A Cytokine-like Protein DKK3 Is Atheroprotective

Running Title: Yu et al.; DKK3 is Atheroprotective

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Abstract

Background—Dickkopf-related protein (DKK) 3 is a secreted protein that is involved in the regulation of cardiac remodeling and vascular smooth muscle cell differentiation, but little is known about its role in atherosclerosis.

Methods—We tested the hypothesis that DKK3 is atheroprotective using both epidemiological and experimental approaches. Blood DKK3 levels were measured in the Bruneck Study in 2000 (n=684) and then in 2005 (n=574). DKK3-deficient mice were crossed to ApoE−/− mice to evaluate atherosclerosis development and vessel injury-induced neointimal formation. Endothelial cell migration and the underlying mechanisms were studied using in vitro cell culture models.

Results—In the prospective population-based Bruneck Study, the level of plasma DKK3 was inversely related to carotid artery intima-media thickness and five-year progression of carotid atherosclerosis, independently from standard risk factors for atherosclerosis. Experimentally, we analyzed the area of atherosclerotic lesions, femoral artery injury-induced re-endothelialization and neointima formation in both DKK3+/−/ApoE−/− and DKK3+/+/ApoE−/− mice. It was demonstrated that DKK3 deficiency accelerated atherosclerosis and delayed re-endothelialization with consequently exacerbated neointima formation. To explore the underlying mechanisms, we performed transwell and scratch migration assays using cultured human endothelial cells, which exhibited a significant induction in cell migration in response to DKK3 stimulation. This DKK3-induced migration was associated with activation of ROR2 and DVL1, activated Rac1 GTPases and upregulated JNK and c-jun phosphorylation in endothelial cells. Knockdown of ROR2 receptor using specific siRNA or transfection of a dominant negative form of Rac1 in endothelial cells markedly inhibited cell migration and downstream JNK and c-jun phosphorylation.

Conclusions—This study provides the evidence for a role of DKK3 in the protection against atherosclerosis involving endothelial migration and repair, with great therapeutic potential implications against atherosclerosis.

Key Words: atherosclerosis; population studies; animal model cardiovascular disease; endothelial cell; DKK3
Clinical Perspective

What is new?

- We found that plasma level of dickkopf-related protein 3 (DKK3), a member of the dickkopf family is negatively correlated with atherosclerosis in human subjects.
- We demonstrated that DKK3 promotes re-endothelialization in murine models of atherosclerosis and wire-induced femoral artery injury, thus revealing its atheroprotective role.
- We explored the mechanism of DKK3-induced endothelial cell migration, i.e. via non-canonical Wnt signaling pathway.

What are the clinical implications?

- The present finding of an inverse association between plasma DKK3 level and atherosclerosis may provide a novel biomarker for endothelial integrity and repair.
- DKK3 exhibits atheroprotective characteristics, which may bear clinical potential for treatment of atherosclerosis.
The dickkopf-related protein (DKK) family, composed of DKK1, 2, 3, 4 and Soggy, is a group of secreted glycoproteins, of which DKK3 is highly expressed in endothelium and muscles. DKK3 appears to have a decisive function in myogenic cell fate since it is also highly expressed in different skeletal muscle subtypes. DKK3 has also been established as a potential tumor biomarker expressed in many cancer cell lines and an effective tumor suppressor in numerous human cancers. It has been reported that DKK3 plays a role in promoting angiogenesis in different types of tumors. Recently, several studies have discovered that DKK3 prevented the progression of cardiac hypertrophy and was also involved in vascular smooth muscle cell differentiation. However, the involvement of DKK3 in vascular diseases such as atherosclerosis remains unknown.

Atherosclerosis is characterized by endothelial dysfunction, inflammation, progressive lipid deposition and vessel stiffness with potential complications such as myocardial infarction or stroke. The endothelium, as a crude restrictive barrier of the vessel wall, can protect the vessel from inflammation. Once endothelial cells are impaired, they will become the initial sensors of a complex cascade of events. Many studies have demonstrated that the underlying pathophysiology of atherosclerosis is initiated by endothelial dysfunction, which is caused by physical or chemical offences such as hypertension, shear stress of disturbed laminar flow, reactive oxygen species in the circulation, decreased nitric oxide bioactivity, hyperlipidemia and hyperglycemia. These factors can directly or indirectly induce endothelial dysfunction/death in arteries followed by cell regeneration in situ. In this process, neighbouring endothelial cells have been proven to contribute to re-endothelialization by migration and proliferation. In previous studies, vascular endothelial growth factor (VEGF) has been identified as a potent soluble factor for acceleration of re-endothelialization and prevention...
of neointima formation. However, whether some novel soluble molecules are also playing a role in endothelial repair remains under investigation. In the present study, we take advantage of relevant human samples, transgenic animals and in vitro cell biology models to elucidate the potential impact of DKK3 in atherosclerosis. In humans, we observed an inverse correlation between blood DKK3 level and development of atherosclerosis. In addition, we utilized genetic knockout mouse models combined with ApoE−/− mouse to assess the effects of DKK3 on atherosclerosis, re-endothelialization and neointima formation after femoral artery injury. We found that DKK3 promoted re-endothelialization and inhibited lesion formation in DKK3+/+ApoE−/− mice. Our in vitro studies also revealed that DKK3 can induce endothelial cell migration via non-canonical Wnt signaling pathway.

Methods
An expanded Methods is available in the online-only Data Supplement.

Study Population
Population recruitment was performed as part of the prospective community-based Bruneck Study. The survey area was located in the north of Italy (Bolzano Province). Special features of the study design and protocol have been described previously in detail and are provided in the online-only Data Supplement. The current study focused on the evaluation in 2000 (n=684) and follow-up between 2000 and 2005. The appropriate ethics committees approved the study protocol and all study subjects gave their written informed consent before entering the study.

Enzyme-Linked Immunosorbent Assay (ELISA) for Plasma DKK3
The levels of DKK3 in human plasma were detected using R&D DKK3 ELISA kit (R&D, DY1118). DKK1 levels were measured in serum with a commercial ELISA (Biomedica, Vienna,
Austria): Intra- and inter-assay CVs were low at 3% each and the lower detection limit was 1.6 pmol/L.

Animals

All animal experiments were performed according to the protocols approved by the Institutional Committee for the Use and Care of Laboratory Animals. ApoE−/− mice were purchased from the Jackson Laboratory. DKK3−/− mice were generated as described previously. Three genotypes of DKK3−/−, DKK3+/- and DKK3+/+ mice were identified using PCR (primers: 5'-GATAGCTTTCCGGGACACAC-3'; 5'-TCCATCAGCTCCTCACTCTCCA CCTCT-3'; 5'-TAAGTTGGGTAACGCCAGGGT-3'). ApoE−/− mice were crossed with DKK3−/− mice in our laboratory, and heterozygous offsprings were mated to produce ApoE−/− mice lacking DKK3 (DKK3−/−ApoE−/−). The genetic background of all mice used in the present study was C57BL/6.

Creation of Chimeric Mice

The procedure used for creating chimeric mice was similar to previously described. In brief, bone marrow transplantation was carried out on the DKK3+/- mice and DKK3−/− mice separately. Bone marrow cells were obtained from the femurs and tibias of either DKK3+/- or DKK3−/− mice (donors) and injected (1x10^7 cells in 0.2ml) into the tail veins of the 6-8 week old DKK3−/− mice or DKK3+/- mice (recipients), which received lethal irradiation (950 Rads) before. The measurement of DKK3 level in peripheral blood was performed 3 weeks after bone marrow transplantation.

Tissue Harvesting and Lesion Analysis

Mice were anesthetized by intraperitoneal injection of pentobarbital atrium (50mg/kg b.w.). Blood was obtained from inferior vena cava for lipid analysis. The heart was harvested intact and stored immediately in liquid nitrogen and the whole length of the aorta was stored in formalin at
4°C. 8-μm thick frozen sections were obtained from the heart and were stained with Oil Red O as described elsewhere. Aortas were opened longitudinally and fixed on a silicon bed with stainless steel pins (Fine Science Tool, USA) with the intima exposed. Oil Red O staining was performed. Lesion areas were measured and quantified using a computer software AxioVision™ as described previously.

**Transwell Chemotaxis Assay**

Migration chemotaxis assay was performed by applying 24-well Boyden chambers with 8 μm pore size polycarbonate membranes (Corning) as described previously. HUVECs were seeded onto the upper chamber at 1x10⁵ cells in 0.1% FBS EBM-2 basal medium, while the bottom chamber contained either 0.1% FBS EBM-2 basal medium with indicated concentrations of recombinant human DKK3 or Adeno-DKK3-HA/Adeno-CMV null overexpressed CHO cells supernatant. 0.1% FBS EBM-2 basal medium served as negative control for the comparison with recombinant human DKK3. After incubation for 6 hours at 37°C, the cells remained on the upper side of the filters were removed by a cotton swab. The migrated cells on the underside of the membrane were fixed with 4% paraformaldehyde prior to staining with 0.1% crystal violet solution for 15 minutes. Data was expressed as the fold of migrated HUVECs compared to their corresponding controls.

**Statistical Analysis**

*Population study*

The data were analyzed using the SPSS 24 software package. Levels of variables according to DKK3 tertile groups were presented as mean values ± SD or as medians with corresponding 25th and 75th percentiles (continuous variables), and percentages (dichotomous variables). Associations between DKK3 level (predictor variable) and vascular risk factors, life style and
demographic variables, IMT and atherosclerosis progression were assessed using linear and logistic regression analysis. Levels of C-reactive protein and triglycerides were log_{e}-transformed to satisfy the assumption of normality and constant variance of the residuals. The multivariate models focusing on IMT or atherosclerosis progression included the following covariates: age (years), sex (female, male), smoking (cigarettes/day), hypertension, HDL and LDL cholesterol, triglycerides, hsCRP, creatinine, body mass index, wait-to-hip ratio, chronic infections, fasting glucose, and physical activity (sports score). A two-sided p value < 0.05 was considered significant.

**In vivo and in vitro studies**

Data for *in vivo* and *in vitro* studies are presented as the mean ± standard error of the mean (S.E.M.) of at least three separate experiments. The analysis was performed using Graphpad Prism V.6 (GraphPad Software, San Diego CA) using t-test between two groups and analysis of variance (one-way ANOVA) followed by Dunnett’s multiple comparison test for more than two groups. A p value < 0.05 was considered significant.

