

A Cytokine-like Protein DKK3 Is Atheroprotective

Running Title: *Yu et al.; DKK3 is Atheroprotective*

Baoqi Yu, PhD^{*1}; Stefan Kiechl, MD^{*2}; Dan Qi, MB³; Xiaocong Wang, PhD¹;
Yanting Song, MB³; Siegfried Weger, MD⁴; Agnes Mayr, MD⁴; Alexandra Le Bras, PhD¹;
Eirini Karamariti, PhD¹; Zhongyi Zhang, MSc¹; Ivan del Barco Barrantes, PhD⁵;
Christof Niehrs, PhD^{5,6}; Georg Schett, MD⁷; Yanhua Hu, MD¹; Wen Wang, PhD⁸;
Johann Willeit, MD²; Aijuan Qu, PhD³; Qingbo Xu, MD, PhD¹

¹Cardiovascular Division, King's College London BHF Centre, London, United Kingdom;
²Department of Neurology, Medical University of Innsbruck, Innsbruck, Austria; ³Department of
Physiology and Pathophysiology, Capital Medical University, Beijing, China; ⁴Department of
Internal and Laboratory Medicine, Bruneck Hospital, Bruneck, Italy; ⁵Division of Molecular
Embryology, DKFZ-ZMBH Alliance, Heidelberg, Germany; ⁶Institute of Molecular Biology (IMB),
Mainz, Germany; ⁷Department of Internal Medicine 3, Institute for Clinical Immunology, Friedrich-
Alexander-University Erlangen-Nuremberg, Erlangen, Germany; ⁸Institute of Bioengineering,
Queen Mary University of London, London, United Kingdom

*Both authors contributed equally

Address for Correspondence:

Qingbo Xu, MD, PhD
Cardiovascular Division
King's College London BHF Centre
125 Coldharbour Lane
London SE5 9NU, UK
Tel: (+44)20 7848-5322
Fax: (+44)20 7848-5296
Email: qingbo.xu@kcl.ac.uk

Aijuan Qu, MD, PhD
Department of Physiology and Pathophysiology
Capital Medical University
No.10 Xitoutiao Road
YouAnmen, Beijing 100069, China
Tel/Fax: (+86)10 83950091
Email: aijuanqu@ccmu.edu.cn

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.



Circulation

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 muscles¹⁻⁴. DKK3 appears to have a decisive function in myogenic cell fate since it is also highly expressed in different skeletal muscle subtypes⁵. DKK3 has also been established as a potential tumor biomarker expressed in many cancer cell lines and an effective tumor suppressor in numerous human cancers⁶⁻⁸. It has been reported that DKK3 plays a role in promoting angiogenesis in different types of tumors^{9, 10}. Recently, several studies have discovered that DKK3 prevented the progression of cardiac hypertrophy^{11, 12} and was also involved in vascular smooth muscle cell differentiation^{2, 4}. 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^{13, 14}. 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 events¹⁵. Many studies have demonstrated that the underlying pathophysiology of atherosclerosis is initiated by endothelial dysfunction¹⁶, which is caused by physical or chemical offences such as hypertension¹⁷, shear stress of disturbed laminar flow^{18, 19}, reactive oxygen species in the circulation²⁰, decreased nitric oxide bioactivity²¹, hyperlipidemia and hyperglycemia²². These factors can directly or indirectly induce endothelial dysfunction/death in arteries²³ followed by cell regeneration *in situ*²⁴. In this process, neighbouring endothelial cells have been proven to contribute to re-endothelialization by migration and proliferation²⁵. 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^{26,27}. 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^{28,29}. 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 detail²⁸⁻³⁰ 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 previously³¹. Three genotypes of DKK3^{-/-}, DKK3^{+/-} and DKK3^{+/+} mice were identified using PCR (primers: 5'-GATAGCTTTCCGGGACACAC-3; 5'-TCCATCAGCTCCTCCA 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 described³². 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 arium (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- μ m thick frozen sections were obtained from the heart and were stained with Oil Red O as described elsewhere³³. 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 μ m pore size polycarbonate membranes (Corning) as described previously³⁴. HUVECs were seeded onto the upper chamber at 1×10^5 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.

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 \pm 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, waist-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. \geq 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 ($P_{\text{interaction}}=0.17$).

