Binding of Dickkopf-3 to CXCR7 Enhances Vascular Progenitor Cell Migration and Degradable Graft Regeneration

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ABSTRACT

Rationale: Vascular progenitor cells (VPCs) play key roles in physiological and pathological vascular remodelling, a process that is crucial for the regeneration of acellular biodegradable scaffolds engineered as vital strategies against the limited availability of healthy autologous vessels for bypass grafting. Therefore, understanding the mechanisms driving VPCs recruitment and differentiation could help the development of new strategies to improve Tissue-Engineered Vessel Grafts (TEVGs) and design drug-targeted therapy for vessel regeneration.

Objective: In this study we sought to investigate the role of Dickkopf-3 (Dkk3), recently identified as a cytokine promotor of endothelial repair and smooth muscle cell differentiation, on VPC cell migration and vascular regeneration and to identify its functional receptor that remains unknown.

Methods and Results: Vascular stem/progenitor cells were isolated from murine aortic adventitia and selected for the Stem cell antigen-1 (Sca-1) marker. Dkk3 induced the chemotaxis of Sca-1+ cells *in vitro* in transwell and wound healing assays, and *ex vivo* in the aortic ring assay. Functional studies to identify Dkk3 receptor revealed that overexpression or knockdown of chemokine receptor CXCR7 in Sca1+ cells resulted in alterations in cell migration. Co-Immunoprecipitation experiments using Sca1+ cells extracts treated with Dkk3 showed the physical interaction between DKK3 and CXCR7, and specific saturation binding assays identified a high affinity Dkk3-CXCR7 binding with a dissociation constant (Kd) of 14.14 nM. Binding of CXCR7 by Dkk3 triggered the subsequent activation of ERK1/2, PI3K/AKT, Rac1 and RhoA signalling pathways involved in Sca1+ cell migration. TEVGs were fabricated with or without Dkk3 and implanted to replace the rat abdominal aorta. Dkk3-loaded TEVGs showed efficient endothelization and recruitment of VPCs, which had acquired characteristics of mature smooth muscle cells. CXCR7 blocking using specific antibodies in this vessel graft model hampered stem/progenitor cell recruitment into the vessel wall, thus compromising vascular remodelling.

Conclusions: We provide a novel and solid evidence that CXCR7 serves as Dkk3 receptor, which mediates Dkk3-induced vascular progenitor migration *in vitro* and in tissue-engineered vessels, hence harnessing patent grafts resembling native blood vessels.

Key words: Dkk3, stem/progenitor cells, migration, tissue-engineered vessel grafts, vessel remodelling.

NON-STANDARD ABBREVIATIONS AND ACRONYMS

AKT or PKB: Protein Kinase B

AP: Alkaline Phosphatase

ApoE: Apolipoprotein E

BrdU: Bromodeoxyuridine

Dkk3: Dickkopf-3

EC: Endothelial cell

ERK1/2: Extracellular signal–regulated kinases 1/2

GFP: Green fluorescent protein

Kd: Dissociation constant

LRP5/6: Low-density lipoprotein receptor-related protein 5 or 6

MLC: Myosin light chain

PCL: Polycacoprolactone

PI3K: Phosphatidylinositol 3-kinase

Rac1: Ras-related C3 botulinum toxin substrate 1

RhoA: Ras homolog gene family, member A

ROCK: Rho-associated protein kinase

Sca-1: Stem cell antigen-1

Sdf-1α: Stromal cell-derived factor 1 alpha

SEM: Scanning Electron Microscope

SMC: Smooth muscle cell

SMPC: Smooth muscle progenitor cells

SM-MHC: Smooth muscle myosin heavy chain

TEVGs: Tissue engineered vessel grafts

VPCs – Vascular progenitor cells

vWF: Von Willebrand factor

α-SMA: alpha smooth muscle actin

INTRODUCTION

Coronary and peripheral vascular bypass surgeries are one of the most effective treatments for cardiovascular diseases, in which autografts constitute the standard clinical approach for blood vessel replacement[1]. However, due to disease or previous harvesting, autologous vessels have limited availability. As an alternative, tissue-engineered vessel grafts (TEVGs) composed of synthetic or biodegradable polymers have been developed[2, 3]. Although with apparent potential, their clinical application has been challenged by unsuccessful long-term patency due to intimal hyperplasia and thrombosis. The design of patent vascular grafts, capable of inducing vascular remodelling, adequately integrating into the native blood vessel, and displaying the required mechanical strength to withstand the blood pressure, greatly relies on host cell recruitment, particularly of vascular progenitor cells (VPCs)[4, 5].