**Results**

**Plasma DKK3 Level is Negatively Correlated with Atherosclerosis in the Population-based Bruneck Study**

The Bruneck study is a prospective survey on atherosclerosis and its risk factors, as well as protective mechanisms against it^{28,29}. Plasma was collected from a random sample of the general community. A total of 611 samples from the year 2000 and 554 samples from the year 2005 were analyzed for DKK3 concentrations using ELISA. DKK3 levels in both assessments were highly correlated indicating a low intra-individual variability of a five-year interval (Spearman’s rank
correlation coefficient r=0.500) (Supplement Figure 1). Population characteristics according to DKK3 tertile groups are depicted in Table 1. Age increased across DKK3 tertile groups (p<0.001). After adjustment for age and sex, several standard and emerging cardiovascular risk factors showed association with DKK3 level (creatinine, body mass index, waist-to-hip ratio, and fasting glucose) and all these associations were inverse (Table 1). Of note, common carotid artery intima-media thickness (CCA IMT), a surrogate marker of early vessel pathology, was reduced in the top DKK3 tertile group (p=0.008) (Figure 1A). There was also a gradual decrease in the risk of incident atherosclerosis (incident plaques, n=59 out of 259 subjects free of carotid atherosclerosis at baseline, Figure 1B) and carotid artery stenosis (advanced plaques, n=63 out of 332 subjects with pre-existent carotid atherosclerosis, Figure 1C) across DKK3 tertile groups (p<0.05 each).

We next calculated the odd ratios of incident atherosclerosis and stenosis for a 1-SD unit higher DKK3 level (logistic regression analysis, p=0.035 and 0.004, Model 1, Table S1) and confirmed the associations to be independent of a broad panel of established and putative vascular risk factors including those significantly related to DKK3 level in Table 1 (Model 2, Supplement Table 1). Similar findings were obtained with CCA-IMT (Supplement Table 1). Overall, there is strong evidence of an inverse association between DKK3 level and both early and advanced stages of atherosclerosis. To scrutinize the correlation between DKK3 and other circulating factors in the blood, G-CSF, MMP9, VEGF, SDF-1, sRANKL, osteoprotegerin and angiogenic cells levels were measured. SDF-1 was significantly related to DKK3 (p=0.021, adjusted for age and sex) (Supplement Table 2).

Interestingly, the level of DKK1, another member of the Dickkopf family, showed a significant positive association with CCA-IMT (age- and sex-adjusted regression coefficient
[95% CI] for a 1-SD unit higher DKK1 level, 0.031 [0.008 to 0.054]). The inverse association between DKK3 and CCA-IMT was more pronounced in subjects with high (≥44.1 pmol/L i.e. ≥ median) DKK1 level (age- and sex-adjusted regression coefficient [95% CI] for a 1-SD unit higher DKK3 level, -0.018 [-0.037 to 0.001]) than in those with low (<44.1 pmol/L) DKK1 level (age- and sex-adjusted regression coefficient [95% CI] for a 1-SD unit higher DKK3 level, -0.011 [-0.027 to 0.004]) but this interactive effect between DKK1 and DKK3 did not reach statistical significance (P_interaction=0.17).

Deficiency of DKK3 Promotes Atherosclerosis in Mice

To investigate the role of DKK3 in the development of atherosclerosis, we crossed DKK3-deficient mice35 with ApoE−/− animals to generate DKK3+/−/ApoE−/− mice (Supplement Figure 2). As observed with the measurement of atherosclerotic lesions by en face staining (oil red O) of aortas (Figure 2C and Supplement Figure 3), cross-sectional analysis of aortic root samples in male mice fed a normal chow diet for 16 weeks revealed smaller lesion in DKK3+/−/ApoE−/− than in DKK3−/−/ApoE−/− mice (Figure 2A and 2B). Analysis by immunofluorescence showed a significant increase of αSMA staining in lesions from DKK3+/−/ApoE−/− mice (Figure 2A and 2D) compared to DKK3−/−ApoE−/−, suggesting these smaller lesions were more stable and less advanced.

Furthermore, a staining against CD68 marker revealed a reduction in the number of lesional macrophages in DKK3+/−/ApoE−/− mice (Figure 2A and 2D). All together, these data suggest that DKK3 could have a protective role against atherosclerosis in ApoE−/− mice.

DKK3 Deficiency Leads to Endothelial Dysfunction in ApoE−/− Mice

It is well established that endothelial cells play an essential role in the homeostasis of vascular wall36. To investigate the possible mechanism associated with the accelerated atherosclerosis in DKK3−/−/ApoE−/− mice, we first analyzed the integrity of the endothelium by injecting Evans blue
in DKK3\(^{-/-}\)/ApoE\(^{-/-}\) and DKK3\(^{+/+}\)/ApoE\(^{-/-}\) mice. The aortas harvested from DKK3\(^{-/-}\)/ApoE\(^{-/-}\) mice exhibited a larger blue area, indicating more endothelial damage (Figure 3A). Scanning electron microscopy analysis demonstrated apparent endothelium loss in the aortas from DKK3\(^{-/-}\)/ApoE\(^{-/-}\) mice (Figure 3B). To further confirm the impaired endothelial integrity in DKK3\(^{-/-}\)/ApoE\(^{-/-}\) mice, immunofluorescence staining of endothelium marker eNOS was performed. A significant decrease in the number of eNOS-positive cells was observed in DKK3\(^{-/-}\)/ApoE\(^{-/-}\) mice (Figure 3C). These data indicate DKK3-deficient mice display increased endothelium damage, suggesting a protective role of DKK3 on the endothelium in the context of atherosclerosis.

**DKK3 Deficiency Delays Re-endothelialization and Aggravates Neointima Formation in Wire-Injured Murine Femoral Arteries**

To test the hypothesis that DKK3 is involved in post-injury endothelium recovery, we performed wire injury in femoral arteries of DKK3\(^{-/-}\)/ApoE\(^{-/-}\) and littermate DKK3\(^{+/+}\)/ApoE\(^{-/-}\) mice. Re-endothelialization of the arteries was quantified one-week post injury by en face staining of endothelial marker eNOS. Endothelial cells were recovered by 80% after one week in DKK3\(^{+/+}\) mice. In contrast, re-endothelialization in DKK3\(^{-/-}\) mice was only about 50% recovery one-week post-injury (Supplement Figure 4). Subsequent neointimal hyperplasia was dramatically aggravated in DKK3\(^{-/-}\) mice 2 and 3 weeks post-injury, as demonstrated by increased intima area and reduced lumen area (Figure 4A). To test whether the expression of DKK3 in hematopoietic cells contributed to this process, we performed bone marrow transplantation experiments. In the wild-type chimaera, DKK3 level in mice serum was significantly increased after 2 weeks despite transplantation with DKK3\(^{-/-}\) bone marrow when compared to a non-transplanted wild-type mouse control, indicating that DKK3 is mostly released from non-bone marrow tissues (Supplement Figure 5). ApoE\(^{-/-}\)/DKK3\(^{+/+}\) chimeric mice
transplanted with \( DKK3^{+/−} \) bone marrow showed comparable neointimal hyperplasia to \( ApoE^{−/−}/DKK3^{+/+} \) mice with wild-type bone marrow 3 weeks after femoral artery wire injury (Figure 4B), indicating that DKK3 expression in hematopoietic-derived cells does not contribute to protection from atherosclerosis and neointima formation post-injury.

**Exogenous DKK3 Induces Endothelial Cell Migration**

To study whether DKK3 can directly stimulate endothelial cell migration in vitro, both transwell and scratch-wound healing migration assays were performed. Data showed that endothelial cell (HUVEC) migration was significantly induced by human recombinant DKK3 and peaked at 25 ng/ml (Figure 5A, B). Similarly, mouse DKK3 induced mouse lung endothelial cell migration indicating a conserved mechanism (Supplement Figure 6A, B). To further investigate whether the glycosylated form of DKK3 contributes to endothelial cell migration, CHO cell lines, which do not express native DKK3, were transduced with human HA-tagged DKK3 adenovirus to produce the protein. Initially, qPCR and ELISA analysis confirmed DKK3 expression in both CHO cell lysates and their supernatant in a dose-dependent manner (Supplement Figure 6C, D). Additionally, Western blot analysis was performed to detect glycosylated DKK3 (65KD) in CHO cell supernatant and de-glycosylated DKK3 (50KD) in cell lysate (Supplement Figure 6E, F). Furthermore, the supernatant of DKK3-adenovirus induced CHO cells (ADV-DKK3-SN) was utilized in migration assays to confirm endothelial cell movement (Figure 5C, D). Together, these results suggested that glycosylated DKK3 present in ADV-DKK3-SN was responsible for the effects observed endothelial cell migration. To rule out the possibility that residual adenoviral particles present in ADV-DKK3-SN could lead to overexpression of DKK3 in endothelial cells and affect cells migration, a qPCR analysis was performed and showed that the increase of DKK3 expression in ADV-DKK3-SN treated-endothelial cells was negligible in comparison.
with the induction of DKK3 expression in endothelial cells directly transduced with DKK3 adenovirus (Supplement Figure 6G, H, I). Moreover, endothelial migration upon DKK3 protein stimulation was also confirmed by phospho-FAK and paxillin staining (Figure 5E, F). A murine subcutaneous Matrigel plug assay showed that DKK3 also induced endothelial cell migration in vivo (Supplement Figure 7). On the other hand, BrdU cell proliferation and Annexin V apoptosis assays on HUVECs incubated with the recombinant DKK3 protein or ADV-DKK3-SN revealed that DKK3 had no effect on cell proliferation or apoptosis, (Supplement Figure 8A, B). Taken together, the above results support the notion that exogenous DKK3 (especially glycosylated DKK3) significantly induce endothelial cell migration.

**DKK3 Induces Endothelial Cell Migration through the Wnt Pathways**

β-catenin has been implicated in the regulation of several DKK3 functions through the Wnt/β-catenin signaling pathway. However, in DKK3 treated-endothelial cells, β-catenin was not activated as indicated by Western blot analysis or immunofluorescence staining (Supplement Figure 9A-C). Distinct from a role of the canonical Wnt/β-catenin pathway in regulation of cell proliferation and development, the non-canonical Wnt pathway is involved in cell polarity and convergent extension movements. Recent studies have indicated that the tyrosine kinase receptor ROR2 plays an important role in non-canonical Wnt pathway to mediate cell migration. To investigate whether DKK3 induces cell migration through the β-catenin-independent non-canonical Wnt-PCP pathway, firstly, we performed co-immunoprecipitation analysis which revealed the binding of DKK3 to ROR2 in endothelial cells after stimulation with either recombinant DKK3 or DKK3-CHO SN (Figure 6A). This result implicates that transmembrane receptor ROR2 could be a potential binding site for DKK3. As previous studies have demonstrated, members of the Dishevelled family can mediate the Wnt-PCP signaling
pathway following activation of ROR2. Hence, we further investigated Dvl1, 2, 3 gene expression levels, and found that only Dvl1 displayed a 5-fold reduction upon stimulation with DKK3 after 6 hours (Figure 6B). Further immunofluorescence staining of DVL1 revealed its translocation from cytoplasm to the nucleus after DKK3 treatments for 6 hours (Figure 6C).

