siRNA could be effectively delivered to the vascular wall. To the best of our knowledge, this is the first report to demonstrate that DDR2 siRNA could effectively prevent neointimal hyperplasia induced by vascular injury.

We designed 4 DDR2 siRNAs to study the silencing effect of DDR2 protein expression. The silencing effect of DDR2 protein expression ranged from 54% to 86% (Figure 2). DDR2 siRNA-2 was the most active inhibitor and was chosen as the therapeutic agent. We have demonstrated a dose-response of the DDR2 siRNA-2 on silencing DDR2 protein expression and a negative effect of the scramble siRNA-2. Furthermore, DDR2 siRNA did not affect the DDR1 protein expression. This result indicates the specificity of the DDR2 siRNA to knock down DDR2 gene expression.

Recently, DDR2 has been shown to regulate immune function in dentritic cells. Innate immunity or INF response may be stimulated by siRNA. In this study, we measured serum INF-γ levels after applying DDR2 siRNA. The serum
INF-γ levels were similar between siRNA-treated animal and control animals. The macrophage content in the arterial wall was not affected by DDR2 siRNA. These results implicate that local delivery of DDR2 siRNA to the injured carotid artery does not affect immune response in our study model. The immune response does not contribute to the attenuation of neointimal formation by DDR2 siRNA in the carotid injury model.

The toxic effect of DDR2 siRNA was not investigated in the present study. However, the DDR2 siRNA was not chemically modified. siRNAs are highly effective without any chemical modifications, resulting in a dramatically lower toxicity profile.24,25 Off-target effect of siRNA is a major biological restriction.9,19 We used scramble siRNA to confirm the specificity of our designed siRNA. The off-target effect of DDR2 siRNA may be minimal.

In conclusion, we demonstrate here that DDR2 increases migration of SMCs and the expression of DDR2 in the carotid artery significantly increases after balloon injury. RNA interference for DDR2 decreases DDR2 expression and attenuates neointimal formation after carotid injury. DDR2 plays a pivotal role in the pathogenesis of intimal thickening after mechanical injury. Because neointimal hyperplasia is the main cause of atherosclerosis and restenosis, using DDR2 siRNA as a therapeutic agent for management of atherosclerosis and restenosis warrants further study.

Sources of Funding
This study was supported by a grant from National Science Council, Taiwan and Taiwan Society of Cardiology.

Disclosures
None.

References
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It was brought to our attention that several of the figure panels in the above article, published in 2008 by *Arteriosclerosis, Thrombosis, and Vascular Biology*, appear to contain possible instances of questionable manipulation. Specifically, the bottom control panel of Figure 2A has very similar parts to the bottom panel of DDR2 siRNA. Upon request, the institution, Taipei Medical University, conducted an investigation and found that the corresponding author, Dr Hang Chang, committed serious mistakes in manuscript preparation and raised ethical concerns. Therefore, in accordance with the AHA's ethical policy, the editors, therefore, hereby retract the article.
Supplemental Fig. II
Supplemental Fig. III
Supplemental Fig. IV
Supplemental Fig. V
DDR2 mRNA level (fold of sham)

Sham day 0
Sham 7 days
Sham 14 days
7 days
14 days
14 days + 1 μM
1 μM
5 μM
10 μM
20 μM

Supplemental Fig. VII
Supplemental Figure Legend

Supplemental Figure I: Recombinant DDR2 protein enhances DDR2 density in cultured VSMCs. Representative microscopic image of cultured VSMCs with or without DDR2 recombinant treatment. Left panel indicates VSMCs morphology and right panel indicates fluorescence image for DDR2 labeling. Similar result was found in another two independent experiments.

Supplemental Figure II: Recombinant DDR2 protein and collagen I increases DDR2 mRNA expression in cultured VSMCs. Quantitative analysis of DDR2 mRNA by real-time PCR under recombinant DDR2 protein and collagen I treatment in the absence or presence of DDR2 siRNA. *P<0.001 vs. control. **P<0.01 vs. control. N=3.