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 α SMA 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 pathway³. 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 development³⁷, the non-canonical Wnt pathway is involved in cell polarity³⁸ and convergent extension movements³⁹. Recent studies have indicated that the tyrosine kinase receptor ROR2 plays an important role in non-canonical Wnt pathway to mediate cell migration⁴⁰⁻⁴². 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. In

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 responses⁵¹, including post-angioplasty neointima formation and restenosis⁵². 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^{53, 54}. 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^{3, 48, 55}. 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^{9, 10} and that overexpression of DKK3 did not affect proliferation and migration of endothelial colony-forming cell¹⁰. 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^{3, 56}. It has been demonstrated that DKK1 and DKK2 have important functions in endothelial function⁵⁷⁻⁵⁹, including the role of DKK1 plays in accelerating pro-inflammatory response and atherosclerosis⁵⁹. 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 Kremen²⁰ and LRP5⁶⁰. 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^{61, 62}. 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^{38, 63}. 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.

Sources of Funding

This work was partly supported by the British Heart Foundation (RG/14/6/31144) and the Oak Foundation, National Natural Science Foundation of China (81370521, 81320157, and 81670400), The Importation and Development of High-Caliber Talents Project of Beijing Municipal Institutions (CIT&TCD20150325), The Key Science and Technology Project of Beijing Municipal Institutions (KZ201610025025), The Fok Ying-Tong Education Foundation (151041) and Beijing Haiju Project. S.K., J.W., A.M., and S.W. were supported by the excellence initiative (Competence Centers for Excellent Technologies) of the Austrian Research

Promotion Agency: Research Center of Excellence in Vascular Ageing, Tyrol (K-Project No. 843536) funded by the Wirtschaftsagentur Wien and Standortagentur Tirol.

Disclosures

None

References

1. Cruciat CM, Niehrs C. Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harb Perspect Biol.* 2013;5:a015081.
2. Karamariti E, Margariti A, Winkler B, Wang X, Hong X, Baban D, Ragoussis J, Huang Y, Han JD, Wong MM, Sag CM, Shah AM, Hu Y, Xu Q. Smooth muscle cells differentiated from reprogrammed embryonic lung fibroblasts through DKK3 signaling are potent for tissue engineering of vascular grafts. *Circ Res.* 2013;112:1433-1443.
3. Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci.* 2003;116:2627-2634.
4. Wang X, Karamariti E, Simpson R, Wang W, Xu Q. Dickkopf homolog 3 induces stem cell differentiation into smooth muscle lineage via ATF6 signalling. *J Biol Chem.* 2015;290:19844-19852.
5. de Wilde J, Hulshof MF, Boekschoten MV, de Groot P, Smit E, Mariman EC. The embryonic genes Dkk3, Hoxd8, Hoxd9 and Tbx1 identify muscle types in a diet-independent and fiber-type unrelated way. *BMC Genomics.* 2010;11:176.
6. Yue W, Sun Q, Dacic S, Landreneau RJ, Siegfried JM, Yu J, Zhang L. Downregulation of Dkk3 activates beta-catenin/TCF-4 signaling in lung cancer. *Carcinogenesis.* 2008;29:84-92.
7. Lee EJ, Jo M, Rho SB, Park K, Yoo YN, Park J, Chae M, Zhang W, Lee JH. Dkk3, downregulated in cervical cancer, functions as a negative regulator of beta-catenin. *Int J Cancer.* 2009;124:287-297.
8. Veeck J, Wild PJ, Fuchs T, Schuffler PJ, Hartmann A, Knuchel R, Dahl E. Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer. *BMC Cancer.* 2009;9:217.
9. Fong D, Hermann M, Untergasser G, Pirkebner D, Draxl A, Heitz M, Moser P, Margreiter R, Hengster P, Amberger A. Dkk-3 expression in the tumor endothelium: a novel prognostic marker of pancreatic adenocarcinomas. *Cancer Sci.* 2009;100:1414-1420.
10. Untergasser G, Steurer M, Zimmermann M, Hermann M, Kern J, Amberger A, Gastl G, Gunsilius E. The Dickkopf-homolog 3 is expressed in tumor endothelial cells and supports capillary formation. *Int J Cancer.* 2008;122:1539-1547.
11. Akazawa H, Komuro I. Dickkopf-3: a stubborn protector of cardiac hypertrophy. *Cardiovasc Res.* 2014;102:6-8.
12. Zhang Y, Liu Y, Zhu XH, Zhang XD, Jiang DS, Bian ZY, Zhang XF, Chen K, Wei X, Gao L, Zhu LH, Yang Q, Fan GC, Lau WB, Ma X, Li H. Dickkopf-3 attenuates pressure overload-induced cardiac remodelling. *Cardiovasc Res.* 2014;102:35-45.