To study the downstream signaling pathways activated after the binding of DKK3 to ROR2, we measured the level of GTP-Rac1 and GTP-RhoA using pull-down assays. The level of GTP-Rac1 (Figure 6D) but not GTP-RhoA (Supplement Figure 10) was significantly up-regulated by both recombinant human DKK3 or CHO supernatant, which indicated that Rac1 but not RhoA can be activated by DKK3. A time course Western blotting analysis showed phosphorylation of JNK and c-jun occurred as early as 5 minutes after treatment with DKK3 in endothelial cells (Figure 6E). siRNA-mediated knockdown of ROR2 in DKK3 treated HUVECs (Figure 6F) not only significantly reduced downstream DVL1 protein level, JNK and c-jun phosphorylation (Figure 6G) but also inhibited cell migration, therefore placing ROR2 at the top of the signaling cascade mediating DKK3-induced migration (Figure 6J). Successful transfection of plasmids coding for eGFP-labeled constitutive Rac1 mutants were shown by quantitative FACS analysis (Supplement Figure 10). Moreover, phosphorylation of JNK was markedly upregulated in response to DKK3 treatment in cells transfected with constitutively active Rac1, but not with constitutively negative mutant (Figure 6H). Importantly, migratory abilities of endothelial cells were enhanced in Rac1 constitutively activated cells in response to DKK3 treatment (Figure 6). Finally, migration of endothelial cells towards DKK3 stimulation was markedly reduced in the presence of SP 600125 (an inhibitor of the JNK) (Figure 6K).

Therefore, these data indicate that DKK3 induces endothelial cell migration by activating Wnt-PCP signaling pathway via Rac1/JNK but not RhoA.
**DKK3 Has no Effect on Leukocyte Migration.** Leukocyte migration is critical for atherosclerosis development\(^{43, 44}\). As DKK3 is a potent attractant for endothelial cell migration, it would be crucial to know whether it can also induce leukocyte migration towards the lesion area during atherogenesis. To investigate this issue, the different cell populations contained in peritoneal cells from mice after DKK3 injection into the abdominal cavity were analyzed by FACS, and compared with the saline group (control). Unlike thioglycollate stimulation (positive control) in which cell number was markedly augmented, DKK3 treatment did not significantly increase the number of macrophages, T or B cells in total peritoneal cell population (Supplement Figure 11A, B). Furthermore, *in vitro* migration assay revealed that there was no significant difference of macrophage subpopulation migration towards various concentrations of mouse recombinant DKK3 (Supplement Figure 11C). Thus, we conclude that DKK3 does not induce leukocyte recruitment and migration *in vitro* and *in vivo*.

**Discussion**

In previous reports, the role of DKK3 as a potential tumor suppressor has been well studied in several human cancers\(^{45, 46}\). In recent years, DKK3 was found to act as a potent protector of cardiac hypertrophy via Wnt signaling pathway\(^{11, 12, 47}\), and has been associated with stem cell differentiation into vascular smooth muscle cells\(^{2, 4}\). In the present study, we found that plasma DKK3 level was inversely and independently associated with CCA-IMT and incident carotid atherosclerosis and stenosis over a five-year follow-up suggesting that DKK3 confers protection against both early and advanced stages of atherogenesis.

DKK3, as a secretory glycoprotein, can be released from a variety of tissues in mouse under physiological conditions, which explains the ubiquitous expression of DKK3 *in vivo*\(^ {48}\).
our study, we created chimeric mice models to further investigate which source of circulating DKK3 takes part in the protection from atherosclerosis and neointima formation. In the DKK3+/− chimeric mouse with wild-type bone marrow model, the DKK3 level in serum was barely increased despite transplantation with wild-type bone marrow, indicating that DKK3 is mostly released from non-hematopoietic cells. Interestingly, the wild-type chimeric mice with DKK3+/− bone marrow, exhibited even higher level of DKK3, implying that the bone marrow transplantation procedure may induce even more non-hematopoietic cell release of DKK3 into circulation. Given the evidence that endothelial cells express DKK3, the endothelium could be a source of circulating DKK3. When endothelial cells are damaged or dysfunctional, their ability to release DKK3 might also be decreased. Our findings support the notion that the lower levels of DKK3 which were found in the blood of patients with atherosclerosis, could be explained by lower DKK3 release due to endothelial dysfunction. While the specific cellular and molecular mechanisms of DKK3 release remain unknown, further studies would be needed to clarify how DKK3 is released into the blood.

After being released into the blood, DKK3 may exert its effect on endothelial cells and subsequently the development of atherosclerosis. The endothelium is an indispensable barrier inside the vessel wall and its integrity has been viewed as a determinant in atherogenesis, especially in the early stages.49, 50 As a progressive chronic disease, once initiated by risk factors, atherosclerosis provokes a cascade of pathophysiological responses, including post-angioplasty neointima formation and restenosis.52 In our study, different experimental models have confirmed that the endothelium was apparently dysfunctional or damaged in aortas of mice with DKK3 deficiency, suggesting that DKK3 plays a protective role in endothelial integrity. In DKK3+/−/ApoE−/− mice the atherosclerotic lesion area was smaller compared with DKK3+/−/ApoE−/−
mice. In the femoral artery wire injury model, DKK3 displayed not only properties of protection of endothelium integrity by accelerating the re-endothelialization at the early stage, but also of reduction of neointima formation at the late phase. The data derived from experimental models provide the direct evidence that DKK3 could act as a chemokine-like protein in endothelial migration and thus be protective of atherosclerosis.

In the early stage of atherogenesis, endothelial regeneration is a critical protective mechanism to repair damaged cells and to prevent the progression of atherosclerosis. As mentioned above, an inverse correlation between blood DKK3 level and atherosclerosis in humans and a reduction of post-injury arterial re-endothelialization in DKK3−/− mouse model have been observed. It is rational to investigate if the effect of DKK3 on endothelial cell migration could contribute to endothelial repair. In vitro migration assays showed that exogenous DKK3 significantly induced endothelial cell migration. It was reported that DKK3 is a secreted glycoprotein with four potential N-glycosylation sites, and endogenous DKK3 will be glycosylated prior to its release into the supernatant. In our study, enhanced endothelial migration was induced by secreted/released DKK3 produced by Adeno-DKK3-CHO cells. The glycosylated form of DKK3 protein could be detected in the supernatant, suggesting that glycosylated-DKK3 is the main actor on endothelial cell migration. DKK3-induced endothelial migration could play a part in atherogenesis, but other potential effects of DKK3 on the development of the disease are still unknown. Further studies would be needed to understand its roles in the pathogenesis of atherosclerosis.

Previous studies described that DKK3 was expressed in various tumor endothelial cells and that overexpression of DKK3 did not affect proliferation and migration of endothelial colony-forming cell. Similarly, our data also showed that DKK3 within the cell following
Adeno-DKK3 transduction did not induce cell migration, indicating that DKK3 may need to interact with its receptor(s) on the cell surface to exert its effects on cell movements. As Wnt signaling pathway inhibitors, DKK-family members DKK1, DKK2 and DKK4 were proved to antagonize canonical Wnt/β-catenin signaling via Frizzled family receptors and LRP5/LRP6 co-receptors. It has been demonstrated that DKK1 and DKK2 have important functions in endothelial function, including the role of DKK1 in accelerating pro-inflammatory response and atherosclerosis. In the present study, we found that DKK1 level in human blood showed a significant positive association with atherosclerosis independently of DKK3 levels. In vitro study of cultured endothelial cells pre-treated with DKK3 did induce changes in DKK1-induced IL-6 and MCP-1 expression (Supplement Figure 12). Importantly, LRP5 or LRP6 antagonist sclerostin and draxin did not inhibit DKK3-induced activation of noncanonical Wnt signaling pathway (Supplement Figure 13). Thus, it seems that DKK3 exerts its effect on endothelial functions related to atherosclerosis independently of other members of DKK family proteins.

In contrast with the other DKK-family members, the specific receptor(s) and the relevant signaling pathway(s), with which DKK3 interacts remain controversial. Several studies have demonstrated that DKK3 exerted its functions through canonical Wnt/β-catenin signaling pathway via Kremen and LRP5. On the other hand, other studies found no evidence that DKK3 binds to LRP5/6 or Krm1/2 or inhibits the canonical Wnt signaling pathway. In comparison with the canonical Wnt pathway, which is mainly involved in cell proliferation and differentiation, the non-canonical Wnt-planar cell polarity pathway interferes with cell adhesion, motility, and polarity. In the present study, we found that neither β-catenin expression nor its cellular location was changed in endothelial cells after DKK3 treatment. Instead, our data
revealed that DKK3 induced cell migration through activation of GTPase Rac1 but not RhoA and phosphorylation of JNK and c-jun. These results suggest that DKK3-induced cell migration is mediated via a ROR2/Dvl1/Rac1/JNK signaling pathway (Supplement Figure 14).

In summary, in the current study, we have provided the first evidence that DKK3 potentially confers protection against atherosclerosis in human subjects and established that DKK3 affects atherosclerosis progression and neointimal formation in mouse models by influencing re-endothelialization. Furthermore, we identified ROR2/Dvl1/Rac1/JNK as the potential signaling pathway which relays DKK3 signal in endothelial cells in vitro to induce cell migration. Taken together, the findings indicate that DKK3 could be an atheroprotective cytokine that might serve as a biomarker of endothelial integrity and repair and a potential therapeutic agent e.g. for improving both early stage reendothelialization and long-term outcome of patients post-angioplasty. While substantial knowledge on vascular risk factors has accumulated over the past years, insights into protective mechanisms are limited and require more extensive studies.

