Dickkopf1 destabilizes atherosclerotic plaques and promotes plaque formation by inducing apoptosis of endothelial cells through activation of ER stress

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Several clinical studies reported that Dickkopf1 (DKK1) plasma levels are correlated with atherosclerosis. However, the impact of DKK1 on the formation and vulnerability of atherosclerotic plaques remains elusive. This study investigated DKK1’s effects on enlargement and destabilization of plaques by targeting endothelial cells and assessing the possible cellular mechanisms involved. The effects of DKK1 on atherogenesis and plaque stability were evaluated in ApoE−/− mice using lentivirus injections to knockdown and knock-in the DKK1 gene. The presence of DKK1 resulted in enlarged and destabilized atherosclerotic lesions and increased apoptosis, while silencing of DKK1 alleviated plaque formation and vulnerability in the whole progression of atherosclerosis. DKK1 expression was upregulated in response to ox-LDL treatment in a time- and concentration-dependent manner on human umbilical vein endothelial cell (HUVEC). The interference of DKK1 reversed ox-LDL-induced apoptosis in HUVECs. The mechanism underlying this effect was DKK1’s activation of the JNK signal transduction pathway and inhibition of canonical Wnt signaling, following by activation of the IRE1α and eif2α/CHOP pathways. In conclusion, DKK1 promotes plaque formation and vulnerability partly by inducing apoptosis in endothelial cells, which partly through inducing the JNK-endoplasmic reticulum stress pathway and inhibiting canonical Wnt signaling.

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Recent studies have found that acute coronary syndrome (ACS) is associated with the sudden rupture of atherosclerotic plaques and the rapid development of these plaques. While atherogenesis remains incompletely understood, studies of atherogenesis pathology suggest that multiple cellular malfunctions, including endothelial cell (EC) dysfunction and vascular integrity disruption, are involved, as well as increases in inflammatory cell numbers, the production of cytokines, the proliferation and migration of vascular smooth muscle cells (VSMCs), the activation of monocytes and macrophages, and neovascularization. Pathological biomechanical and haemodynamic changes (e.g., oxidative damage, shear stress) result in these events, which are closely correlated with plaque stability.1 EC dysfunction has been observed in atherosclerotic lesions in both humans and animals and eventually leads to apoptosis and the development of atherosclerosis.2,3 Endothelial dysfunction leads to proinflammatory activation, generates autocrine, and paracrine signaling loops, and influences other type of cells that are involved in atherogenesis.4 EC apoptosis occurs throughout the early stages of atherosclerosis and plays important roles in plaque regression and plaque instability,5,6 which are caused by various factors, particularly by oxidized low-density lipoprotein (ox-LDL).1,7 Thus, inhibition of EC apoptosis may be a useful therapeutic strategy for ameliorating plaque instability.

Dickkopf1 (DKK1), a secretory glycoprotein, can block the Wnt pathway by competitively binding to receptors (e.g., LRP5/6, Kremen) on the cell membrane.8 Ueland et al. found that DKK1 expression was stronger in von Willebrand factor (vWF)-positive ECs and in CD68-positive macrophages than in other areas of plaques.9 They also found that DKK1 participated in platelet-induced EC activation, indicating that DKK1 promotes inflammation in atherosclerotic plaques and is an atherogenic factor.9 In a previous clinical study of patients with ACS, we found that DKK1 plasma levels were not only correlated with disease severity but also served as a prognostic predictor. Thus, DKK1 concentration may reflect the severity and stability of coronary atherosclerosis.10 Several studies have indicated that DKK1 plays an important role in atherosclerosis; however, the underlying mechanisms have yet to be elucidated. Furthermore, the knockout mouse is not an ideal model for DKK1 research in disease, because the homozygous DKK1 (−/−) mutation is lethal.11 Therefore, in this investigation, we used a lentivirus to overexpress or silence DKK1 in ApoE−/− mice.

A previous study found a strong association between endoplasmic reticulum stress (ERS) markers, such as CCAAT/fi
enhancer-binding protein-homologous protein (CHOP) and glucose-regulated protein 78 (GRP78), and the presence of atherosclerotic plaques in human coronary artery lesions, suggesting that ERS is involved in the development of plaque instability in humans. Disrupting the secretion of Wnt5a, a Wnt pathway agonist, has been shown to induce ERS in mammalian cells, indicating that a correlation exists between Wnt secretion and ERS. DKK1 is an important regulator of the Wnt pathway, yet, its role in ERS-associated apoptosis in atherosclerosis remains unclear.