Accumulating evidence has shown that stem/progenitor cells play an active role in vascular remodelling[6-9]. Amongst these cells, it has been demonstrated that vessel wall resident VPCs are able to migrate, proliferate and differentiate into different cell lineages, including endothelial and smooth muscle cells[10-12]. Our group has identified a population of multipotent and lineage committed progenitor cells, e.g. Sca-1+, c-kit+, CD34+ and Flk1+ cells, resident in the aortic adventitia of ApoE-deficient mice[13]. In experimental vein graft and wire-induced arterial injury mouse models, the adventitia-derived Sca-1+ progenitor cells transferred to the perivascular side of the vessel were able to migrate to the neointima and to differentiate into smooth muscle cells (SMCs), with contribution to vascular remodelling and atherosclerotic lesion enhancement[13, 14]. More recently, a study demonstrated that a subpopulation of Sca-1+ cells originates from differentiated medial SMCs and that these cells not only exhibit a multipotent phenotype but can also expand and contribute to adventitial remodelling following vascular injury[15]. However, little is known about the molecular mechanisms of vascular progenitor cell recruitment *in vivo*.

Dickkopf-3 (Dkk3) is a secreted glycoprotein, highly expressed in endothelial cells (ECs), SMCs and platelets[16-19]. Functionally, Dkk3 was found to play important roles in cardiovascular biology. Previous reports had already demonstrated that Dkk3 can protect against cardiac hypertrophy[20, 21] and our group recently showed that Dkk3 is an atheroprotective cytokine. Indeed, in a human population-based prospective study, Dkk3 serum level was inversely correlated with carotid artery intimal thickening[19]. These findings, together with the potential of Dkk3 to induce SMC differentiation of stem cells[16, 17] led us to investigate its effect on VPCs and vascular regeneration, which remains unknown. Mechanistically, a great controversy surrounds the identification of Dkk3 receptor. Some studies have shown that, contrary to Dkk1, Dkk2 and Dkk4, Dkk3 is not able to bind to Wnt pathway components Kremen1, Kremen2, LRP5 and LRP6[22-26]. On the other hand, contradictory reports have indicated that Dkk3 could interact with these receptors[16, 25, 27]. We hypothesized that Dkk3 could act as a chemokine for VPCs possibly via binding to a chemokine receptor. In this work, we identify CXCR7 as the functional chemokine receptor for Dkk3 and elucidate the downstream migration-related pathways ERK1/2, PI3K/AKT and Rho GTPases. We also designed Dkk3-loaded TEVGs and showed that they exhibit long-term patency with controlled recruitment and differentiation of VPCs, thus harnessing neoarteries resembling native blood vessels. CXCR7 blocking in this model inhibits VPCs recruitment and compromises graft remodelling.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Details of materials and experimental procedures are available in the Online Data Supplement.

Sca-1+ Adventitial Progenitor Cell Isolation and Dkk3 Binding

Mouse vascular progenitor cells were isolated from the outgrowth of aortic adventitial tissues as previously described[13]. Sca-1+ VPCs were treated with 25 ng/mL of Dkk3 for 3 hours. The lysate was pre-cleared by using the Control Agarose Resin column. The eluted immune complexes and input samples were separated on a 4-12% Bis-Tris gel and the immunoblot was probed with Dkk3 antibody. His-tag pull-down assay was performed according to the instructions provided in MagneHis Protein Purification System (Promega). Receptor affinity binding assay was done as described previously[22].