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Disclosures
None

References


Table 1. Levels of study characteristics according to DKK3 tertile groups in the Bruneck Study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>DKK3 Tertile I (&lt;65 ng/mL)</th>
<th>DKK3 Tertile II (65-74 ng/mL)</th>
<th>DKK3 Tertile III (≥75 ng/mL)</th>
<th>P value*</th>
<th>P value†</th>
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<tr>
<td>Age</td>
<td>61.8±8.6</td>
<td>66.7±10.1</td>
<td>70.0±10.0</td>
<td>&lt;0.001</td>
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<td>Male sex (%)</td>
<td>51.3</td>
<td>45.4</td>
<td>44.9</td>
<td>0.208</td>
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<td>Systolic BP (mmHg)</td>
<td>137.0±17.5</td>
<td>141.6±20.6</td>
<td>140.5±18.3</td>
<td>0.062</td>
<td>0.503</td>
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<tr>
<td>Diastolic BP (mmHg)</td>
<td>83.9±7.8</td>
<td>84.0±8.2</td>
<td>83.8±8.9</td>
<td>0.911</td>
<td>0.781</td>
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<td>Hypertension (%)</td>
<td>48.3</td>
<td>60.3</td>
<td>60.0</td>
<td>0.018</td>
<td>0.860</td>
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<tr>
<td>Urinary albumin (g/dl)</td>
<td>23.2±53.7</td>
<td>19.4±43.5</td>
<td>62.8±282.5</td>
<td>0.188</td>
<td>0.537</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.85±0.12</td>
<td>0.88±0.16</td>
<td>0.92±0.29</td>
<td>0.001</td>
<td>0.004</td>
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<td>Leukocyte count (10⁶/L)</td>
<td>6.2±1.6</td>
<td>6.1±1.6</td>
<td>6.2±1.8</td>
<td>0.788</td>
<td>0.954</td>
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<tr>
<td>hsCRP (mg/L)</td>
<td>1.8 (0.9-3.8)</td>
<td>1.7 (0.8-3.9)</td>
<td>1.8 (0.9-4.6)</td>
<td>0.974</td>
<td>0.064</td>
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<td>Fibrinogen (mg/dl)</td>
<td>288.9±56.2</td>
<td>287.9±62.3</td>
<td>294.1±62.7</td>
<td>0.392</td>
<td>0.058</td>
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<td>Chronic infections (%)</td>
<td>14.1</td>
<td>24.2</td>
<td>31.8</td>
<td>0.001</td>
<td>0.537</td>
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<td>Ferritin (μg/L)</td>
<td>124.5±155.1</td>
<td>115.3±137.0</td>
<td>113.4±110.0</td>
<td>0.415</td>
<td>0.422</td>
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<tr>
<td>LDL (mg/dl)</td>
<td>148.5±40.0</td>
<td>151.8±35.8</td>
<td>144.8±35.1</td>
<td>0.317</td>
<td>0.311</td>
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<tr>
<td>HDL (mg/dl)</td>
<td>57.3±17.0</td>
<td>56.4±14.5</td>
<td>57.6±14.4</td>
<td>0.868</td>
<td>0.759</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>130 (88-173)</td>
<td>121 (92-165)</td>
<td>117 (78-157)</td>
<td>0.023</td>
<td>0.055</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>26.3±3.7</td>
<td>25.2±4.1</td>
<td>24.6±4.1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>WHR (cm/cm)</td>
<td>0.92±0.1</td>
<td>0.92±0.1</td>
<td>0.91±0.1</td>
<td>0.043</td>
<td>0.003</td>
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<td>Physical activity (sports score)</td>
<td>2.4±0.8</td>
<td>2.3±0.7</td>
<td>2.3±0.8</td>
<td>0.059</td>
<td>0.110</td>
</tr>
<tr>
<td>Fast glucose (mg/dl)</td>
<td>103.7±27.6</td>
<td>102.0±25.8</td>
<td>99.2±19.1</td>
<td>0.063</td>
<td>0.001</td>
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<td>Hba1c (mmol/mol)</td>
<td>4.1±0.7</td>
<td>4.1±0.7</td>
<td>4.1±0.6</td>
<td>0.315</td>
<td>0.120</td>
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<td>Diabetes mellitus (%)</td>
<td>11.9</td>
<td>13.8</td>
<td>11.8</td>
<td>0.799</td>
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<td>Homocysteine (μmol/L)</td>
<td>12.5±12.1</td>
<td>13.0±5.0</td>
<td>13.7±5.2</td>
<td>0.162</td>
<td>0.846</td>
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<td>Smoking (%)</td>
<td>19.9</td>
<td>13.8</td>
<td>15.3</td>
<td>0.256</td>
<td>0.759</td>
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<td>Smoking (cigarettes/day)</td>
<td>2.6±6.3</td>
<td>1.9±5.5</td>
<td>1.2±3.7</td>
<td>0.005</td>
<td>0.243</td>
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<tr>
<td>Alcohol consumption (g/day)</td>
<td>24.9±32.2</td>
<td>20.9±31.1</td>
<td>18.4±23.7</td>
<td>0.050</td>
<td>0.179</td>
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P values were derived from linear regression analyses (continuous variables) and logistic regression analyses (categorical variables) comparing levels of study characteristics (outcome) across DKK3 tertile groups (predictor). Analyses were either unadjusted (*) or adjusted for age and sex (†).
Figure Legends

Figure 1. Plasma DKK3 level is inversely correlated with atherosclerosis in the prospective population-based Bruneck Study. A, Age- and sex-adjusted mean common carotid artery intima-media thickness (CCA IMT) was grouped according to DKK3 tertile groups. Odd ratios of incident carotid atherosclerosis (early atherogenesis) (B) and incident carotid stenosis manifesting between 2000 and 2005 (advanced atherogenesis) (C) according to baseline (2000) DKK3 tertile groups. The bottom tertile group served as the reference category. Analyses were adjusted for age and sex.

Figure 2. Atherosclerotic lesions in DKK3−/−/ApoE−/− mice. Mice receiving normal chow diet were killed at 20 weeks old, and the heart and aorta were harvested. The aortic root was sectioned, and aortas were mounted, stained with oil Red O. Lesional areas in the aortic sinus and the surface were measured and quantified as described in the Methods. The sections were immunostained with anti-α-smooth actin and CD68 antibodies, respectively. Positive cells were quantified under the microscope. A, Representative pictures of aortic sinus sections stained for oil Red O, α-smooth actin to identify smooth muscle cells and CD68 to identify macrophages; Measurement of lesions size on aortic sinus sections (B), on en face stained-lesion areas (C; % of total surface areas) and quantification of αSMA and CD68 positive cells (D; % of total cells). *Significant difference between DKK3−/−/ApoE−/− and DKK3+/+/ApoE−/− groups, p<0.05 (n=11).

Figure 3. Increased endothelial damage in DKK3−/−/ApoE−/− mice. A, Evans blue dye leaking study. Ten-week-old DKK3−/−/ApoE−/− and DKK3+/+/ApoE−/− mice received an injection of 1%
Evans blue dye via the tail vein. Mice were sacrificed, and aortas harvested and washed. Blue areas, representing the damaged area on the surface of aortas were observed. B, Scanning electron microscopy analysis of aortic samples from DKK3−/−/ApoE−/− and DKK3+/+/ApoE−/− mice. Arrows indicate the damaged cell. C, En face preparation of the vessel was stained for endothelial marker eNOS and visualized following incubation with secondary Cy3 conjugated antibody. Quantitative data analysis of the number of endothelial eNOS positive cells around the bifurcation (bi) areas are presented in the graph (n=6), *p<0.01, indicating significant difference between the two groups.

Figure 4. Increased neointimal lesions in DKK3−/−/ApoE−/− mice after vessel injury. Femoral arteries were wire-injured, harvested at different times after surgery, and cross-sectioned for morphological analysis. A, Representative Hematoxylin and Eosin (HE) stained sections of femoral arteries from DKK3−/−/ApoE−/− and DKK3+/+/ApoE−/− mice (upper panel). Means ±SEM from 6 mice for each group of the neointimal area, neontima/media ratio and luminal area are presented (lower panel). *p<0.01, indicating a significant difference between the two groups. B, Representative HE stained sections of femoral arteries from a chimeric mouse, which is DKK3+/+/ApoE−/− mouse receiving DKK3−/−/ApoE−/− or DKK3+/+/ApoE−/− bone marrow after irradiation 3 weeks after vessel injury. The graph shows means±SEM of lesion areas (n=6). No significant difference was noted between the two groups.

Figure 5. DKK3 can induce endothelial cell migration. Chemotaxis of human umbilical vein endothelial cells (HUVECs; 1×10^5 cells/well) across 8.0 μm transwells toward either human recombinant DKK3 (A) or Adeno-DKK3-overexpressed CHO supernatant (C) was counted 6
hours after crystal violet staining. 0.1% FBS EBM-2 medium or Adeno-CMV null-overexpressed CHO supernatant was used as controls respectively, n=5. Scale bars, 100μm.

**B.** Scratch-wound assay was performed on HUVECs. The chemotaxis index of each quantification was defined by the average of 9 fields of view from each well and was presented as fold increase compared to the corresponding controls, n=5. All graphs are shown as mean±SEM, **p<0.01, ***p<0.001. Null-CHO SN, Adeno-null overexpressed CHO supernatant; DKK3-CHO SN, Adeno-DKK3 overexpressed CHO supernatant; Scale bars, 100μm.

**E, F.** HUVECs were treated with human recombinant DKK3 for indicated time points before immunofluorescence staining of phosphorylated FAK and paxillin. ctrl, control; p-FAK, phosphorylated FAK. Scale bars, 50μm.

**Figure 6. DKK3 induces endothelial cell migration via ROR2/Dvl1/Rac1/JNK signaling pathway. A.** Western blot analysis showed the binding of ROR2 and DKK3 after immunoprecipitation of either DKK3 or HA-binding protein in HUVECs stimulated with DKK3 or DKK3-CHO SN. **B.** The real-time quantitative PCR showed the fold changes in mRNA levels of DVL1, 2, 3 after DKK3 treatment. **C.** Immunofluorescence staining showed the translocation of ROR2 and DVL1 in the nucleus after DKK3 treatments. **D.** Pull-down assays were performed on HUVECs treated with DKK3 from 1 to 30 minutes to analyze GTP-Rac1 activation. **E.** Western blot analysis was performed on DKK3-treated HUVECs for the detection of JNK and c-jun phosphorylation and their total expression. **F.** The gene and protein levels of ROR2 were analyzed by qPCR and FACS after knockdown by ROR2 siRNA. Western blotting showed the levels of ROR2, DVL1 proteins and JNK, c-jun phosphorylation in HUVECs after either control siRNA or ROR2 siRNA transfection (**G**), or phosphorylated JNK and c-jun levels in HUVECs.
after transfection of constitutive mutants of Rac1 (H). Transwell assay was performed on HUVECs that were either transfected with constitutive mutants of Rac1 (I) or control, ROR2 siRNA (J) before migration toward DKK3, 6 hours with either crystal violet staining or direct fluorescence observation of plasmid-transfected cells. K. Transwell assay was performed on HUVECs which migrated towards DKK3 in the presence of JNK inhibitor (SP600125) for 6 hours. All the blots shown are representative of 3 separate experiments. All graphs are shown as mean±SEM, n=3, *p<0.05, **p<0.01, ***p<0.001. DMSO, dimethyl sulfoxide; p-JNK, phosphorylated JNK; t-JNK, total JNK; p-c-jun, phosphorylated c-jun; t-JNK, total c-jun; act, constitutively active mutant; wt, constitutively wt mutant; neg, constitutively negative mutant.