On the basis of these findings, we hypothesized that DKK1 promotes plaque formation and instability in part by stimulating EC apoptosis. To accomplish this, we investigated the effect of modulated DKK1 expression on atherosclerosis plaques in ApoE−/− mice and EC apoptosis; and explored the underlying mechanisms in endothelial cells using human umbilical vein endothelial cells (HUVECs).

Results

**DKK1 influenced the formation and vulnerability of aortic plaques and caused vascular endothelium dysfunction in** ApoE−/− **mice.** Intense GFP staining was observed in aortic plaques and carotid artery plaques (Figure 1b). The results of Western blotting to reveal aorta-containing proteins, immunohistochemistry and analysis of plasma DKK1 further demonstrated that DKK1 protein expression was significantly lower in the shDKK1 group and higher in the DKK1 group than in the NS and GFP groups (Figures 1c–f), which established that overexpression and silencing vectors were effective.

Endomucin is a marker for endothelial cells, and MOMA-2 is a marker for monocyte-macrophage. Similar to the findings of a previous study, atherosclerotic lesions in the aortic plaques in the ApoE−/− mice showed strong DKK1 expression in all endomucin-positive ECs and in small regions containing MOMA-2-positive macrophages (Figure 1g). Co-localization immunofluorescence staining further confirmed that the majority of DKK1 was expressed in ECs other than smooth muscle cells and macrophages.

Vascular endothelium dysfunction plays an important role in atherosclerosis. When the mice were fed atherogenic chow for 4 weeks, the shDKK1 group showed recuperative acetylcholine-induced endothelium-dependent relaxation in the thoracic aorta ring after pre-contraction with norepinephrine, whereas the DKK1 group showed damaged endothelial relaxation (Supplementary Figure 1).

In mice fed atherogenic chow for 12 weeks, both the en face lesion areas and the cross-section lesion areas measured in the aortic root were decreased in the shDKK1 group and increased in the DKK1 group compared with the areas in the NS and GFP groups (Figures 2a–c).

In both aortic (Figure 2d) and carotid (Figure 2h) plaques, the relative content of VSMCs and collagen fibers were lower in the DKK1 group but higher in the shDKK1 group than in the NS and GFP groups. Conversely, the relative contents of macrophages and lipids were higher in the DKK1 group but lower in the shDKK1 group than in the NS and the GFP groups. Accordingly, the plaque vulnerability index was higher in the DKK1 group but lower in the shDKK1 group. Moreover, fibrotic cap thickness was substantially lower in the DKK1 group and higher in the shDKK1 group than in the NS and the GFP groups (Figures 2d and g). Although plaques were completely filled in most carotid arteries, except in the shDKK1 group, the carotid plaques were shown the similar trend (Figures 2h–j). Moreover, vascular outward remodeling was more significant in the DKK1 group than in the other groups (Figure 2k). In mice fed atherogenic chow for 4 or 8 weeks, the shDKK1 group displayed substantially increased fibrous caps of plaque, while the DKK1 group showed increased plaque size and vulnerability (Supplementary Figure 2). DKK1 can aggravate plaque vulnerability not only during the short-term feeding of atherogenic diet, but also in the long-term atherogenic diet. These results reveal that DKK1 augments plaque formation and instability in both aortic and carotid plaques and that silencing DKK1 attenuates these effects.

**DKK1-induced inflammatory factors and apoptosis in aortic atherosclerotic plaques in** ApoE−/− **mice.** Several studies showed that inflammation and apoptosis contribute to the instability of atherosclerotic plaques. The content of inflammatory factors (e.g., IL-6, IL-1β, MCP-1, and TNF-α) was lower in the shDKK1 group and higher in the DKK1 group (P<0.05, Figure 3a).

Expression of the anti-apoptotic factor Bcl-2 was significantly upregulated in the shDKK1 group, while expression of the pro-apoptotic factor Bax and cleaved caspase-3 was significantly downregulated compared to the NS and GFP groups (P<0.05, Figure 3b). The opposite results were found in the DKK1 group (P<0.05, Figure 3b).

The percentage of TUNEL (+) cells was lower in the shDKK1 group and higher in the DKK1 group than in the NS and GFP groups (P<0.05, Figures 3c and d). The percentage of TUNEL (+) endomucin (+) cells showed a similar trend (P<0.05, Figure 3e). Accordingly, the results from immunofluorescence analysis and TUNEL assay co-localization revealed that DKK1 promoted apoptosis in atherosclerotic plaques, mostly due to its effects on ECs.