13. Murray CJ, Lopez AD. Evidence-based health policy--lessons from the Global Burden of Disease Study. *Science*. 1996;274:740-743.
14. Writing Group Members, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Després JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jiménez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB; American Heart Association Statistics Committee; Stroke Statistics Subcommittee. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation*. 2016;133:e38-360.
15. Tesfamariam B. Platelet function in intravascular device implant-induced intimal injury. *Cardiovasc Revasc Med*. 2008;9:78-87.
16. Foteinos G, Hu Y, Xiao Q, Metzler B, Xu Q. Rapid endothelial turnover in atherosclerosis-prone areas coincides with stem cell repair in apolipoprotein E-deficient mice. *Circulation*. 2008;117:1856-1863.
17. Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, Ferraro A, Chello M, Mastroroberto P, Verdecchia P, Schillaci G. Prognostic significance of endothelial dysfunction in hypertensive patients. *Circulation*. 2001;104:191-196.
18. Chiu JJ, Chien S. Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol Rev*. 2011;91:327-387.
19. Xu Q. Disturbed flow-enhanced endothelial turnover in atherosclerosis. *Trends Cardiovasc Med*. 2009;19:191-195.
20. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation*. 2001;104:2673-2678.
21. Flavahan NA. Atherosclerosis or lipoprotein-induced endothelial dysfunction. Potential mechanisms underlying reduction in EDRF/nitric oxide activity. *Circulation*. 1992;85:1927-1938.
22. Ceriello A, Taboga C, Tonutti L, Quagliaro L, Piconi L, Bais B, Da Ros R, Motz E. Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: effects of short- and long-term simvastatin treatment. *Circulation*. 2002;106:1211-1218.
23. Tricot O, Mallat Z, Heymes C, Belmin J, Leseche G, Tedgui A. Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques. *Circulation*. 2000;101:2450-2453.
24. Xu Q. The impact of progenitor cells in atherosclerosis. *Nat Clin Pract Cardiovasc Med*. 2006;3:94-101.
25. Blann AD, Woywodt A, Bertolini F, Bull TM, Buyon JP, Clancy RM, Haubitz M, Hebbel RP, Lip GY, Mancuso P, Sampol J, Solovey A, Dignat-George F. Circulating endothelial cells. Biomarker of vascular disease. *Thromb Haemost*. 2005;93:228-235.
26. Hutter R, Carrick FE, Valdiviezo C, Wolinsky C, Rudge JS, Wiegand SJ, Fuster V, Badimon JJ, Sauter BV. Vascular endothelial growth factor regulates reendothelialization and neointima formation in a mouse model of arterial injury. *Circulation*. 2004;110:2430-2435.

27. Hayashi K, Nakamura S, Morishita R, Moriguchi A, Aoki M, Matsumoto K, Nakamura T, Kaneda Y, Sakai N, Ogihara T. In vivo transfer of human hepatocyte growth factor gene accelerates re-endothelialization and inhibits neointimal formation after balloon injury in rat model. *Gene Ther.* 2000;7:1664-1671.
28. Willeit J, Kiechl S. Prevalence and risk factors of asymptomatic extracranial carotid artery atherosclerosis. A population-based study. *Arterioscler Thromb.* 1993;13:661-668.
29. Xu Q, Willeit J, Marosi M, Kleindienst R, Oberhollenzer F, Kiechl S, Stulnig T, Luef G, Wick G. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis. *Lancet.* 1993;341:255-259.
30. Kiechl S, Willeit J. The natural course of atherosclerosis. Part II: vascular remodeling. Bruneck Study Group. *Arterioscler Thromb Vasc Biol.* 1999;19:1491-1498.
31. Barrantes Idel B, Montero-Pedrazuela A, Guadano-Ferraz A, Obregon MJ, Martinez de Mena R, Gailus-Durner V, Fuchs H, Franz TJ, Kalaydjiev S, Klempt M, Hölter S, Rathkolb B, Reinhard C, Morreale de Escobar G, Bernal J, Busch DH, Wurst W, Wolf E, Schulz H, Shtrom S, Greiner E, Hrabé de Angelis M, Westphal H, Niehrs C. Generation and characterization of dickkopf3 mutant mice. *Mol Cell Biol.* 2006;26:2317-2326.
32. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest.* 2004;113:1258-1265.
33. Hu Y, Davison F, Zhang Z, Xu Q. Endothelial replacement and angiogenesis in arteriosclerotic lesions of allografts are contributed by circulating progenitor cells. *Circulation.* 2003;108:3122-3127.
34. Wong MM, Chen Y, Margariti A, Winkler B, Campagnolo P, Potter C, Hu Y, Xu Q. Macrophages control vascular stem/progenitor cell plasticity through tumor necrosis factor- α -mediated nuclear factor- κ B activation. *Arterioscler Thromb Vasc Biol.* 2014;34:635-643.
35. Pinho S, Niehrs C. Dkk3 is required for TGF- β signaling during *Xenopus* mesoderm induction. *Differentiation.* 2007;75:957-967.
36. Gimbrone MA, Jr., Topper JN, Nagel T, Anderson KR, Garcia-Cardena G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci.* 2000;902:230-239.
37. Gough NR. Focus issue: Wnt and beta-catenin signaling in development and disease. *Sci Signal.* 2012;5:e2.
38. Katoh M, Katoh M. WNT signaling pathway and stem cell signaling network. *Clin Cancer Res.* 2007;13:4042-4045.
39. Yamanaka H, Moriguchi T, Masuyama N, Kusakabe M, Hanafusa H, Takada R, Takada S, Nishida E. JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep.* 2002;3:69-75.
40. Oishi I, Suzuki H, Onishi N, Takada R, Kani S, Ohkawara B, Koshida I, Suzuki K, Yamada G, Schwabe GC, Mundlos S, Shibuya H, Takada S, Minami Y. The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. *Genes Cells.* 2003;8:645-654.
41. Zhang C, Hu Y, Wan J, He H. MicroRNA-124 suppresses the migration and invasion of osteosarcoma cells via targeting ROR2-mediated non-canonical Wnt signaling. *Oncol Rep.* 2015;34:2195-2201.