Scale bars, 100μm.
Tertile group of DKK3 Level

Incidnt stenosis: $0.4$ $0.6$ $0.8$ $1.0$

Incidnt atherosclerosis: $0.4$ $0.6$ $0.8$ $1.0$

CCA IMT: $0.31$ (0.14-0.68)

OR: $0.79$ (0.39-1.41) $0.40$ (0.17-0.90)

P-values:
- P = 0.008 for CCA IMT
- P = 0.027 for incident atherosclerosis
- P = 0.003 for incident stenosis
A  

IB  

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<th>Ab</th>
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B  

mRNA expression (Fold of Control)

ctrl  

DVL1  

DVL2  

DVL3  

C  

ctrl  

DKK3  

D  

GTP-Rac1

Total-Rac1  

E  

DKK3  

F  

Ror2 gene expression level

Normalized to mode

G  

si-ctrl  

si-Ror2

H  

Act  

WT  

Neg

I  

Act  

WT  

Neg

J  

si-ctrl  

si-Ror2

K  

DMSO  

JNK inhibitor
A Cytokine-like Protein DKK3 Is Atheroprotective
Baoqi Yu, Stefan Kiechl, Dan Qi, Xiaochong Wang, Yanting Song, Siegfried Weger, Agnes Mayr, Alexandra Le Bras, Eirini Karamariti, Zhongyi Zhang, Ivan del Barco Barrantes, Christof Niehrs, Georg Schett, Yanhua Hu, Wen Wang, Johann Willeit, Aijuan Qu and Qingbo Xu

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Expanded Methods

Study Population. Population recruitment was performed as part of the prospective community-based Bruneck Study\textsuperscript{1,2}. The survey area was located in the north of Italy (Bolzano Province). Special features of the study design and protocol have been described previously in detail\textsuperscript{1-3}. At the study baseline in 1990, we recruited an age- and sex-stratified random sample from all inhabitants of Bruneck aged 40-79 years (125 women and 125 men each from the 5th to 8th decades of age, overall n=1000). A total of 919 subjects participated and had complete data assessment. Follow-up examinations were performed every five years with participation rates among survivors exceeding 90%: n=826 in 1995, n=684 in 2000, and n=574 in 2005. The current study utilized data and blood samples collected in the 2000 and 2005 examinations. The appropriate ethics committees approved the study protocol and all study subjects gave their written informed consent before entering the study.

Clinical Examination and Laboratory Methods. Body mass index was calculated as weight divided by height squared (kg/m\textsuperscript{2}). Diabetes was diagnosed according to American Diabetes Association (ADA) criteria. Hypertension was defined as blood pressure $\geq$140/90 mm Hg (mean of three independent measurements obtained with a standard mercury sphygmomanometer after at least ten minutes of rest) or the use of anti-hypertensive drugs\textsuperscript{1-5}. All other parameters were assessed by standard procedures as detailed previously\textsuperscript{1-5}. Blood samples were collected from antecubital vein after subjects had fasted and abstained from smoking for $\geq$12 hours. Blood was collected in EDTA tubes and processed within 3 hours of collection. The APC-CFU assay and the APC culture assay were then performed promptly. Plasma was aliquoted and frozen for ELISA and other future use.

Enzyme-Linked Immunosorbent Assay (ELISA) for Plasma Soluble Molecules Measurements. The levels of DKK3 in human plasma were detected using R&D DKK3 ELISA kit (R&D, DY1118). The capture antibody was diluted to a working concentration without carrier protein. A 96-well ELISA microplate (R&D, DY990) was immediately coated with 100µl of capture antibody, sealed (sealer, R&D, DY992), and incubated overnight at 4 oC. The next day the solution was removed and the wells were washed 3 times with wash buffer (provided in the kit). Each well was then blocked with 300µl of blocking reagent (provided in the kit) and maintained at room temperature for at least 1 hour. The wells were washed with wash buffer 3 times. 100µl of samples (diluted 1:10-1:100) or standards were added. The plate was covered with an adhesive strip and incubated at room temperature for 2 hours. Each
well was washed 3 times with wash buffer. 100µl of detection antibody was added and the plate was covered again with an adhesive sealer and incubated at room temperature for 1 hour and then removed. 100µl of streptavidin-HRP (provided in the kit) was added per well followed by 3 washes with wash buffer and incubation at room temperature for 20 minutes (avoiding direct exposure to light). 100µl of substrate solution (provided in the kit) consisting of equal volumes of color reagent A (H2O2) and color reagent B (Tetramethylbenzidine) was added per well and incubated at room temperature for another 15-25 minutes. The reaction was stopped by adding in 50µl stop solution (2N sulphuric acid). The optical density of each well was determined immediately using a Tecan microplate reader set to 450 nm. The standard curve was created by plotting the concentration for each standard on the y-axis against the mean absorbance for each standard on the x-axis and drawing a best-fit curve through the points on the graph. The DKK3 concentration was calculated according to the standard curve. DKK1 levels were measured in serum with a commercial ELISA (Biomedica, Vienna, Austria): Intra- and inter-assay CVs were low at 3% each and the lower detection limit was 1.6 pmol/L.

The levels of plasma G-CSF, SDF1-α, VEGF and MMP-9 were also determined by using corresponding ELISA kits (Quantikine, R&D Systems, UK). All ELISA tests were carried out at room temperature on freshly thawed plasma samples. The concentrations of all cytokines were determined by comparison with a standard curve, following manufacturer's instruction. Other laboratory parameters were all examined by standard methods.

Angiogenic Promoting Cell (APC) Colony Forming Unit Assay (APC-CFU) and APC Culture Assay (APC number). Angiogenic promoting cell (APC) number and APC-CFU were assessed in 571 and 542 participants. Subjects with and without APCs number and APC-CFU did not differ in age, sex and expression of cardiovascular risk factors. Venous blood samples were collected as described above and the APC-CFU assay performed as described previously6-8. Briefly, peripheral blood mononuclear cells were isolated by density gradient centrifugation over lymphoprep (Axis-Shield, Oslo, Norway) at 800 G for 20 minutes. Peripheral blood mononuclear cells were resuspended in APC culture medium (M199 with 20%FCS and antibiotics) and then plated on fibronectin-coated 6-well plates at a concentration of 5 million cells per well. This step was designed to remove mature circulating endothelial cells which are rapidly adherent to fibronectin. Following culture for 48 hours, non-adherent cells were then aspirated, counted and, plated on fibronectin-coated 24-well plates at a concentration of 1 million cells per well. On day 5 of the assay, the medium was changed and on day 7 the endothelial colonies were counted manually. Strict guidelines were followed to ensure consistent counting of APC colonies. Two senior investigators who were blinded to the subjects’ clinical status counted colonies. Reproducibility was
assessed over 50 samples comparing colony counts by the two individuals. The coefficient of variance was <10% in each case. The APCs in a minimum of two wells were counted and the average was then recorded. Reproducibility was assessed over 50 samples by comparing the APC numbers from the two individuals. The coefficient of variance was <10% in each case.

**Scanning Protocol and Definition of Ultrasound End Points.** The ultrasound protocol involves the scanning of the internal (bulbous and distal segments) and common carotid arteries (proximal and distal segments) of either side with a 10-MHz imaging probe\(^3, 9\). Atherosclerotic lesions were defined according to two ultrasound criteria: (1) wall surface (protrusion or roughness of the arterial boundary) and (2) wall texture (echogenicity). The maximum axial diameter of plaques (in millimeters) was assessed on the near and far walls at each of the eight vessel segments. For the follow-up between 2000 and 2005 two stages of atherosclerosis development/progression were differentiated: (1) early atherogenesis was defined as the occurrence of new plaques in previously normal vessel segments, and (2) advanced atherogenesis (incident stenosis) was assumed whenever the relative increase in the maximum plaque diameter exceeded twice the measurement error for the method and a lumen narrowing > 40% (diameter stenosis) was achieved. The two progression categories were highly reproducible [kappa coefficients > 0.8 (n=100)]. Further details on imaging procedures and measurement errors have been published elsewhere\(^3, 9, 10\). The intima-media thickness (IMT) was quantified at the far wall of plaque-free sections of the common carotid arteries as the distance between the lumen-intima and media-adventitia interface (intra-observer coefficient of variation, 7.9 percent (n=100))\(^3, 9\).

**Animals.** All animal experiments were performed according to the protocols approved by the Institutional Committee for the Use and Care of Laboratory Animals. ApoE\(^{-}/-\) mice were purchased from the Jackson Laboratory. For ApoE\(^{-}/-\) mice genotyping, the following primers were employed: oIMR180 (5\'-GCC TAG CCG AGG GAG AGC CG-3\'), oIMR181 (5\'-TGT GAC TTG GGA GCT CTG CAG C-3\'), and oIMR182 (5\'-GCC GCC CCG ACT GCA TCT-3\'). DKK3\(^{-}/-\) mice were generated as described previously\(^11\). Three genotypes of DKK3\(^{-}/-\), DKK3\(^{-/+}\) and DKK3\(^{++}\) mice were identified using PCR (primers: 5\'-GATAGCTTTCCGGACACAC-3\'; 5\'-TCCATCAAGCTCTCCA CCTCT-3\'; 5\'-TAAGTTGGTTACGCCCAGGT-3\'). ApoE\(^{-}/-\) mice were crossed with DKK3\(^{-}/-\) mice in our laboratory, and heterozygous offspring were mated to produce ApoE\(^{-}/-\) mice lacking DKK3 (DKK3\(^{-/-}\)ApoE\(^{-/-}\)). The mice were maintained on a light/dark (12/12 h) cycle at 22°C and received food and water ad libitum. The genetic background of all mice used in the present study was C57BL/6.
Creation of Chimeric Mice. The procedure used for creating chimeric mice was similar to previously described. In brief, bone marrow transplantation was carried out on the DKK3++/+ mice and DKK3--/-- mice separately. Bone marrow cells were obtained from the femurs and tibias of either DKK3++/+ or DKK3--/-- mice (donors) and injected (1x10⁷ cells in 0.2ml) into the tail veins of the 6-8 week old DKK3--/-- mice or DKK3++/+ mice (recipients), which received lethal irradiation (950 Rads) before. The measurement of DKK3 level in peripheral blood was performed 3 weeks after bone marrow transplantation.