Supplemental Figure III: DDR2 increases migration of VSMCs. Upper panel, VSMCs migrated through filter were stained. Lower panel, migration of VSMCs were quantified by staining and counting the number of cells that migrated to the bottom of the filter in 5 fields under a X400 high-power field (HPF) *P<0.001 vs. control. (n=4 per group).

Supplemental Figure IV. Effect of DDR2 on proliferation activity of VSMCs. Proliferation of VSMCs was quantified by [\textsuperscript{3}H]Thymidine incorporation assay. *P<0.001. Data are from 4 independent experiments.

Supplemental Figure V: Effect of recombinant DDR2 and collagen I on VSMCs proliferation. Quantitative analysis of VSMCs cell numbers after DDR2 recombinant
protein or collagen I treatment for 24 h in the presence or absence of siRNA.

*P<0.001 vs. control.

**Supplemental Figure VI.** DDR2 siRNA reduces DDR2, cyclic D1 and PCNA protein after carotid injury. Upper panel, Representative Western blots for DDR1, DDR2, cyclic D1 and PCNA in the injured carotid artery. Middle and lower panels, Quantitative analysis DDR1, DDR2, cyclic D1, and PCNA protein levels. The values from injured group have been normalized to matched GAPDH measurement and then expressed as a ratio of normalized values to protein in the sham group. (n=4 per group).

**Supplemental Figure VII:** DDR2 siRNA reduces DDR2 mRNA expression after carotid injury. Quantitative analysis of DDR2 mRNA levels. The values from injured group have been normalized to matched GAPDH measurement and then expressed as a ratio of normalized values to mRNA in the sham group. (n=4 per group). *P<0.001 vs. 14 days.
Supplemental Method

Vascular smooth muscle cell culture: Cells were cultured in medium 199 containing 20% fetal calf serum, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 4 mmol/L L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 5%CO₂/95% air in a humidified incubator. When confluent, VSMC monolayers were passaged every 6 to 7 days after trypsinization and were used for experiment from the third to fourth passages.

Proliferation assay: Cells were pre-incubated with siRNA for 16 hours before performing migration and proliferation assay. Thymidine uptake was studied by addition of 500 nCi/ml [³H]thymidine (Perkin Elmer, Boston, MA) for 6 to 24 h. Cells were washed twice with PBS. Nonspecific uptake was studied in the presence of 10 μM cytochalasin B and subtracted from the measured value. MicroScint-20 (50 μl) was added, and the plate was read with TopCount (Packard Instrument).

PCR primer sequences: PCR primer sequences were chosen as the followings: DDR2, forward, 5’-GATCATGTTTGAATTTGACCGA-3’ (NM_031764:816-837); reverse, 5’-GCACTGGGGTTCACATC-3’ (NM_031764:982-998).

Immunohistochemical analysis: For immunohistochemical stain, the slides were postfixed in 4% paraformaldehyde for 20 min, treated in 3% hydrogen peroxide/PBS for 25 min, blocked in 5% normal rabbit serum for 20 min, blocked with biotin/avidin for 15 min each, and incubated with fluorescent isothiocyanate (FITC)-conjugated
goat polyclonal anti-DDR2 antibody (Santa-Cruz Biotechnology), mouse monoclonal anti-MMP2 antibody (Calbiochem®, San Diego, CA), or mouse monoclonal anti-SMC actin antibody (Calbiochem®). for 2 hours at room temperature, biotinylated rabbit-anti mouse IgG at 1:400 for 30 min, and Vector Elite ABC biotin-avidin-peroxidase complex for 30 min. To stain the collagen, direct red 80 (Sigma-Aldrich Co., Buchs SG, Switzerland) was used. Sections were then developed with diaminobenzidine and diaminobenzidine enhancer (Vector), counterstained with hematoxylin. Images were examined with a fluorescent microscope.