42. Tseng JC, Lin CY, Su LC, Fu HH, Yang SD, Chuu CP. CAPE suppresses migration and invasion of prostate cancer cells via activation of non-canonical Wnt signaling. *Oncotarget*. 2016;7:38010-38024.
43. Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med*. 2006;203:1273-1282.
44. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011;145:341-355.
45. Kobayashi K, Ouchida M, Tsuji T, Hanafusa H, Miyazaki M, Namba M, Shimizu N, Shimizu K. Reduced expression of the REIC/Dkk-3 gene by promoter-hypermethylation in human tumor cells. *Gene*. 2002;282:151-158.
46. Abarzua F, Sakaguchi M, Takaishi M, Nasu Y, Kurose K, Ebara S, Miyazaki M, Namba M, Kumon H, Huh NH. Adenovirus-mediated overexpression of REIC/Dkk-3 selectively induces apoptosis in human prostate cancer cells through activation of c-Jun-NH2-kinase. *Cancer Res*. 2005;65:9617-9622.
47. Lu D, Bao D, Dong W, Liu N, Zhang X, Gao S, Ge W, Gao X, Zhang L. Dkk3 prevents familial dilated cardiomyopathy development through Wnt pathway. *Lab Invest*. 2016;96:239-248.
48. Zhang K, Watanabe M, Kashiwakura Y, Li SA, Edamura K, Huang P, Yamaguchi K, Nasu Y, Kobayashi Y, Sakaguchi M, Ochiai K, Yamada H, Takei K, Ueki H, Huh NH, Li M, Kaku H, Na Y, Kumon H. Expression pattern of REIC/Dkk-3 in various cell types and the implications of the soluble form in prostatic acinar development. *Int J Oncol*. 2010;37:1495-1501.
49. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med*. 1999;340:115-126.
50. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol*. 2003;23:168-175.
51. Ross R, Agius L. The process of atherogenesis--cellular and molecular interaction: from experimental animal models to humans. *Diabetologia*. 1992;35 Suppl 2:S34-40.
52. Rajagopal V, Rockson SG. Coronary restenosis: a review of mechanisms and management. *Am J Med*. 2003;115:547-553.
53. Zampetaki A, Kirton JP, Xu Q. Vascular repair by endothelial progenitor cells. *Cardiovasc Res*. 2008;78:413-421.
54. Bai X, Wang X, Xu Q. Endothelial damage and stem cell repair in atherosclerosis. *Vascul Pharmacol*. 2010;52:224-229.
55. Zenzmaier C, Untergasser G, Hermann M, Dirnhofer S, Sampson N, Berger P. Dysregulation of Dkk-3 expression in benign and malignant prostatic tissue. *Prostate*. 2008;68:540-547.
56. Cheng SL, Ramachandran B, Behrmann A, Shao JS, Mead M, Smith C, Krehma K, Bello Arredondo Y, Kovacs A, Kapoor K, Brill LM, Perera R, Williams BO, Towler DA. Vascular smooth muscle LRP6 limits arteriosclerotic calcification in diabetic LDLR^{-/-} mice by restraining noncanonical Wnt signals. *Circ Res*. 2015;117:142-156.
57. Cheng SL, Shao JS, Behrmann A, Krehma K, Towler DA. Dkk1 and MSX2-Wnt7b signaling reciprocally regulate the endothelial-mesenchymal transition in aortic endothelial cells. *Arterioscler Thromb Vasc Biol*. 2013;33:1679-1689.
58. Min JK, Park H, Choi HJ, Kim Y, Pyun BJ, Agrawal V, Song BW, Jeon J, Maeng YS, Rho SS, Shim S, Chai JH, Koo BK, Hong HJ, Yun CO, Choi C, Kim YM, Hwang KC, Kwon