Results

Dkk3 Induces Migration of Resident Sca-1+ Vpcs *In Vitro* And *Ex Vivo*

To investigate the chemotactic potential of Dkk3 in Sca-1+ cells, we first performed *in vitro* migration assays. Sdf-1α chemokine was used as a positive control for cell migration in the experiments[28, 29]. Transwell and wound healing assays revealed that Dkk3 markedly induced the migration of Sca-1+ cells over a range of concentrations of 0-100 ng/mL (Figure 1a, b, d and e), indicating that Dkk3 possesses chemokine-like properties comparable to Sdf-1α (Figure 1a, c, d and f). To rule out a potential contribution of Dkk3-induced Sca-1+ cell proliferation in our observations, we performed BrdU incorporation cell proliferation assay at 16, 24 and 48 hours of treatment. No significant differences were observed in the cell proliferation rate of the cells between control and treatment groups, when the dose of 25 ng/mL of Dkk3 was used (Online Figure Ia). Dkk3 treatment at higher concentration (50 ng/mL) promoted cell proliferation only after 48 hours of treatment. The effect of Dkk3 on cell death by apoptosis and necrosis was also determined. During the 24 hours of treatment (25 ng/mL), no significant difference in cell death rate was found between control and Dkk3 groups (Online Figure Ib). We thus concluded that Dkk3 induces the chemotaxis of Sca-1+ cells *in vitro*. To further assess the chemotactic effect of Dkk3 in a setting that better mimics the *in vivo* 3-dimensional conditions and to study the effect of Dkk3 on endogenous resident VPCs of the vessel wall, the aortic ring assay was performed with aortas of wild type and ApoE$^{-/-}$ mice. In wild type explants, although Dkk3 induced cell outgrowth, no difference with statistical significance was observed between control and Dkk3 treated group (Figure1 g-h). However, in ApoE$^{-/-}$ explants, Dkk3 remarkably promoted cell outgrowth from the aortic rings, analogously to Sdf-1α (Figure 1g and i). The explanation could be that vascular cells derived from the native atherosclerosis model ApoE$^{-/-}$ mice are potentially more responsive or sensitive to stimuli than the cells derived from wild type mice. We have shown in a previous study that, compared to wild type cells, ApoE$^{-/-}$ Sca1+ cells exhibit an altered expression of genes implicated in cell migration, including matrix metallopeptidases and integrins, and

possess an intrinsically enhanced migratory potential[30]. As cell outgrowth from the aortic rings comprises different cell types, we next sought to examine the contribution of Sca-1+ progenitor cells in Dkk3-induced outgrowth. Aortic rings from transgenic Sca-1-GFP ApoE$^{-/-}$ mice were isolated and cultured, as this mouse model allows rapid detection of Sca-1+ cells, through green fluorescence protein (GFP) expression. Dkk3 treatment triggered an increase in Sca-1+-GFP+ cell outgrowth compared to control (Figure 1j-k). Moreover, the percentage of Sca-1+-GFP+ cells in the total number of outgrown cells was also higher in Dkk3 treatment group than in control group (Figure1j and l). These results further confirmed the chemotactic role of Dkk3 in resident Sca-1+ VPCs with an efficiency comparable to Sdf-1α.