Tissue Harvesting and Lesion Analysis. Mice were anesthetized by intraperitoneal injection of pentobarbital atrium (50mg/kg b.w.). Blood was obtained from inferior vena cava for lipid analysis. The heart was harvested intact and stored immediately in liquid nitrogen and the whole length of the aorta was stored in formalin at 4°C. 8-µm thick frozen sections were obtained from the heart and were stained with Oil Red O as described elsewhere. Aortas were opened longitudinally and fixed on a silicon bed with stainless steel pins (Fine Science Tool, USA) with the intima exposed. Oil Red O staining was performed. Lesion areas were measured and quantified using a computer software AxioVision™ as described previously.

Lipid Measurement. Blood from the mice was collected, centrifuged and the serum was kept for analysis. Serum cholesterol and triglyceride were measured with the Infinity™ Kit (Sigma) for cholesterol and triglyceride respectively.

Endothelial Damage Assays in vivo. Evans blue dye stains blue in the areas where endothelium is damaged or dysfunctional. Mice received intravenous Evans blue dye injection, and their aortas harvested, opened and blue areas were measured using a computer software AxioVision™. For scanning electron microscope analysis, mice were killed, and the aortas were fixed in situ with 2% formaldehyde plus 2% glutaraldehyde and harvested. Aortic samples were fixed and processed as described previously. The samples were observed using a scanning electron microscopy in our central facility laboratory.

Mouse Femoral Artery Denudation Injury. The mice were anesthetized and the surgical procedure was similar to that described previously. Removal of the endothelium of the common femoral arteries was achieved by 3 passages of a 0.25 mm angioplasty guidewire (Advanced Cardiovascular Systems). After removal of the wire, the branch of the femoral artery was ligated, and the wound was closed.

Morphometric Analysis and Quantification of Lesion Formation. The femoral arteries were harvested at different times after the operation. The specimens were fixed in 4% formaldehyde for H&E staining. Sections (5µm)
were collected at 100µm intervals (10 sections per fragment), mounted on slides, and stained with H&E for morphometric analysis. The procedure used for lesion quantification was similar to that described previously. Briefly, using Axiosvision software, the area of neointima in a given image was highlighted and quantified (mm²) automatically. 10 sections were analyzed per vessel sample. Meanwhile, the thickness of the neointima and media of the vessel was examined. Because native media thickness is variable (reflecting the diameter of the artery), neointima thickness was assessed in the ratio of intima to intima plus media thickness. Re-endothelialization was defined by endothelial coverage of the luminal surface and assessed by x400 microscopic examination.

**En Face Staining.** The mice were sacrificed after 48 hours, their aortas were harvested, processed and fixed as described previously and probed for 1 hour at room temperature with rat anti-mouse antibodies against eNOS (BD Biosciences; 1:20 dilution). The secondary antibody used was rabbit anti-rat conjugated with Cy3 (DAKO Corp; 1:50 dilution).

**In vivo Matrigel Plug Assay.** DKK3 or BSA (50 µl, 100µg/ml) were mixed well with and 250 µl Matrigel on ice, and injected subcutaneously into the mice. 6 injections were conducted for each group. The plugs were harvested 14 days later. Samples were fixed in liquid nitrogen, cryosections were prepared and immunofluorescence staining was then performed.

**Immunofluorescence Staining.** Frozen or paraffin sections on HUVECs were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma) and blocked with 5% normal donkey serum (Dako) for 1 hour at room temperature. Incubation of tissue sections or cells with primary antibodies was performed at 4°C overnight, followed by incubation with secondary antibodies (Corresponding fluorescent-conjugated IgG antibodies were used as secondary antibodies (Invitrogen)) for 45 mins at 37°C. Then tissue sections or cells were counterstained with DAPI (Sigma) for 5 mins at room temperature and mounted in fluorescent mounting media (Dako). Images were acquired using an Olympus IX81 microscope and assessed using Volocity software (PerkinElmer) or a Leica SP5 confocal microscope and LAS AF lite software. Total positive cells were counted using images obtained from the microscope, and these images were also used for calculating the percentage of positive cells.

**Isolation and Culture of Cells.** HUVEC. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (C2519A pooled donor) and cultured in complete Endothelial Growth Medium, EGM-2 Bullet kit (Lonza, CC3162). The cells were placed in a gelatin-coated plastic flask, incubated at
37°C, 5% CO2 and were passaged every other day at a 1 to 3 ratio. The experiments were carried on HUVECs of passage 2-5.

**MLEC.** Mouse lung endothelial cells (MLEC) were isolated using collagenase/dispase digestion method as previously described. Briefly, 8-10 days old pups of C57BL/6J mice were euthanized before excision of three lung lobes of each mouse aseptically. The tissue was minced finely followed by enzymatic digestion with collagenase/dispase for 45 minutes at 37°C on a rotator. A single cell suspension was obtained by triturating tissue clumps and passing through cell strain before collecting cell pellets by centrifugation at 400g for 5 minutes. Then the cells were resuspended and sorted with anti-PECAM-1 immunomagnetic microbeads to obtain MLEC. According to the instruction of magnetic cell sorting kits (MACS) (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany), briefly, the cells were incubated with the PECAM-1-conjugated/coated microbeads at 4°C for 15 minutes and then sorted using MACS columns (Miltenyi Biotec). The PECAM-1 positive cells were plated on a gelatin-coated T75 flask and changed EGM-2 medium 4 hours later to remove non-adherent cells and platelets.

**Mouse Peritoneal Macrophages.** The isolation of mouse peritoneal macrophages was described as in previous studies14. Briefly, C57BL/6J mice were received an intraperitoneal (IP) injection of either 0.9% saline, 3% thioglycollate medium (Sigma-Aldrich) or recombinant mouse DKK3 protein (Thermo Fisher Scientific) in each mouse (1µg/20g). After 3 days, all the mice in different groups were euthanized by cervical dislocation, and 10 ml of cold sterile 3% Fetal Bovine Serum (FBS) PBS was injected through the peritoneal wall into each mouse. After injection, the peritoneal fluid was then withdrawn and centrifuged at 200g for 10 minutes at 4°C. The supernatant was discarded and cell components were analyzed by FACs analysis.

**CHO-K1 Cells.** Chinese hamster ovary, CHO-K1 cell line was purchased from ATCC (CCL-61), and cultured in Nutrient Mixture F-12 Ham medium (Sigma N4888) supplemented with 2mM Glutamine and 10% Fetal Bovine Serum (FBS). The cells were passaged every other day at a 1 to 6 ratio.

**FACS Analysis.** Mouse peritoneal macrophages were blocked with CD16/CD32 (Mouse BD Fc Block™) (BD Biosciences) for 5 minutes on ice before addition of antibodies CD11b, CD3, B220 or their corresponding IgG control for 30 minutes on ice before analysis by FACS in order to test the populations of macrophage, T cell and B cell in isolated peritoneal macrophages after stimulation with saline, thioglycollate or DKK3 recombinant protein in different mice. Data analysis was carried out using FlowJo software.
**Antibodies and Inhibitors.** Primary antibodies were goat anti-DKK3 (R&D Systems, AF1118), rabbit anti-HA tag antibody (ab9110), rabbit anti-Phospho-SAPK/JNK (Thr183/Tyr185) (cell signaling 4668), rabbit anti-Phospho-JNK (Ser63/73) (SC16312-R), rabbit anti-c-jun (sc-1694), rabbit anti-FAK (phosphor Y397) antibody (ab4803), rabbit anti-paxillin (ab32084) and rabbit anti-ROR2 (ab92379), rabbit Anti-Dishevelled/Dvl1 antibody (ab106844), mouse anti-Rac1 (Millipore 05-389), mouse anti-RhoA (Cytoskeleton ARH04), mouse anti-active-β-catenin (Millipore 05-665), rabbit anti-β-catenin (ab32572), mouse anti-α-tubulin (Sigma T9026). Conjugated flow cytometry antibodies FITC anti-CD11b antibody (101205), FITC rat IgG2b, κ Isotype ctrl antibody (400605), PE anti-CD3 antibody (100205), PE rat IgG2b, κ Isotype ctrl antibody (400607) and APC anti-CD45R/B220 antibody (103211), APC rat IgG2a, κ Isotype ctrl antibody (400511) were purchased from Biolegend. Secondary antibodies for immunostaining were anti-mouse Alexa Fluor 546, anti-rabbit Alexa Fluor 546 and anti-rat Alexa546 and were purchased from Invitrogen. Human DKK1 (5439-DR-010), Draxin (6148-DR-025) and SOST/Sclerostin (1406-ST-025) were purchased from R&D System. Cells were also counterstained with Alexa Fluor® 488 Phalloidin (Life technologies A12379) and 10µg/ml 4’, 6-diamidino-2-phenylindole (DAPI). Secondary antibodies for Western Blotting were purchased from Dako. JNK inhibitor SP600125 (C14H8N2O) was purchased from Calbiochem.

**Plasmids and Cell Transfection.** pcDNA3-EGFP-Rac1-Q61L (plasmid 12981), pcDNA3-EGFP-Rac1-T17N (plasmid 12982), pcDNA3-EGFP-RhoA-Q63L (plasmid 12968), pcDNA3-EGFP-RhoA-T19N (plasmid 12967) were purchased from Addgene. HUVECs were transfected with above plasmids using Lipofectamine 3000 (Thermo Fisher) in Opti-MEM™ (Thermo Fisher) medium according to the manufacturer’s instructions. After incubation at 37°C for 6 hours, the medium was changed to complete EGM-2 medium and migration assays were performed on transfected HUVECs 24 hours post transfection.

**Adenovirus and Cell Transfection.** Human DKK3-HA adenovirus (Abm 085672A) and Adeno CMV-null (Abm 000047A) were purchased from Abm and amplified in HEK293 cells as described previously1. The empty vector virus (Adeno CMV-null) was applied as a negative control in experiments. For overexpression of DKK3 in either CHO cells supernatant or HUVECs, 70% confluent CHO cells or HUVECs were infected with Adeno-DKK3-HA or Adeno-CMV null at a multiplicity of infection (MOI) of 10 for 6 hours before replacement of fresh serum free F-12 basal medium. The supernatant from CHO cells was harvested 48 hours after transduction and in which DKK3 concentration was determined by human DKK3 DuoSet ELISA kit (R&D
DY1118). The evaluations of DKK3 overexpression in HUVECs were performed 48-72 hours after transfection.