- YG. The WNT antagonist Dickkopf2 promotes angiogenesis in rodent and human endothelial cells. *J Clin Invest*. 2011;121:1882-1893.
59. Ueland T, Otterdal K, Lekva T, Halvorsen B, Gabrielsen A, Sandberg WJ, Paulsson-Berne G, Pedersen TM, Folkersen L, Gullestad L, Oie E, Hansson GK, Aukrust P. Dickkopf-1 enhances inflammatory interaction between platelets and endothelial cells and shows increased expression in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2009;29:1228-1234.
 60. Hoang BH, Kubo T, Healey JH, Yang R, Nathan SS, Kolb EA, Mazza B, Meyers PA, Gorlick R. Dickkopf 3 inhibits invasion and motility of Saos-2 osteosarcoma cells by modulating the Wnt-beta-catenin pathway. *Cancer Res*. 2004;64:2734-2739.
 61. Mao B, Wu W, Li Y, Hoppe D, Stanek P, Glinka A, Niehrs C. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature*. 2001;411:321-325.
 62. Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stanek P, Walter C, Glinka A, Niehrs C. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature*. 2002;417:664-667.
 63. Veeck J, Bektas N, Hartmann A, Kristiansen G, Heindrichs U, Knuchel R, Dahl E. Wnt signalling in human breast cancer: expression of the putative Wnt inhibitor Dickkopf-3 (DKK3) is frequently suppressed by promoter hypermethylation in mammary tumours. *Breast Cancer Res*. 2008;10:R82.



Circulation

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

Variable	DKK3 Tertile I (<65 ng/mL)	DKK3 Tertile II (65-74 ng/ml)	DKK3 Tertile III (≥75 ng/ml)	P value*	P value†
Age	61.8±8.6	66.7±10.1	70.0±10.0	<0.001	
Male sex (%)	51.3	45.4	44.9	0.208	
Systolic BP (mmHg)	137.0±17.5	141.6±20.6	140.5±18.3	0.062	0.503
Diastolic BP (mmHg)	83.9±7.8	84.0±8.2	83.8±8.9	0.911	0.781
Hypertension (%)	48.3	60.3	60.0	0.018	0.860
Urinary albumin (g/dl)	23.2±53.7	19.4±43.5	62.8±282.5	0.188	0.537
Creatinine (mg/dl)	0.85±0.12	0.88±0.16	0.92±0.29	0.001	0.004
Leukocyte count (10 ⁹ /L)	6.2±1.6	6.1±1.6	6.2±1.8	0.788	0.954
hsCRP (mg/L)	1.8 (0.9-3.8)	1.7 (0.8-3.9)	1.8 (0.9-4.6)	0.974	0.064
Fibrinogen (mg/dl)	288.9±56.2	287.9±62.3	294.1±62.7	0.392	0.058
Chronic infections (%)	14.1	24.2	31.8	0.001	0.537
Ferritin (µg/L)	124.5±155.1	115.3±137.0	113.4±110.0	0.415	0.422
LDL (mg/dl)	148.5±40.0	151.8±35.8	144.8±35.1	0.317	0.311
HDL (mg/dl)	57.3±17.0	56.4±14.5	57.6±14.4	0.868	0.759
Triglycerides (mg/dl)	130 (88-173)	121 (92-165)	117 (78-157)	0.023	0.055
Body mass index (kg/m ²)	26.3±3.7	25.2±4.1	24.6±4.1	<0.001	<0.001
WHR (cm/cm)	0.92±0.1	0.92±0.1	0.91±0.1	0.043	0.003
Physical activity (sports score)	2.4±0.8	2.3±0.7	2.3±0.8	0.059	0.110
Fast glucose (mg/dl)	103.7±27.6	102.0±25.8	99.2±19.1	0.063	0.001
HbA1c (mmol/mol)	4.1±0.7	4.1±0.7	4.1±0.6	0.315	0.120
Diabetes mellitus (%)	11.9	13.8	11.8	0.799	0.073
Homocystein (µmol/L)	12.5±12.1	13.0±5.0	13.7±5.2	0.162	0.846
Smoking (%)	19.9	13.8	15.3	0.256	0.759
Smoking (cigarettes/day)	2.6±6.3	1.9±5.5	1.2±3.7	0.005	0.243
Alcohol consumption (g/day)	24.9±32.2	20.9±31.1	18.4±23.7	0.050	0.179

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 α SMA 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 \pm SEM from 6 mice for each group of the neointimal area, neointima/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 \pm 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 μ m 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, 100 μ m. **B**, **D**. 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 \pm 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, 100 μ m. **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, 50 μ m.