CXCR7 Is Involved In Dkk3-Driven Migration Of Sca-1+ Cells

The similarity of action between Dkk3 and Sdf-1α prompted us to investigate if the receptors of Sdf-1α, CXCR4 and CXCR7, could participate in Dkk3-mediated migration of Sca-1+ cells. Flow cytometry analysis revealed that most of Sca-1+ cells expressed CXCR7 (77.9%±6.2%), whereas few Sca-1+ cells displayed CXCR4 on their surface (0.9%±0.2%) (Figure 2a and b). Few cells were positive for both receptors (1.1%±0.2%) and all the cells expressing CXCR4 also expressed CXCR7. Western blot analysis further confirmed the high expression of CXCR7 in Sca-1+ cells (Figure 2c). Noticeably, contrary to Sca-1+ cells, SMCs and ECs significantly expressed both CXCR4 and CXCR7. We speculate that CXCR7 may potentially be a vascular stem cell receptor, which is supported by previous studies that report a high expression of CXCR7 in progenitor cells[31-33]. To assess the potential involvement of CXCR7 in Dkk3- and Sdf-1α-driven Sca-1+ cell migration, we performed transwell migration assays on progenitors following efficient knockdown of CXCR7 by siRNA targeting. Downregulation of CXCR7 was confirmed by real time qPCR (Figure 2d) and western blot (Figure 2e) analysis. CXCR7 downregulation induced strong decrease in Dkk3-driven Sca-1+ cell migration (Figure 2f and g), comparable to the decrease observed in Sdf-1α-driven cell migration (Figure 2h and i). Our findings proved that CXCR7 was involved in Sca-1+ cell migration induced by Dkk3. Due to the low expression of CXCR4, a different strategy was employed to inhibit its functional activity. Migration assays were performed using CXCR4 inhibitor AMD3100, at three different concentrations: 10, 25 and 50 μM (Online Figure IIa-b). Inhibition of CXCR4 had no effect on the migration rate of Sca-1+ cells in the presence of either Dkk3 or Sdf-1α. This suggested that CXCR4 receptor was not implicated in either Dkk3- or Sdf-1α-mediated migration of Sca-1+ cells.

CXCR7 Is A High-Affinity Binding Receptor Of Dkk3

To verify the physical interaction between Dkk3 and CXCR7 we performed a co-immunoprecipitation assay. CXCR7 from lysates of Sca-1+ cells treated with Dkk3 was pulled down using anti-CXCR7 antibody and co-precipitated with Dkk3, as revealed by Western blot, with a corresponding band displayed in the input sample and not when an IgG control antibody was used (Figure 3a). We also conducted a reverse pull-down assay, in which Dkk3-His tagged was pulled-down from Sca-1+ cell lysate and eluted with any bound CXCR7 (Figure 3b). Noticeably, contrarily to CXCR7, CXCR4 was not pulled-down with Dkk3. To measure the affinity of the interaction between Dkk3 and CXCR7, saturation binding experiments were performed as previously described[22, 23]. The measurement of Sdf-1α binding affinity to CXCR4 and CXCR7 was used to validate the design of the experiment. Dkk3 and Sdf-1α were conjugated with alkaline phosphatase (AP) and the efficiency of AP conjugation was confirmed by determining the AP activity following serial dilutions (Online

Figure IIIa-b). Next, CXCR7 and CXCR4 receptors (HA-tagged) were overexpressed in HEK 293T cells (Figure 3c-d and Online Figure IIc-d) and the corresponding cell lysates were loaded onto the wells of high-affinity binding ELISA plates previously coated with anti-HA antibody. Sdf-1α-AP bound with high affinity to CXCR4 and CXCR7, as characteristic hyperbolic curves were obtained for each receptor (Figure 3e). The dissociation constant (Kd) for CXCR4 (10.05 nM) was comparable to that calculated for CXCR7 (4.93 nM), indicating comparable strength of affinity for Sdf-1α. Dkk3-AP also bound with high affinity to CXCR7, which was represented by the typical hyperbolic binding curve and a Kd of 14.14 nM (Figure 3f). The binding curve observed for CXCR4 was also indicative of absence of Dkk3 binding. To rule out the possibility that AP itself could bind to CXCR7, a serial dilution of AP alone was performed. The results showed only a residual binding (Figure 3e-f).As Kremen1 and Kremen2 were identified as functional receptors for members of the Dkk family[22], we also assessed their binding affinity to Dkk3. Sca-1+ VPCs express Kremen1, but display a low expression of Kremen2 (Online Figure IVa-b). In addition, Kremen1 was neither pulled-down with Dkk3-His-tagged (Online Figure IVc), nor it co-immunoprecipitated with CXCR7 (Online Figure IVd), in Sca-1+ VPCs. In the saturation binding assay, ambiguous binding curves were obtained for Kremen1 and Kremen2, suggesting that Dkk3 could not bind to these receptors (Figure 3e). . Kremen1 downregulation by SiRNA transfection also did not affect significantly Dkk3-induced migration of Sca-1+ cells (Online Figure IVe-g). Finally, Dkk3 also increased the migration rate of CXCR7-overexpressing HEK cells in comparison with the non-treated control group (Figure 3g-h), whereas overexpression of Kremen1 and Kremen2 did not modify Dkk3-mediated HEK cell migration (Online Figure IVh-m). Treatment with Sdf-1α also resulted in an increase of the migration rate of CXCR7 overexpressing cells (Figure 3i-j). Not surprisingly, Sdf-1α induced the migration of cells overexpressing CXCR4, when compared to non-treated control group (Online Figure IIg-h). However, Dkk3 treatment had no effect on the migration rate of CXCR4 overexpressing cells (Online Figure IIe-f). We thus concluded that Dkk3 not only binds with high affinity to CXCR7, but also that CXCR7 is a functional receptor for Dkk3.