**DKK1 expression was upregulated following ox-LDL treatment in a time- and concentration-dependent manner, and interference of DKK1 inhibited ox-LDL-induced apoptosis in** HUVECs. Oxidized low-density lipoprotein (ox-LDL) may play a pre-eminent function in atherosclerotic lesion formation. In vitro, we used ox-LDL to mimic the stimulation to the endothelial cells during atherosclerosis. HUVECs treated with ox-LDL (150 μg/ml) for various lengths of time (0 h, 0.5 h, 1 h, 3 h, 6 h, or 12 h) exhibited time-dependent increases in DKK1 protein and mRNA levels as well as in DKK1 levels in the culture supernatant. DKK1 expression gradually increased at the mRNA level starting from 1h, at the protein level starting from 3h, and in the culture supernatant from 6h (P<0.05, Figures 4a–c). HUVECs treated for 6h with various concentrations of ox-LDL (0, 25, 50, 100, 150, or 200 μg/ml) exhibited concentration-dependent increases in DKK1 mRNA and protein expression and in DKK1 levels in the culture supernatant. DKK1 expression was consistently significantly higher in cells
treated with 150 and 200 μg/ml ox-LDL than in cells treated with 0 μg/ml (P<0.05, Figures 4d–f). These results indicate that DKK1 expression increases in HUVECs in response to treatment with ox-LDL. Based on these results, we chose to treat cells with 150 μg/ml ox-LDL for 6 h to investigate how DKK1 affects EC function.

We first examined the effect of DKK1 on apoptosis in HUVECs treated with ox-LDL. To accomplish this, cells were transfected with either negative control siRNA (NC) or DKK1 siRNA. The DKK1 siRNA significantly reversed the effects of ox-LDL on the expression of cleaved caspase-3 and the protein ratio of Bcl-2 to Bax (P<0.05, Figure 4g). Flow cytometry and TUNEL assay results consistently indicated that the percentage of cells undergoing apoptosis was significantly lower in the DKK1 siRNA group than in the NC group (P<0.05, Figures 4h–k).

Figure 1 Efficiency of lentivirus transfection in ApoE /– mice. (a) Flow charts showing the experimental protocol used in the in vivo studies. (b) Fluorescence images of the aortic root and carotid plaques two weeks after lentivirus transfection in the lenti-GFP group. (c,d) DKK1 protein expression in the aortic root in four groups of mice as determined by Western blotting. n = 6. (e) Areas stained positive for DKK1 in four groups of mice as determined by immunohistochemical staining in the aortic root and carotid plaques. n = 6. (f) Plasma levels of DKK1 in four groups of mice. (n = 11). (g) Co-localization of DKK1 (red) and endomucin (green) or MOMA-2 (green) expression in aortic plaques in the NS group. DAPI (blue) indicates nuclei. Data are shown as the mean ± S.D., *P<0.05 versus NS; #P<0.05 versus GFP.
Figure 2  Influence of DKK1 on plaque formation and stability in ApoE−/− mice. (a) En face Oil Red O staining of aortas and cross-sectional aortic root lesions with H&E staining in four groups of mice (NS, GFP, shDKK1, and DKK1). (b) Quantitative analysis of en face aortic lesions expressed as percentage lesion area relative to total aorta area. $n = 6$. (c) Quantitative analysis of cross-sectional plaque areas in aortic roots. $n = 6$. (d–e, h–i) Representative immunohistochemical staining and quantification of plaque content in aortic plaques and carotid artery plaques. (g) Quantitative analysis of plaque fibrotic cap thickness of aortic plaques. $n = 5$. (k) Quantitative analysis of plaque area and total lumen area in the carotid artery. $n = 6$. Data are shown as the mean ± S.D. *P < 0.05 versus NS; #P < 0.05 versus GFP.
Figure 3  Effects of DKK1 on inflammatory factors and plaque apoptosis in ApoE−/− mice. (a) Representative immunohistochemical staining and quantification of plaque content of inflammatory factors (IL-6, IL-1β, MCP-1, and TNF-α). n = 6. (b) Representative immunohistochemical stained images and quantification of Bax, Bcl-2, cleaved caspase-3 and caspase-12 levels in atherosclerotic plaques of ApoE−/− mice. n = 6. (c) Co-localization and quantitative analysis of TUNEL staining (green) and endomucin-positive areas (red) to measure cellular apoptosis in aortic plaque. DAPI (blue) indicates nuclei. n = 3. Data are shown as the mean ± S.D., *P < 0.05 versus NS; #P < 0.05 versus GFP.
DKK1-induced apoptosis in HUVECs by activating ERS. We next monitored changes in the expression of caspase-12, which is considered to be a marker of ERS-associated apoptosis in mice. Caspase-12 expression was reduced in the shDKK1 group and enhanced in the DKK1 group ($P<0.05$, Figure 3a). To further investigate whether DKK1-induced
apoptosis through ERS, we treated cells with 4-phenylbutyric acid (4-PBA), an ERS inhibitor. Treatment with 4-PBA significantly attenuated the increase in cleaved caspase-3 expression and the decrease in the Bcl-2/Bax ratio observed in rDKK1-stimulated HUVECs (P<0.05, Figure 5a) or lenti-DKK1-transfected HUVECs (P<0.05, Supplementary Figure 3a). Notably, the TUNEL assay and flow cytometry results were consistent with those obtained by Western blotting (P<0.05, Figures 5b–e). In summary, we demonstrated that DKK1 induces apoptosis partly via the ERS pathway both in vivo and in vitro.