**BrdU Proliferation Assay.** HUVECs were treated with/without human DKK3 recombinant protein in 0.1% FBS EBM-2 medium or Adeno-DKK3-HA/Adeno-CMV null transfected CHO cells supernatant at indicated time points on a gelatine coated 96-well culture plate. The proliferation assay was performed using a Cell Proliferation ELISA, BrdU (colorimetric) (Roche). According to the manufacturer’s instructions, the BrdU labeling solution was incubated with cells for 2 hours at 37°C prior to fixation of cells. Then the fixed cells were incubated with BrdU peroxidase-conjugated antibody for 90 minutes at room temperature followed by incubation of substrate solution and stop solution until the color development was sufficient to be detected. The absorbance was measured at 450nm with correction at 690nm. The proliferation of HUVECs was expressed as the fold of mean values of absorbance compared to their corresponding controls.

**Annexin V Apoptosis Assay.** Apoptosis assay was performed on HUVECs using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen™) after treatment with/without human DKK3 recombinant protein in 0.1% FBS EBM-2 medium or Adeno-DKK3-HA/Adeno-CMV null transfected CHO cells supernatant at indicated time points. As directed by manufacture’s instruction, 1x10^5 cells of each sample were incubated with FITC Annexin V and Propidium Iodide (PI) together for 15 minutes at room temperature protected from light. Unstained cells, cells stained with FITC Annexin V or PI only severed as controls. The apoptotic cells were identified by FACS immediately after staining.

**Transwell Chemotaxis Assay.** Migration chemotaxis assay was performed by applying 24-well Boyden chambers with 8 µm pore size polycarbonate membranes (Corning) as described previously19. HUVECs were seeded onto the upper chamber at 1x10^5 cells in 0.1% FBS EBM-2 basal medium, while the bottom chamber contained either 0.1% FBS EBM-2 basal medium with indicated concentrations of recombinant human DKK3 or Adeno-DKK3-HA/Adeno-CMV null overexpressed CHO cells supernatant. 0.1% FBS EBM-2 basal medium served as negative control for the comparison with recombinant human DKK3. After incubation for 6 hours at 37°C, the cells remained on the upper side of the filters were removed by a cotton swab. The migrated cells on the underside of the membrane were fixed with 4% paraformaldehyde prior to staining with 0.1% crystal violet solution for 15 minutes. Data was expressed as the fold of migrated HUVECs compared to their corresponding controls.
**Scratch-Wound Assay.** In each well of a 24-well plate, a straight scratch was made by a pipette tip to stimulate a 'wound' through the middle of 100% confluent HUVECs monolayer. The cells were treated with 0.1% FBS EBM-2 basal medium with/without indicated concentrations of recombinant human DKK3 or Adeno-DKK3-HA/Adeno-CMV null overexpressed CHO cells supernatant. After 6 hours incubation, the migration of HUVECs into the “wound” area was quantified as the mean number of the migrated cells.

**RT-PCR and Quantitative Real Time Polymerase Chain Reaction (qPCR).** Total RNA was isolated from HUVECs using a QIAGEN RNeasy Mini kit according to the manufacturer’s instructions. 1 µg RNA was reverse transcribed into cDNA using QuantiTect® Reverse Transcription Kit (Qiagen) in a 20 µl reaction. Then the qPCR was performed using 20ng of cDNA per sample with a SYBR Green Master Mix in a 20 µl reaction. Ct values were measured using the Eppendorf Mastercycler ep Realplex and GAPDH was used as an endogenous control to normalize the amounts of RNA in each sample. The primer sets are:

- ROR2, Forward Primer 5’-GTGCCGTTGCTAAAGAATGAT-3’
  Reverse Primer 5’-ATTCGCAGTCGTGAACCATATT-3’
- DVL1, Forward Primer 5’-GAGGGTGCTCACTCGGATG-3’
  Reverse Primer 5’-GTCCTGTCTCGTTGTCCA-3’
- DKK3, Forward 5’-AGGACACGCACAAATG-3’
  Reverse 5’-CCAGTCTGGTGGGTATCTT-3’
- IL-6, Forward 5’-TTG CCT TCT TGG GAC TGA TGC T-3’
  Reverse 5’-GTA TCT CTC TGA AGG ACT CTG G-3’
- MCP-1, Forward 5’-AGTAGGCTGGAGAGCTACAA-3’
  Reverse 5’-GTATGTCTGGACCCATTCTTC-3’

**Western Blot Analysis.** Harvested cells were lysed with RIPA lysis buffer (Thermo Fisher) plus cOmplete Protease Inhibitor Cocktail Tablets (Roche), PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche) and proteins were sequentially measured using DC™ Protein Assay (Bio-Rad). 40 µg of total protein lysate for each sample was loaded into each well of 4-12% Bis-Tris Protein gels (NuPAPE, Novex) before being transferred to an Immobilon®-FL transfer membrane (Millipore), followed by a standard western blotting procedure.

**Rac1, RhoA GTPase Activation Assay.** The activation assay of GTP-bound Rac1 or GTP-bound RhoA were carried out according to the manufacturer’s instructions of Rac1 Pull-down Activation Assay Kit (Cytoskeleton) and RhoA Pull-down Activation Assay Kit (Cytoskeleton) respectively. Briefly, cells were stimulated with the indicated treatments then harvested within cell lysis buffer
supplemented with protease inhibitor cocktail (reagents all supplied by kits). Then Rac1 and RhoA activity were assessed using GST-tagged p21 binding domain (PBD) of PAK agarose beads or glutathione S transferase (GST)-tagged Rho-binding domain (RBD) of Rhotekin beads to pull-down GTP-Rac1 or GTP-RhoA from lysates separately. Aliquots were collected for total Rac1 or Cdc42 (input) and GTPγS (positive) and GDP (negative) control analysis. Samples were separated by 4–12% Bis-Tris gels, transferred to PVDF membranes, and blotted with Rac1 (Millipore, 05-389) or RhoA antibody (Cytoskeleton, ARH03).

**Statistical Analysis**

**Population study.** The data were analyzed using the SPSS 24 software package. Levels of variables according to DKK3 tertile groups were presented as mean values ± SD or as medians with corresponding 25th and 75th percentiles (continuous variables), and percentages (dichotomous variables). Associations between DKK3 level (predictor variable) and vascular risk factors, life style and demographic variables, IMT and atherosclerosis progression were assessed using linear and logistic regression analysis. Levels of variables with a markedly skewed distribution were loge-transformed to satisfy the assumption of normality and constant variance of the residuals. The multivariate models focusing on IMT or atherosclerosis progression included the following covariates: age (years), sex (female, male), smoking (cigarettes/day), hypertension, HDL and LDL cholesterol, triglycerides, hsCRP, creatinine, body mass index, wait-to-hip ratio, chronic infections, fasting glucose, and physical activity (sports score). A two-sided p value < 0.05 was considered significant.

**In vivo and in vitro studies.** Data for in vivo and in vitro studies are presented as the mean ± standard error of the mean (S.E.M.) of at least three separate experiments. The analysis was performed using Graphpad Prism V.6 (GraphPad Software, San Diego CA) using t-test between two groups and analysis of variance (one-way ANOVA) followed by Dunnett’s multiple comparison test for more than two groups. A p value<0.05 was considered significant.

**References**


17. Torsney E, Mayr U, Zou Y, Thompson WD, Hu Y, Xu Q. Thrombosis and
neointima formation in vein grafts are inhibited by locally applied aspirin through

splicing in endothelial cells is crucial for angiogenesis. *Circulation.*
2013;127:1712-1722.

Macrophages Control Vascular Stem/Progenitor Cell Plasticity Through Tumor
Necrosis Factor-alpha-Mediated Nuclear Factor-kappaB Activation. *Arterioscler
Thromb Vasc Biol.* 2014;34:635-643.
Table S1. Association of DKK3 level with common carotid artery IMT and progression of carotid atherosclerosis.

<table>
<thead>
<tr>
<th>Incident atherosclerosis (2000 to 2005)</th>
<th>OR (95% CI) for a 1-SD higher DKK3 level</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>0.64 (0.42-0.97)</td>
<td>0.035</td>
</tr>
<tr>
<td>Model 2</td>
<td>0.61 (0.39-0.95)</td>
<td>0.030</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Incident stenosis (2000 to 2005)</th>
<th>OR (95% CI) for a 1-SD higher DKK3 level</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>0.56 (0.38 to 0.83)</td>
<td>0.004</td>
</tr>
<tr>
<td>Model 2</td>
<td>0.53 (0.35 to 0.82)</td>
<td>0.004</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Intima-media thickness IMT (mm)</th>
<th>Regression coefficient (95% CI) for a 1-SD unit higher DKK3 level</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>-0.014 (-0.027 to -0.002)</td>
<td>0.020</td>
</tr>
<tr>
<td>Model 2</td>
<td>-0.012 (-0.024 to 0.001)</td>
<td>0.065</td>
</tr>
</tbody>
</table>