ERK1/2 and PI3K/AKT Signalling Pathways Act Downstream Of Sca-1+ Cell Migration

MAPK kinases (such as ERK1/2) and PI3K/AKT pathways are classical signalling cascades involved in cell migration triggered by the activation of chemokine receptors[34, 35]. Having established the chemotactic role of Dkk3 and its effective binding to chemokine receptor CXCR7, we next sought to investigate whether these pathways were involved in Sca-1+ cell migration induced by Dkk3 binding to CXCR7. Changes in the phosphorylation level of ERK1/2 and AKT were examined by Western blot after 0 to 15 minutes of Dkk3 stimulation. Dkk3 treatment induced ERK1/2 and AKT phosphorylation (Figure 4a and b). Stimulation with Sdf-1α also increased ERK1/2 and AKT phosphorylation (Online Figure Va and c). Sdf-1α-mediated ERK1/2 and AKT phosphorylation and consequent induction of cell migration have been shown before in previous studies[36-38]. We provide solid evidence of the implication of ERK1/2 and PI3K/AKT pathways in the mechanisms of cell migration promoted by Dkk3. Treatment with PD98059 and LY294002 inhibited, respectively, ERK1/2 and AKT phosphorylation induced by Dkk3 (Figure 4e and f) and Dkk3-induced cell migration (Figure 4g and h). Specific AKT inhibitor (AKT inhibitor X) also decreased Dkk3-driven migration of Sca-1+ cells (Figure 4i). Furthermore, knockdown of Akt and Erk pathways by SiRNA transfection (Online Figure6). Altogether, our data showed that ERK1/2 and PI3K/AKT signalling pathways were required for Sca-1+ cell migration induced by Dkk3.

Interestingly, Sdf-1α-mediated migration of Sca-1+ cells shared the same signalling mechanisms (Online Figure Va-e). Furthermore, CXCR7 siRNA mediated downregulation in Sca-1+ cells led to a decrease in Dkk3-driven ERK1/2 and AKT phosphorylation (Figure 4c and d), which showed that CXCR7 activation by Dkk3 acts upstream these signalling pathways.