We also examined the effect of DKK1 on ERS. First, cells transfected with DKK1 siRNA were confirmed to have reduced DKK1 expression relative to cells transfected with NC siRNA. In contrast, cells were treated with ox-LDL or rDKK1 to induce the overexpression of DKK1. To determine whether ox-LDL activates ERS through DKK1, we monitored changes in the levels of eukaryotic initiation factor 2α (eif2α), CHOP, inositol-requiring enzyme 1 (IRE1), sliced X-box–binding protein 1 (XBP1s), transcription factor 6 (ATF6), and GRP78,18 which are considered markers of ERS. All of these markers showed significantly increased protein expression in ox-LDL-treated HUVECs compared to control HUVECs. These results suggest that DKK1 may be involved in the activation of ERS in ox-LDL-treated HUVECs.

In summary, our findings indicate that DKK1 induces apoptosis partly via the ERS pathway both in vivo and in vitro. This suggests that DKK1 may be an important mediator of atherosclerotic plaque destabilization and development of atherosclerosis.
expression in HUVECs following treatment with ox-LDL or rDKK1 (P < 0.05, Supplementary Figure 4a). In addition, siRNA-mediated silencing of DKK1 reversed ox-LDL-induced ERS (P < 0.05, Supplementary Figure 4b). On the basis of these findings, we concluded that DKK1 may induce apoptosis in ox-LDL-treated HUVECs partly via ERS.

**DKK1-induced ERS-associated apoptosis through IRE1α and elf2α/CHOP.** We have monitored that DKK1 induced the level of elf2α, CHOP, IRE1, XBP1s, ATF6, and GRP78 in HUVECs. To identify the components playing a main role in DKK1-induced, ERS-associated apoptosis, we transfected HUVECs with NC siRNA, CHOP siRNA, or IRE1α siRNA prior to treatment with rDKK1 or transfection with lenti-DKK1.25 After treatment with rDKK1 or transfection with lenti-DKK1, Bax and cleaved caspase-3 protein expression decreased, while Bcl-2 expression increased in cells transfected with CHOP siRNA or IRE1α siRNA compared to those transfected with NC siRNA (P < 0.05, Figures 6a and b, Supplementary Figure 3b–c). Moreover, the flow cytometry and TUNEL assay results were consistent with those obtained by Western blotting (P < 0.05, Figures 6c–f).

To further verify the role of elf2α in DKK1-mediated effects, we also treated cells with salubrinal, which specifically inhibits eIF2α dephosphorylation.21–23 Salubrinal significantly attenuated the increased cleaved caspase-3 expression and decreased Bcl-2/Bax expression in rDKK1-stimulated HUVECs (P < 0.05, Figure 7a) and lenti-DKK1 transfected HUVECs (P < 0.05, Supplementary Figure 3d). Moreover, the flow cytometry and TUNEL assay results were consistent with those obtained by Western blotting (P < 0.05, Figures 7b–e). Generally, DKK1-induced apoptosis via IRE1α and elf2α/CHOP signaling, independent of other ERS transducers.

**DKK1-induced ERS-associated apoptosis through activation of the JNK pathway and inhibition of Wnt/β-catenin signaling.** We transfected cells with DKK1 siRNA to downregulate DKK1 and infected cells with lentivirus to overexpress DKK1. Western blotting revealed that DKK1 activated JNK, while knockdown of DKK1 led to a decrease in JNK phosphorylation (P < 0.05, Supplementary Fig 5a-5b). To further verify the effect of JNK pathway on ER stress and apoptosis, we pretreated HUVECs with the JNK inhibitor-SP600125 significantly reversed the upward trend in GRP78 and CHOP protein expression observed in HUVECs transfected with lenti-DKK1 (P < 0.05, Figure 8a). These data indicate that DKK1 induces apoptosis and ERS in HUVECs by targeting and upregulating JNK. Furthermore, Bcl-2 expression increased significantly in lenti-DKK1-transfected HUVECs that were pretreated with SP600125, while Bax expression decreased.