ORs (odds ratios) were derived from logistic regression analysis of incident carotid atherosclerosis/stenosis on baseline (2000) DKK3 levels, age and sex (Model 1) plus smoking (cigarettes/day), hypertension, HDL and LDL cholesterol, log-transformed triglycerides, log-transformed hsCRP, creatinine, body mass index, weight-to-hip ratio, chronic infections, fasting glucose and physical activity (sports score) (Model 2). The analysis on incident plaques focused on subjects free of carotid atherosclerosis in 2000 (n=259, incident plaques were observed in 59 individuals) and that on incident stenosis considered subjects with pre-existent carotid atherosclerosis only (n=332, incident stenosis was observed in 63 individuals). Regression coefficients were derived from linear regression models focusing on the common carotid artery intima-media thickness (IMT) measured in 2000 (same adjustment). The SD of DKK3 level is 19.11.
Table S2. Levels of cytokines and APCs according to DKK3 tertile groups in the Bruneck Study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>DKK3 Tertile I (&lt;65 ng/mL)</th>
<th>DKK3 Tertile II (65-74 ng/ml)</th>
<th>DKK3 Tertile III (≥75 ng/ml)</th>
<th>P value*</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC-CFUs (per 10⁶ PBMNC)</td>
<td>104 (0-222)</td>
<td>83 (1-294)</td>
<td>79 (0-314)</td>
<td>0.900</td>
<td>0.633</td>
</tr>
<tr>
<td>APC number (per 10⁶ PBMNC)</td>
<td>611 (285-998)</td>
<td>524 (192-1104)</td>
<td>489 (162-1086)</td>
<td>0.148</td>
<td>0.485</td>
</tr>
<tr>
<td>G-CSF (pg/ml)</td>
<td>8.5 (4.1-15.7)</td>
<td>10.1 (4.3-16.6)</td>
<td>10.0 (4.3-18.3)</td>
<td>0.120</td>
<td>0.511</td>
</tr>
<tr>
<td>MMP9 (ng/ml)</td>
<td>59.0 (34.8-110.0)</td>
<td>67.9 (38.9-102.0)</td>
<td>67.0 (31.7-121.7)</td>
<td>0.530</td>
<td>0.411</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>62.8 (27.3-149.2)</td>
<td>91.9 (32.0-186.5)</td>
<td>66.3 (28.0-147.5)</td>
<td>0.731</td>
<td>0.839</td>
</tr>
<tr>
<td>SDF-1 (pg/ml)</td>
<td>2465 (2247-2703)</td>
<td>2467 (2220-2804)</td>
<td>2693 (2362-3010)</td>
<td>&lt;0.001</td>
<td>0.021</td>
</tr>
<tr>
<td>sRANKL (pmol/l)</td>
<td>1.2±0.9</td>
<td>1.3±1.2</td>
<td>1.2±1.1</td>
<td>0.701</td>
<td>0.356</td>
</tr>
<tr>
<td>Osteoprotegerin (pmol/L)</td>
<td>3.7±1.0</td>
<td>4.1±1.8</td>
<td>4.4±1.5</td>
<td>&lt;0.001</td>
<td>0.130</td>
</tr>
</tbody>
</table>

P values were derived from linear regression analyses (continuous variables) and logistic regression analyses (categorical variables) comparing levels of study characteristics (outcome) across DKK3 tertile groups (predictor). Analyses were either unadjusted (*) or adjusted for age and sex (†).

Angiogenic promoting cell (APC) number and colony forming units (APC-CFUs) were available in 477 and 451 individuals, respectively. Cytokines levels were available as follows: granulocyte colony-stimulating factor (G-CSF) n=218, metalloproteinase 9 (MMP9) n=468, vascular endothelial growth factor (VEGF) n=311, and stromal cell-derived factor 1 (SDF-1) n=477.
Figure S1. DKK3 levels of each human subject in year 2000 and 2005. Scatterplot of DKK3 levels measured in 2000 and 2005 in the same individuals with regression line and 95% CI. The open circles on the upper border of the figure indicate individuals with DKK3 levels higher than the range of the y-axis. r, Spearman’s rank correlation coefficient.
Figure S2. Characterization of DKK3^{-}/ApoE^{-} mice.  
A) Phenotyping of DKK3 and ApoE double knockout mice as identified by RT-PCR.  
B) ELISA measurement of serum DKK3 levels in DKK3 and ApoE double knockout mice. Note that DKK3 is almost undetectable in DKK3^{-}/ApoE^{-} mice.  
C) Serum total cholesterol of DKK3^{-}/ApoE^{-} and DKK3^{+/+}/ApoE^{-} mice with 20 weeks of age was measured with a kit.
Figure S3. Atherosclerotic lesions in DKK3+/−/ApoE+/− mice. Mice receiving normal chow diet were killed at 20 weeks, and aortas were harvested, opended, mounted and stained with oil Red O.
Figure S4. Delayed reendothelialization in injured vessels in DKK3\(^{-/-}\) ApoE\(^{-/-}\) mice. To establish reendothelialization model, femoral arteries of mice were injured. En face preparation of the vessel was stained for endothelial marker eNOS 1 week after wire injury. Dotted lines in images indicate the middle of the injured vessel, and the arrows indicate the direction of cell migration. Quantitative data of endothelial migration were presented in graphic data (n=6), **p<0.01, significant difference between two groups.
**Figure S5. The source of DKK3 in vivo.** A. Using chimeric mice model, 2 weeks after bone marrow transplantation, the level of DKK3 in peripheral blood was measured using a murine DKK3 Quantikine ELISA kit. The quantification in the graph shown mean ± SEM, n=5/group. **p<0.01. Wild-type (WT) to DKK3−/− bone marrow transplantation, wild type mice with DKK3−/− bone marrow. DKK3−/− WT bone marrow transplantation, DKK3−/− mice with wild-type bone marrow.
Figure S6

A

mouse DKK3

0 10 25 50 100

B

MLEC

C

CHO cells

qPCR

D

CHO cells

ELISA

E

supernatant Human DKK3 adenovirus

glycosylated DKK3

HA-DKK3

65kD

F

cell lysate

Human DKK3 adenovirus

deglycosylated DKK3

HA-DKK3

50kD

G

HUVEC

null 6h 24h 48h

DKK3 gene expression level (fold of control)

H

HUVEC

null 10ul 50ul 100ul

DKK3 adenovirus

I

null adenovirus

DKK3 adenovirus

Migrated Cells (Fold)

null DKK3

ns
Figure S6. Exogenous DKK3 induces endothelial cell migration. (A) Transwell assay was performed on mouse lung endothelial cells (MLEC) that migrated toward serum free media containing indicated concentrations of murine recombinant DKK3. Scale bars, 100µm. (B) The quantification of MLEC migration is shown as mean ± SEM, n=3. *p<0.05. **p<0.01. qPCR (C) and ELISA (D) analysis were performed on Adeno-DKK3-transfected CHO cell lysate or supernatant, respectively, to analyze mRNA and protein levels of DKK3 after overexpression with human DKK3 adenovirus in CHO cell. Significant difference from the controls (n=3), ***p<0.001. Glycosylation or deglycosylation of DKK3 were detected by both anti-human DKK3 antibody and anti-HA antibody in either supernatant (E) or CHO cell lysate (F) using Western blotting. (G) qPCR analyzed for DKK3 gene expression in HUVECs treated with Adeno-DKK3-transfected CHO supernatant at indicated time points. Significant difference from the controls (n=3), ***p<0.001. (H) Quantification of DKK3 gene expression in HUVECs after Adeno-DKK3 transfection for 48 hours. Significant difference from the controls (n=3), ***p<0.001. I. Transwell assay was performed to analyze the random migratory ability of HUVECs which transfected with either Null or DKK3 adenovirus. ns, not significance.
Figure S7. DKK3 enhanced endothelial migration in Matrigel plug assay. DKK3 or BSA (100mg/ml) was mixed with Matrigel and injected subcutaneously into mice. Two weeks after injection, Matrigel plugs were harvested and stained for HE (upper panel). Cell numbers within the plug were enumerated. Quantification data are means ± SEM of two groups (n=3), *p<0.05. Frozen sections were stained with EC specific marker eNOS demonstrating that DKK3 plug displayed well-formed endothelial structures and increased cell number compared to the BAS control.
Figure S8. DKK3 has no effect on HUVEC proliferation and apoptosis. (A) Proliferation of HUVECs with treatments of either human recombinant DKK3 or Adeno-DKK3-transfected CHO supernatant at indicated times was evaluated by BrdU incorporation assay. ctrl, control, serum free medium, served as negative control of human recombinant DKK3; Rmpr, recombinant DKK3 protein; Null, Adeno-Null-transfected CHO supernatant, served as negative control of Adeno-DKK3-transfected CHO supernatant; DKK3, Adeno-DKK3-transfected CHO supernatant. (B) FACS analysis was also performed on HUVECs with incubation of either recombinant DKK3 or overexpressed-CHO supernatant at different time points followed by staining of Annexin V antibody or Propidium Iodide. In each dot plot, dots in Q3 (lower right) indicates early apoptotic cells; dots in Q2 (upper right) indicates necrotic cells, the number in each grid indicates cell percentage of the whole population.
Figure S9. DKK3-induced HUVEC migration does not activate β-catenin. (A) Western blotting shows the expression of active and total β-catenin in HUVECs after treatment with human DKK3 at indicated time points. Immunofluorescence staining of DKK3 on HUVECs with either recombinant DKK3 (B) or Adeno-DKK3-transfected CHO supernatant (C) stimulation to identify the location of DKK3 expression.
Figure S10. DKK3 has no effect on RhoA activation. (A) The histograms represent the eGFP positive HUVECs after transfection with eGFP-labeled plasmids. (B) Pull down assay was performed to analyze activated RhoA (GTP-RhoA) in HUVECs after stimulation with DKK3 at different time points.
Figure S11. DKK3 does not show a role in leukocytes recruitment and macrophage migration. (A) The peritoneal cells isolated from mice that 3 days after injection with saline, thioglycollate or mouse recombinant DKK3 were counted under the microscope and the graph shows the cell number per milliliter of each group. (B) Flow cytometry analysis shows the cell components in the whole cell population of each group. (C) Migration towards mouse recombinant DKK3 was evaluated on the peritoneal macrophages which isolated from thioglycollate stimulation in vivo by using Transwell assay. (D) The quantification of peritoneal macrophages migration was shown in mean ± SEM. ns, no significance.
Figure S12. DKK3 pretreatment did not influence DKK1-induced cytokine expression. HUVECs were pre-incubated with DKK3 (50 ng/ml) for 1 hour and then DKK1 (50ng/ml) was added and incubated for 12 hours. The cells were harvested and qPCR for IL-6 and MCP-1 were performed. Data are means±SEM of three experiments. Note there is no significant difference between two groups.
Figure S13. LRP5 and LRP6 antagonists did not block DKK3-induced signalling and cell migration. Panel A, HUVECs were pre-incubated with SOST/Sclerostin or Draxin (50 ng/ml) for 1 hour and then DKK3 (25 ng/ml) was added and incubated for 30 minutes. The cells were harvested and Western blots for JNK and Jun were performed. Panel B, HUVECs were pre-incubated with SOST/Sclerostin or Draxin (50 ng/ml) for 1 hour and then cell migration assay against DKK3 (25 ng/ml) was performed. Note that LRP5 and LRP6 antagonists SOST/Sclerostin or Draxin did not block the activity of DKK3 in endothelial migration.
Figure S14. Schematic representation of the mechanism by which DKK3 induced endothelial cell migration. Soluble DKK3 can binds to Ror2/DVL on the surface of endothelial cells leading to Rac1 activation, which in turn activates JNK. JNK phosphorylates c-Jun that binds to the promoter region of genes, leading to cytoskeleton rearrangement. The overall effect favors endothelial cell migration.