Rac1 and Rhoa GTPases Activation is Required

Rho GTPases are well known for their role in cytoskeleton rearrangement and cell migration. Quantification of the active GTP-bound Rac1 and RhoA forms in Sca-1+ cells was assessed by performing G-LISA activation assay at early time points of Dkk3 stimulation (0-30 minutes) and showed that Dkk3 triggered the activation of Rac1 and RhoA (Figure 5a and e). Transwell migration assays were performed in the presence of Rac1 activation inhibitor NSC23766. NSC23766 efficiently inhibited Rac1 activation (Figure 5c) and resulted in a decrease in the migration rate of Sca-1+ cells induced by Dkk3 (Figure 5d). Treatment of Sca-1+ cells with RhoA inhibitor Rhosin blocked Dkk3-induced RhoA activation (Figure 5g) and abolished Dkk3-mediated Sca-1+ cell migration (Figure 5h). SiRNA mediated knockdown of CXCR7 in the cells abolished the Dkk3-triggered activation of Rac1 and RhoA (Figure 5b and f), which reveals that Rho GTPases activation induced by Dkk3 requires Dkk3 binding to CXCR7. The phosphorylation level of myosin light chain (MLC), a downstream effector of RhoA, in response to Dkk3 stimulation was also assessed. In the presence of Dkk3, MLC phosphorylation at early time points was increased (Figure 5i). Rho associated kinases (ROCK) are activated by RhoA and can induce phosphorylation of MLC. Therefore, we investigated whether ROCK inhibitor Y27632 could repress MLC phosphorylation. Y27632, not only reduced Dkk3-triggered phosphorylation of MLC (Figure 5j), but also decreased the migratory ability of Sca-1+ cells stimulated by Dkk3 (Figure 5k). Finally, as RhoA can also directly phosphorylate MLC, we examined if Rhosin could affect MLC activation. Inhibition of RhoA with Rhosin reduced the Dkk3-triggered phosphorylation of MLC (Figure 5j). Collectively, these results showed that Rac1 and RhoA GTPases are involved in Sca-1+ cell migration mediated by CXCR7 activation induced by Dkk3 binding. Finally, to investigate whether Rac1 and ERK1/2 signalling pathways were interacting with each other, we measured the Dkk3-driven phosphorylation level of ERK1/2 in the presence of Rac1 inhibitor, and the Dkk3-triggered activation of Rac1 upon treatment with ERK inhibitor. Dkk3-induced phosphorylation of ERK1/2 was supressed in response to NSC23766 treatment (Online Figure VIIa). Surprisingly, Rac1 activation triggered by Dkk3 was also repressed upon treatment with PD98059 (Online Figure VIIb). These results indicated a possible feedback mechanism between ERK1/2 and Rac1. In contrast, Rac1 and RhoA activation stimulated by Dkk3 was not affected upon treatment with PI3K/AKT inhibitor LY294002 (Online Figure VIIc-d). Interestingly, treatment with Sdf-1α also promoted the activation of Rac1 and RhoA and phosphorylation of MLC (Online Figure Vf-h).

As Dkk proteins have been associated with the Wnt signalling pathway[39, 40], we assessed whether this pathway was implicated in Dkk3-driven cell migration. Dkk3 stimulation had no effect in the expression of β-catenin, Axin-2 and Tcf1 (Online Figure VIIIa-d). Furthermore, 24 hours of Dkk3 stimulation did not activate the transcription of Wnt target genes, given that the luciferase activity did not modify in the presence of Dkk3 (Online Figure VIIIe). Treatment of Sca-1+ cells with FH535, a specific inhibitor of the canonical Wnt/β-Catenin signalling transduction pathway, resulted in an increase of cell migration, comparable to the increase

obtained when the cells were treated with Dkk3 (Online Figure VIIIf). However, when cells were treated with Dkk3 combined with FH535, no cumulative effect and difference were found in the cell migration rate between this group and the group treated only with Dkk3. Inhibition of Dishevelled protein, a critical component of both canonical and non-canonical Wnt pathways, with Peptide Pen-N3 (Dvl-PDZ), also did not affect Dkk3-triggered migration of Sca-1+ cells (Online Figure VIIIg), as no difference was found between Dkk3-treated group and the group treated with Dkk3 combined with the Peptide Pen-N3. Therefore, we concluded that the Wnt signalling pathway is not involved in Sca-1+ cell migration induced by Dkk3.

Characterisation and Patency of Dkk3-Loaded Tissue Engineered Vessel Grafts (TEVG) In Rat Model