IM-12 activates canonical Wnt signaling, whereas FH535 acts as an inhibitor of canonical Wnt signaling.25 To verify the effect of the canonical Wnt signaling pathway on apoptosis and ERS, we pretreated HUVECs with IM-12 or FH535. HUVECs with pretreated IM-12 significantly reversed the upward trend in GRP78 and IRE1α protein expression in HUVECs transfected with lenti-DKK1 (P < 0.05, Figure 8d), while HUVECs with pretreated FH535 reverted the decrease in GRP78 and IRE1α protein expression in HUVECs transfected with DKK1 siRNA (P < 0.05, Figure 8e). However, the protein levels of JNK and CHOP were not changed in IM-12 or FH535.

**Discussion**

This is the first study to describe how DKK1 affects plaque formation and stability in atherosclerosis in ApoE−/− mice, which was accomplished by using lentivirus-mediated silencing and overexpression of the DKK1 gene. The following conclusions were generated: (1) The overexpression of DKK1 enlarged and destabilized atherosclerotic lesions and increased apoptosis, while inhibition of DKK1 expression hold the formation and vulnerability of atherosclerotic plaques in the whole progression of atherosclerosis; (2) treatment with ox-LDL induces DKK1 expression in HUVECs in a time- and concentration-dependent manner; (3) DKK1 induces ERS through IRE1α and elf2α/CHOP, leading to apoptosis; and (4) DKK1 activates ERS via both the JNK pathway and canonical Wnt signaling.

Our results show that DKK1 dose not influence the circulating levels of total cholesterol, LDL-C or blood glucose (Supplementary Table 1). Classical pathological studies have demonstrated that plaque components, inflammation factors and apoptosis play important roles in modulating the stability of atherosclerotic plaques.26–28 We found that DKK1 silencing reduced macrophage accumulation and increased VSMC numbers in plaques, while DKK1 overexpression augmented plaque vulnerability during the entire process of atherosclerosis.

DKK1 is an antagonist of the Wnt signaling pathway.29–32 Ueland et al.3 found that DKK1 contributes to the activation of ECs by platelets. In a previous clinical study of patients with ACS, DKK1 plasma levels not only correlated with disease severity but also served as a prognostic predictor of disease, suggesting that DKK1 levels reflect coronary atherosclerosis stability.10 In two previous investigations conducted by our group, we found that treatment with ox-LDL promotes DKK1 expression in macrophages, resulting in inhibiting the accumulation of lipids33 and that oscillatory shear stress can induce DKK1 expression in ECs through PAR1/CREB.34 Both of these biomechanical and haemodynamic factors contribute to the development and destabilization of atherosclerosis. In earlier research, we used a partial carotid ligation model to imitate and induce disturbed flow and acute endothelial injury.34,35 Differ from it, we used constrictive silica collars to accelerate atherosclerotic lesion formation.36 And we observed the lesion on both right carotid artery and aorta root 12 weeks’ atherogenic chow after collar surgery. Thus, in the present study, we demonstrated the negative effects of DKK1 on the formation and instability of atherosclerotic plaques.

In this study, we also observed that the expression of inflammatory factors (e.g., IL-6, IL-1β, TNF-α, and MCP-1) decreased in conjunction with the downregulation of DKK1 and increased with upregulation of DKK1. Accumulation of inflammatory factors may induce monocyte recruitment and adhesion to the activated endothelial layer, thereby aggravating plaque instability.6,26–28 Several studies have reported that the exogenous inhibition of DKK1 reduced IL-1β and
Figure 6  DKK1 activates CHOP and IRE1α signaling during ERS to induce apoptosis. HUVECs were transiently transfected with negative control (NC) siRNA, CHOP siRNA (si-CHOP) or IRE1α siRNA (si-IRE1α) for 24 h and then treated with rDKK1 (300 ng/ml) for 6 h. (a,b) Western blotting for quantification of the protein levels of cleaved caspase-3, Bcl-2, and Bax. n = 6. (c,e) HUVECs with stained nuclei (green) were considered TUNEL-positive (arrows). The percentage of TUNEL-positive cells was calculated and quantified. n = 3. (d,f) Flow cytometric analysis for quantification of early apoptotic cells (i.e., Annexin V-positive and 7-AAD-negative cells, lower-right quadrant). n = 3. Data are shown as the mean ± S.D. *P < 0.05 versus NC or control; #P < 0.05 versus only rDKK1 treatment.
TNF-α expression, significantly inhibited TNF-α expression in macrophage sand chondrocytes stimulated by lipopolysaccharide.\(^\text{37}\) Attenuated angiogenesis,\(^\text{38}\) and decreased monocyte adhesion to HUVECs.\(^\text{39}\) DKK1 has also been shown to affect MMP-3 expression and influence collagen degradation in cartilage.\(^\text{40}\) Furthermore, DKK1 upregulation increases neovascularization.\(^\text{41}\) Thus, DKK1 may lead to plaque instability by augmenting inflammation, adhesion, collagen degradation and neovascularization.