Figure 6. DKK3 induces endothelial cell migration via ROR2/Dvl1/Rac1/JNK signaling pathway.

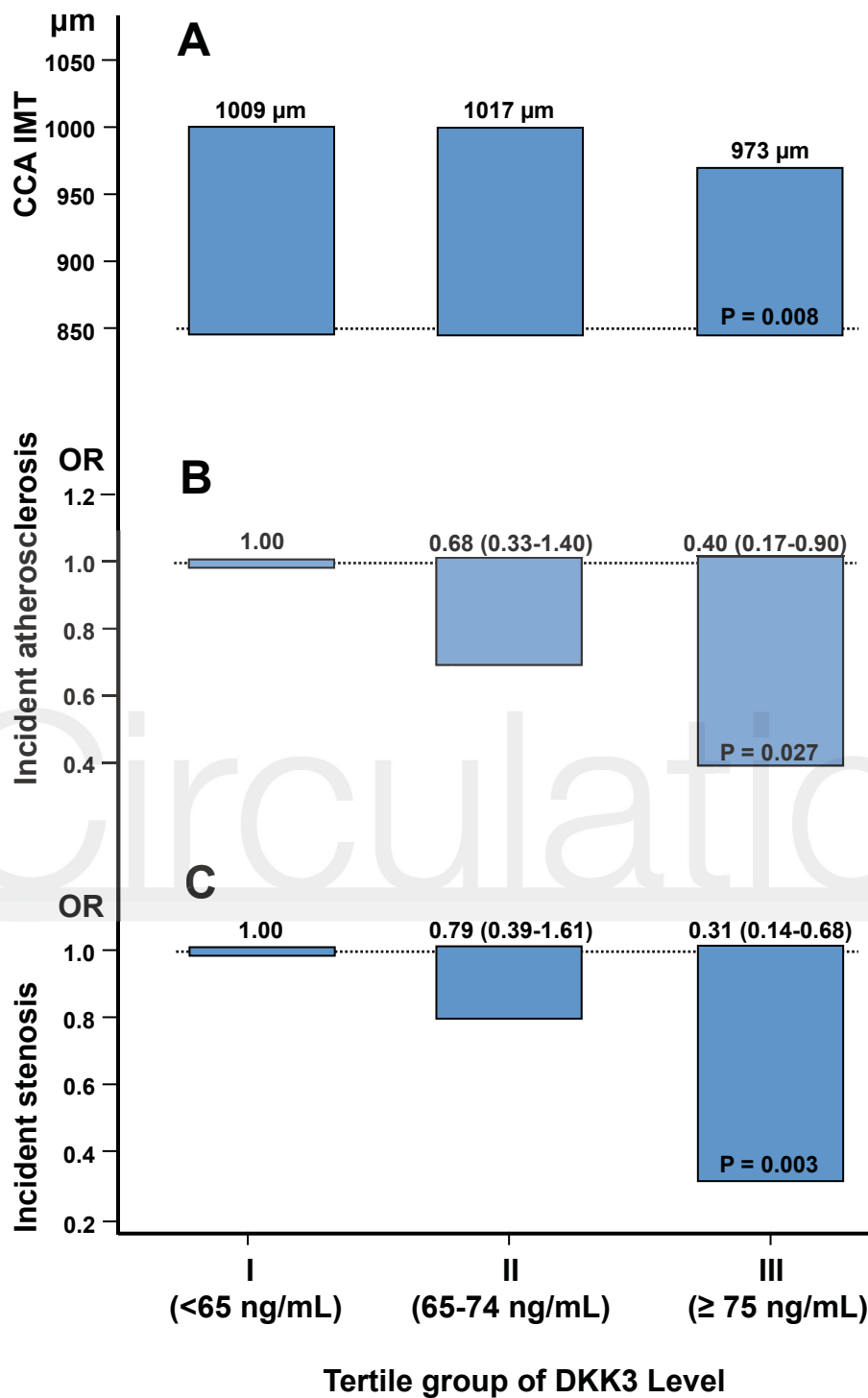
A. Western blot analysis showed the binding of ROR2 and DKK3 after immunoprecipitation of either DKK3 or HA-binding protein in HUVECs stimulated with DKK3 or DKK3-CHO SN. **B**. The real-time quantitative PCR showed the fold changes in mRNA levels of DVL1, 2, 3 after DKK3 treatment. **C**. Immunofluorescence 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