To evaluate the role of Dkk3 in cell migration *in vivo*, TEVGs were evaluated by rat abdominal aorta replacement model (Online Figure IXa left top). Dkk3-loaded TEVGs (2.0 mm in diameter and 500 μm in wall thickness) were fabricated by co-electrospinning technique, then implanted into the abdominal artery of rats and examined at different time points. Scanning electronic microscopy showed homogeneous fibrous structure of the grafts (Online Figure X), consisting of PCL microfibers (~6 μm) and collagen nanofibers (~600 nm). PCL microfibers provided good mechanical support and macroporous structure, while collagen nanofibers introduced a biocompatibility and acted as carriers for Dkk3. With the degradation of the collagen fibers, Dkk3 was released from the grafts in a sustained manner and no burst release was observed during the detection period (Online Figure XI). Unloaded grafts were used as controls for comparison to determine the effect of loaded Dkk3. Ultrasound analysis at 2 weeks, 1 and 3 months (Online Figure IXa right) indicated that most of the implanted grafts were patent and presented sustained blood flow, without aneurysm, bleeding, or occlusion (Online Figure IXc). Some capillaries could be observed from the adventitia of the implanted grafts, which reflects their integration with the surrounding tissues (Online Figure IXa left bottom). Micro-CT revealed that no detectable calcification occurred in both groups at 3 months post-implantation (Online Figure IXb).

Delivery of Dkk3 in Tissue TEGVs Enhances Vascular Regeneration

We then investigated whether Dkk3 could promote vascular cellularisation and regeneration of the vascular grafts. Haematoxylin & Eosin staining showed that the luminal diameter was markedly preserved in patent grafts without narrowing caused by intimal hyperplasia 3 months post-implantation (Figure 6a and Online Figure XII). Moreover, no significant difference in the inner diameter could be seen between Dkk3 and control groups at each time point (Figure 6b). DAPI staining revealed sufficient cell infiltration into the graft wall after 2 weeks, which is crucial for the following vascular regeneration (Online Figure XIII)[3]. After 3 months implantation, electron microscopy images throughout the vessel graft revealed that grafts loaded with Dkk3 were covered by a nearly confluent layer of neo-tissue, uniformly aligned along the blood flow, with a more homogeneous organization, when compared to control group (Figure 6c).

The effect of Dkk3 on vascular smooth muscle regeneration was evaluated by immunofluorescence staining using α-SMA and SM-MHC antibodies, and subsequent measurement of the total area of α-SMA+ and SM-MHC+ cells in the luminal region in control and treated grafts. 2 weeks post-implantation, Dkk3 treated grafts showed a significantly greater SMA+ area within the lumen region compared to the untreated grafts,

but no difference could be measured anymore between the two groups one-month post-implantation, indicating that Dkk3 promoted the recruitment of α-SMA+ cells in the graft at an early stage (Figure 6d-e). Interestingly, at later stages, i.e. 1 ($p<0.01$) and 3 ($p<0.005$) months post-implantation, the SM-MHC+ area within the lumen region was significantly higher in the Dkk3 group in comparison with the control group (Figure 6d and f). These results demonstrated that Dkk3 promoted vascular regeneration by increasing the proportion of mature and functional SMCs, while reducing the over-proliferation of SMCs that may lead to adverse intimal hyperplasia. Dkk3-TEVGs exhibited increased expression of SM-MHC and Calponin and a reduced expression of α-SMA, in comparison to control-TEVGs (Online Figure XIVa). Double immunofluorescence staining of α-SMA and SM-MHC further demonstrated that Dkk3 induced the transition of SMCs toward a contractile phenotype, exhibiting the typical spindle-shaped morphology (Online Figure XIVb).

Graft endothelization was analysed by immunofluorescence staining with vWF antibody. Endothelial cells were first observed at 2 weeks post-implantation in both groups. After 3 months of implantation, the vWF+ cell layer in the Dkk3 group appeared more uniform and continuous, characteristics of a more mature endothelium composed of a thin single sheet of ECs (Online Figure XV). These results are consistent with a recent study published by our group, which shows that Dkk3 accelerated re-endothelization in a femoral artery wire injury mouse model, confirming the role of Dkk3 on vascular endothelium integrity and function[19]. Furthermore, double immunofluorescence staining of vWF and α-SMA also revealed a better tissue organisation in Dkk3 group compared to the control group, as shown by the several layers of SMCs strategically aligned beneath a uniform layer of neo-endothelium (Online Figure XVI). Concomitantly, the detection of extracellular matrix proteins, including elastin and collagen by Masson and Verhoeff-Van Gieson staining, showed a significant increase in the deposition of extracellular matrix content in the Dkk3 group at 1 and 3 months post-implantation (Online Figure XVII). Taken together, these results show that Dkk3 enhances TEVG cellularisation and remodelling, important processes involved in blood vessel maturation.