In addition, we found that inhibition of DKK1 expression can attenuate cleaved caspase-3 expression as well as apoptosis. As a major regulator of the Wnt signaling pathway, DKK1 induces cellular apoptosis in many diseases.\(^\text{29–32}\) Overexpression of DKK1 sensitizes cells in brain tumors,\(^\text{42}\) renal cell carcinoma and thyroid cancers\(^\text{43,44}\) to apoptosis. Weng et al. discovered that DKK1 expression is closely correlated with the expression of pro-apoptotic factors (e.g., Bad and caspase-3) in osteoarthritis and inhibition of DKK1 expression reduced caspase-3 cleavage and alleviated chondrocyte apoptosis by reducing Bax expression and increasing Bcl-2 expression.\(^\text{40,45}\) Cellular apoptosis is ubiquitous in vulnerable plaques; as such, recent studies on vulnerable plaques have mostly focused on inflammation and cellular apoptosis.

Multiple pathophysiological factors, both systemic and localized to the arterial walls, can disturb ER function in ECs, VSMCs, and macrophages during the initiation and progression of atherosclerosis.\(^\text{46}\) Expression of ERS activation markers has been observed in atherosclerotic lesions in humans and animals.\(^\text{47}\) Importantly, ERS-associated apoptosis is correlated with plaque instability and the clinical progression of atherosclerosis.\(^\text{47}\) Expression of ERS activation markers has been observed in atherosclerotic lesions in humans and animals.\(^\text{47}\) Importantly, ERS-associated apoptosis is correlated with plaque instability and the clinical progression of atherosclerosis. CHOP is only robustly expressed in “vulnerable” plaques that show evidence of lesions and apoptosis.\(^\text{47}\) Moreover, Cominacini et al. revealed that persistent ERS is related to abnormal numbers of apoptotic cells in vulnerable plaques.\(^\text{37}\) Consistent with these findings, our results showed that inhibition of DKK1 expression remarkably reduced the expression of ERS-associated apoptotic markers (e.g., caspase-12) in ApoE\(^{-/-}\) mice. Another study found that disrupting the secretion of human Wnt5a, a Wnt pathway agonist, induced ERS in mammalian cells, revealing a correlation between Wnt secretion and
Figure 8: DKK1 induces apoptosis by promoting ERS via activation of the JNK pathway and inhibition of canonical Wnt signaling. (a–c) Cells were pretreated with DMSO or SP600125 (10μM) for 1 h before transfection with lenti-DKK1. Western blotting for quantification of cleaved caspase-3, Bcl-2, Bax, CHOP and GRP78 protein levels. n=6. Data are shown as the mean ± S.D.* P<0.05 versus DMSO; # P<0.05 versus DKK1 treatment only. (d, f, h) Cells were pretreated with DMSO or IM-12 (3μM) for 1 h before and during transfection with lenti-DKK1. Western blotting for quantification of cleaved caspase-3, Bcl-2, Bax, p-JNK, CHOP, IRE1α, and GRP78 protein levels. n=6. Data are shown as the mean ± S.D.* P<0.05 versus DMSO; # P<0.05 versus DKK1 transfection. (e, g, i) Cells were pretreated with DMSO or FH535 (30μM) for 1 h before and during transfection with DKK1 siRNA. Western blotting for quantification of cleaved caspase-3, Bcl-2, Bax, p-JNK, CHOP, IRE1α, and GRP78 protein levels. n=6. Data are shown as the mean ± S.D.* P<0.05 versus DMSO; # P<0.05 versus DKK1 shRNA transfection. (j) Proposed model of the DKK1 signaling pathway responsible for cell apoptosis.
cell death and disease