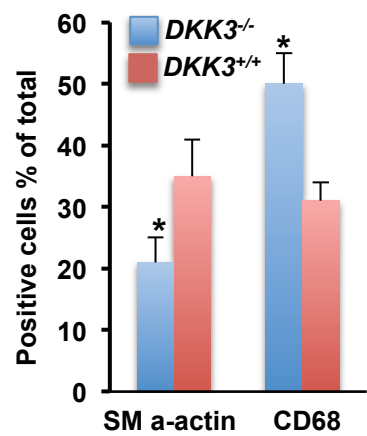
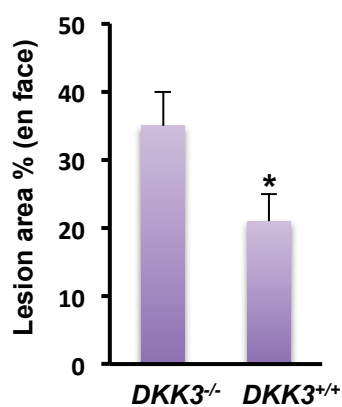
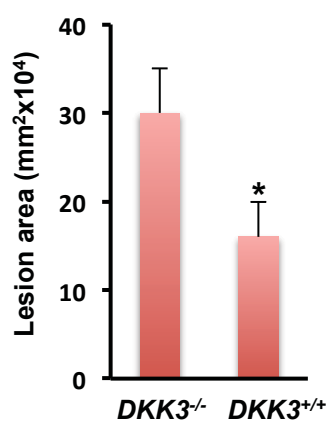
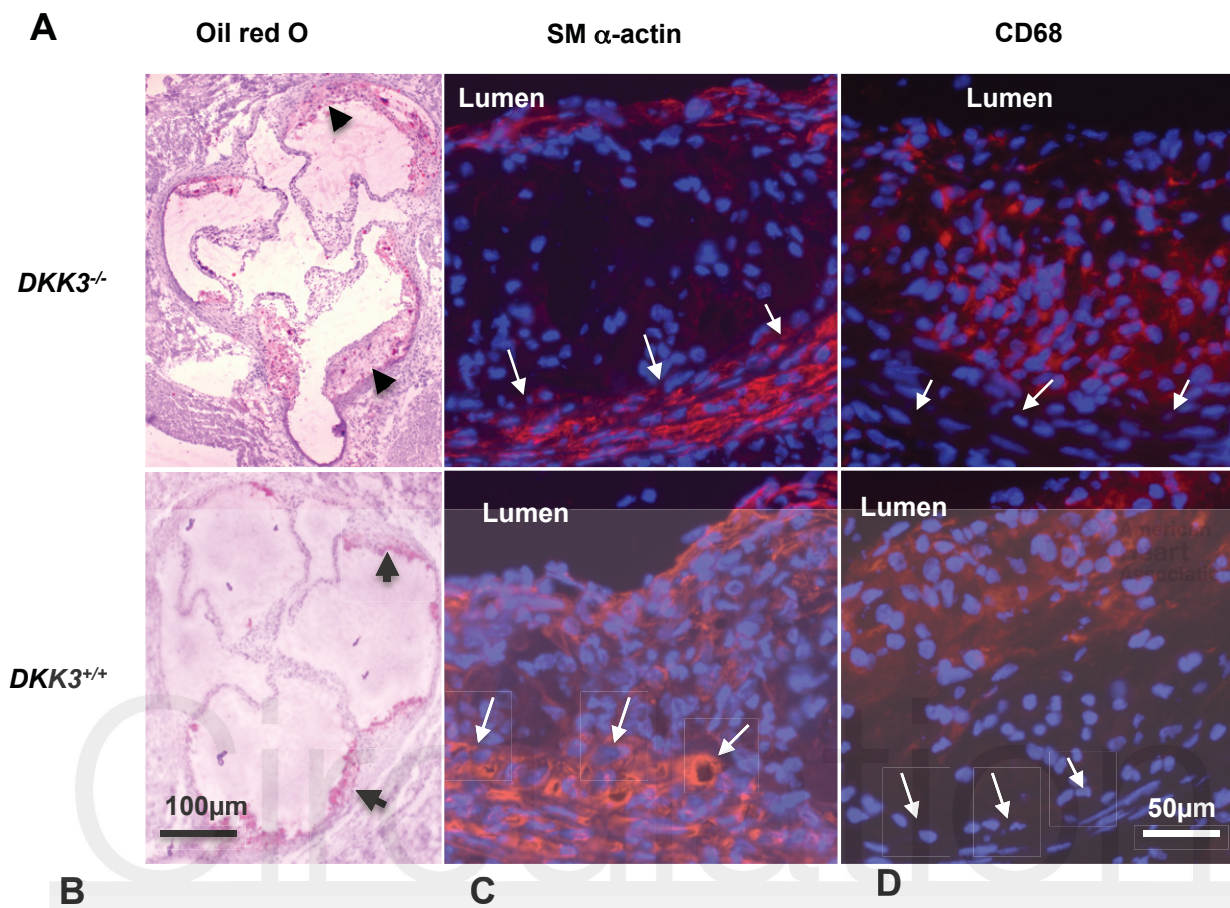
after transfection of constitutive mutants of Rac1 (**H**). Transwell assay was performed on HUVECs that were either transfected with constitutive mutants of Rac1 (**I**) or control, ROR2 siRNA (**J**) before migration toward DKK3, 6 hours with either crystal violet staining or direct fluorescence observation 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 \pm 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, 100 μ m.