12

promotes EC apoptosis via caspase-12 and the mitochondrial
ERS is known to induce apoptosis.55,56 In particular, ERS
inhibited foam cell formation in macrophages. 33 Besides,
variations on EC apoptosis is unclear. In 2005, Boyce
have specific ERS components; however, the effect of these
apoptosis. In the initiation of the atherosclerotic plaque formation57 and the
progression to advanced atherosclerosis, which is vulnerable to
rupture.3,6,7,49–51 We found that both SMCs and macro-
phages take up the GFP-labeled DKK1 from co-cultured
ECs (Supplementary Figure 6a). In previous studies, recom-
binant DKK1 blocked the proliferation of VSMCs52 and
inhibited foam cell formation in macrophages.33 Besides, inhibition of DKK1
expression can attenuate the inflammation of macrophages (Supplementary Figure 6b–c). Thus, the
effects of DKK1 in promoting the dysfunction and apoptosis of endothelial cells may be the “starting point” of DKK1’s
effects on cross-talk with other cells in atherogenesis. ADMA53 and ET-154 are markers of endothelial cell dysfunction. In our
study, we also found that ADMA and ET-1 were positively correlated with DKK1 levels in plasma from healthy controls,
unstable angina pectoris and acute myocardial infarction patients (P<0.05, Supplementary Figure 7). Therefore, we
focused on ECs to experimentally determine the function of

Inhibition of DKK1 expression decreased ox-LDL-induced apoptosis and ERS in HUVECs. Prolonged and unresolvable
ERS is known to induce apoptosis.55,56 In particular, ERS
promotes EC apoptosis via caspase-12 and the mitochondrial
pathway.57,58 ERS has also been induced in ECs via pathological shear stress,59 hypoxia,18,57,58 and increased
GRP78, CHOP and caspase-12 expression. Here, for the first
time, we showed that ox-LDL treatment activates ERS
apoptosis via the JNK pathway, thereby promoting HUVECs
apoptosis.

Numerous studies have shown that different cell types have specific ERS components; however, the effect of these
variations on EC apoptosis is unclear. In 2005, Boyce et al.21 reported that salubrinal, a selective inhibitor of eif2α
dephos-
phorylation, protects cells from ERS. In the present study, we used salubrinal, CHOP siRNA and IRE1α siRNA to inhibit
various components associated with ERS and to investigate the role of eif2α, CHOP and IRE1α in ERS-associated
apoptosis. Ultimately, we found that DKK1-induced ERS-
associated apoptosis via IRE1α and eif2α/CHOP signaling. DKK1 is an antagonist of the canonical Wnt signaling pathway. We also
found DKK1 can influence GRP78, IRE1α and apoptosis factors through Wnt/β-catenin signaling. However, Wnt/β-catenin signaling did not change the protein levels of
JNK and CHOP.

In conclusion, inhibition of DKK1 expression effectively decrease plaque stability by attenuating ERS-mediated cellular
apoptosis through initiating the JNK pathway and
inhibiting Wnt/β-catenin. Moreover, the IRE1α and
eif2α/CHOP pathways were found to participate in the activation of ERS. However, the mechanism underlying the augmentations of
ERS by DKK1 remains to be determined. Notably, lentiviruses have low specificity in vivo. Therefore, in future experiments we will use EC-specific DKK1 KO mice and macrophage-
specific DKK1 KO mice to determine the exact role of DKK1 in
atherosclerosis through its action on various cell types. Although our data are preliminary, these findings might lead
to new and promising methods for the treatment of
atherosclerosis.

Materials and Methods

Ethics statement. All in vivo protocols involving animal care and experiments
were approved by the Guide for Care and Use of Laboratory Animals published by the
United States National Institutes of Health (NIH Publication, 8th Edition, 2011) and
the Animal Management Rules of the Chinese Ministry of Health (Document No. 55, 2001). All in vivo experiments were approved by the by
the Animal Care Committee of Shandong University. All the in vitro experimental protocols were approved by
the Key Laboratory of Cardiovascular Remodeling and Function Research, Qilu
hospital, China. Human plasma samples were obtained from 72 patients of 45–75
years old patients in Qilu hospital. The research protocol was approved by the
ethical committee of Qilu hospital, Shandong University.

Atherosclerosis animal model protocol. A total of 120 ApoE−/− mice
(eight-week-old males) were purchased from the Peking University Animal
Research Center (Beijing). All mice were fed atherogenic chow (i.e., a high-fat diet
with 0.25% cholesterol and 15% cocoa butter) (Figure 1a). The atherosclerotic
animal model protocol was created as previously described.33 We applied constrictive silica collars
with 0.25% cholesterol and 15% cocoa butter. Pentobarbital sodium was used for anesthesia via intraperitoneal injection
(40 mg/kg) when placing the constrictive collars. The mice were randomly divided into four groups (n=30 each): a normal saline group (NS), an empty lentivirus
model (GFP), a DKK1 lentivirus model (shDKK1), and a DKK1 lentivirus model
(DKK1). Eight weeks after the surgery, a 200 μl suspension (4×10^6 TU DKK1 or
DKK1 lentivirus per ml) was injected into each mouse through the tail vein. The
mice were killed 4 weeks post-transfection using pentobarbital sodium (50 mg/kg, i.
p.) before exsanguination by perfusion via the abdominal aorta with PBS.

Cell culture. HUVECs were obtained from ScienCell Research Laboratories
(Carlsbad, CA, USA) and cultivated in endothelial cell medium (ECM) (ScienCell,
Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/
streptomycin. Cell at 37 °C in 5% CO2. Cells from passages 4 to 8 were used for
experiments. THP-1 cells obtained from the American Type Culture Collection
(ATCC). .160 nM phorbol myristate acetate (PMA) was used overnight for THP-1
cell differentiation into macrophages.

Lentiviral silencing and overexpression-vector construction. To
generate a lentivirus-mediated silencing vector, the lentivirus vector pGLV3/H1/GFP
+Puro (pGLV3) was purchased from GeneChem (Shanghai, China), and a
short-hairpin RNA sequence targeting DKK1 and or scrambled control RNA was
cloned into the vector. The following duplexes targeted murine DKK1: sense
5′-TCACATCAAGCCGAAAAT-3′; antisense 5′-TCACATCAAGCCGAAAAT-3′.
To achieve lentivirus-mediated DKK1 overexpression, the lentiviral vector LV5
was purchased from GenePharma Co., Ltd. (Shanghai, China), and the full-length coding
sequence of either human or mouse DKK1 C-terminally tagged with green
fluorescent protein (GFP) was cloned into the vector. A vector cloned with GFP alone
was used as a negative control (NC).

siRNA and RNA interference. Upon reaching 40–60% confluence,
HUVECs were transfected with specific siRNA or negative control siRNA
(GenePharma, Shanghai, China) (shown in Supplementary Table 1) using
Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) in Opti-MEM
(Gibco, Thermo Fisher Scientific, Waltham, MA, USA). After 6 h of transfection, the
medium was replaced with complete ECM, and the cells were cultured for an
additional 24 h. The transfected cells were treated with ox-LDL, recombinant DKK1
(dDKK1) or lentivirus DKK1 at the designated concentrations and for the indicated times.

**Biochemical measurements.** The mice were fasted overnight. Blood samples were collected and centrifuged. Serum levels of total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol and blood sugar were measured via enzymatic assay using an automatic biochemical analyser (Roche Cobas Integra 800, Basel, Switzerland).

**Histopathology and immunohistochemistry.** The whole aorta, the aortic root and the RCA were dissected, removed, fixed in 4% formaldehyde overnight at 4 °C, embedded in OCT compound, prepared into 5-μm-thick sections. The cryosections were stained with haematoxylin and eosin for plaque morphology, oil red O for lipids, picrosirius red for collagen and Masson's trichrome staining for fibrous cap. After blocking in 5% bovine serum albumin (BSA) in PBS, the sections were incubated with primary antibodies (shown in Supplementary Table 2) overnight at 4 °C and then with an HRP Detection System (ZSG-Bio, Beijing, China). Detection was subsequently performed using DAB (3, 3'-diaminobenzidine) (ZSG-Bio). Plaques stained with picrosirius red were viewed under polarized light. The areas of collagen, VSMCs, extracellular lipid deposits and macrophages were recorded as the percentage of positive area divided by the plaque area in 20 high-power fields (20°). The vulnerability index was calculated using the following formula: (macrophage staining%+lipid staining%)/(VSMC staining%+collagen staining%).

**Flow cytometry.** Cell apoptosis was analyzed using an Annexin V/PI-Amino-Actinomycin (7-AAD) Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. The following groups were used to set up compensation and to define quadrants: unstained control cells, cells stained with PE Annexin V (no 7-AAD), and cells stained with 7-AAD (no PE Annexin V). Apoptotic cells were examined using a flow cytometer (Becton-Dickinson, USA) within 1 h, and the percentage of early apoptotic cells (upper-right quadrant) was measured using FlowJo software (Tree Star, Ashland, OR, USA).

**In situ detection of apoptotic cells.** Apoptotic cells and apoptotic ECs in aortic root cryosections were determined using an In Situ Apoptosis Fluorescein Detection Kit (Millipore, Billerica, MA, USA).

**Statistical analysis.** Data were analyzed using SPSS v16.0 (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± S.D. of at least three independent experiments. Normality of variables distribution was tested by the Kolmogorov-Smirnov test. Comparisons were analyzed using Student's t-test or one-way ANOVA followed by Bonferroni post hoc test. P < 0.05 was considered statistically significant.

**Conflict of Interest**

The authors declare no conflict of interest.

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Dickkopf1 destabilizes atherosclerotic plaques

M Di et al

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