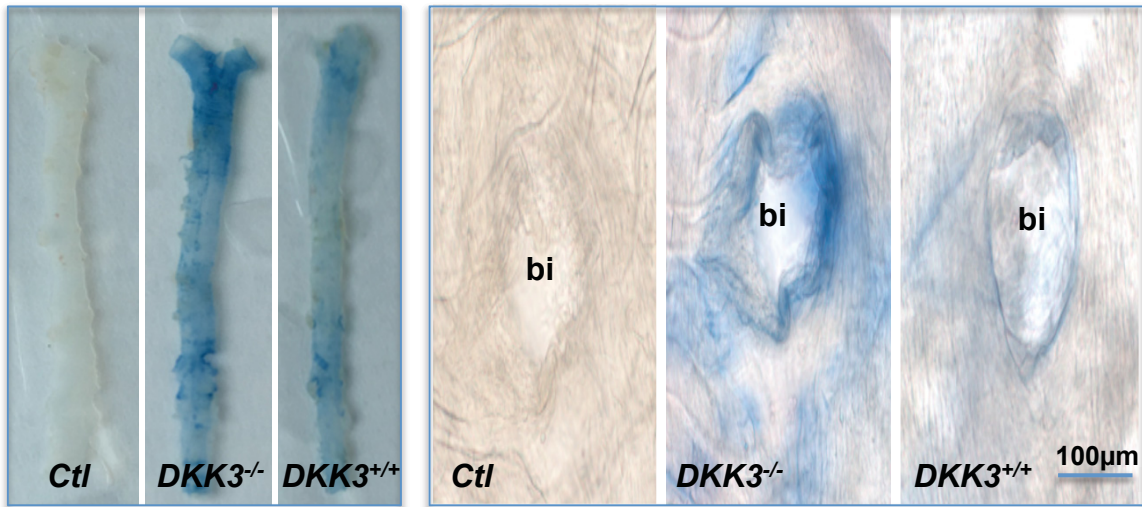


Circulation

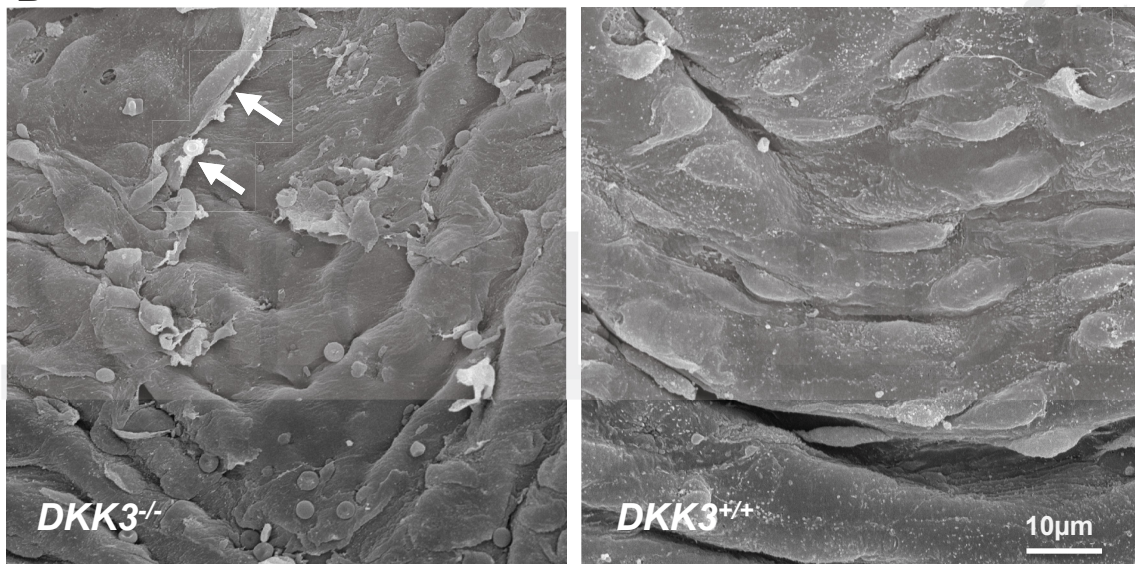




A



B



C