Dkk3 Increases Sca-1+ Progenitor Cell Migration Through CXCR7 Activation And Contributes To Vascular Smooth Muscle Regeneration

Next, the specific effect of Dkk3 on Sca-1+ progenitor cells was further evaluated in our graft model, and the result indicated that incorporation of Dkk3 in the TEVGs remarkably enhanced the infiltration and maintenance of Sca-1+ stem/progenitor cells within the graft wall (Figure 7b-c). Furthermore, double immunofluorescence staining of α-SMA and Sca-1 (Figure 7d) showed a greater number of double positive cells in Dkk3-loaded TEVGs compared with control group, indicating that some mobilized Sca-1+ cells could directly differentiate into SMCs and participate in the regeneration of vascular smooth muscle. To confirm the role of CXCR7 receptor in the Dkk3-driven migration of Sca-1+ progenitor cells, double-layered vascular grafts were designed and evaluated in *in vivo* models. In detail, in addition to the Dkk3-loaded inner layer, a CXCR7 antibody-loaded layer was introduced at the outside of the graft (Figure 7a). Due to the antagonistic effect provided by the CXCR7 antibody, the number of infiltrated Sca-1+ cells was dramatically reduced (Figure 7b-c), confirming that the chemotactic role of Dkk3 is dependent on CXCR7 activation. Furthermore, blocking of CXCR7 decreased the Dkk3-induced number of cells double positive for Sca-1+ and α-SMA cells infiltrated into the grafts at 2-weeks and 1-month post-

implantation (Figure 7d). The effect of CXCR7 blocking in the grafts was also assessed for ECs, SMCs and macrophages. The increased α-SMA+ area in Dkk3-TEVGs was reduced upon CXCR7 blocking at 2-weeks and 1-month post-implantation (Online Figure XVIIIa-b). Likewise, SM-MHC+ area was also decreased in Dkk3-TEVGs when CXCR7 was blocked (Online Figure XVIIIa and c). Regarding the ECs, at 1-month post-implantation, although no difference was observed between control and Dkk3 groups in the endothelial coverage of the grafts, CXCR7 blocking decreased the graft endothelial coverage in the Dkk3-TEVGs (Online Figure XIX). Finally, no difference was detected in the recruitment of macrophages between control and Dkk3 groups or when CXCR7 blocked, at 1-month post-implantation (Online Fig XX). Interestingly, at 1-month post-implantation, Dkk3 does not change *in vivo* the proliferation rate of ECs, SMCs and macrophages (Online Figure XXI). Sdf-1α-TEVGs were also fabricated to further assess if CXCR7 acts as a regulator of regeneration. At 2-weeks post-implantation, similarly to Dkk3, Sdf-1α also induced the recruitment of Sca-1+ VPCs into the grafts, which was reduced upon CXCR7 blocking (Online Figure XXII). Interestingly, macrophage recruitment was higher in Sdf-1α-TEVGs than in control-TEVGs, and CXCR7 blocking decreased the Sdf-1α-induced migration of macrophages. On the other hand, no differences were observed between control and Sdf-1α groups in the recruitment of SMCs and in the endothelial coverage of the grafts. These results confirm that Dkk3-CXCR7 axis is crucial in mediating Sca-1+ cell migration and graft cellularisation and regeneration *in vivo*.

Discussion

Previous studies have reported that Sca-1+ progenitor cells are able to migrate and differentiate into a variety of cells and participate in vascular repairing and remodelling[13, 28, 29, 41]. In this study, we demonstrated that Dkk3 induces chemotaxis for Sca-1+ stem/progenitor cells via binding specifically to CXCR7 resulting in better regeneration of tissue engineered vessel grafts. These conclusions are based on the following observations. Firstly, Dkk3 can induce vascular progenitor migration *in vitro* in both transwell and wound healing assays and *ex vivo* in organ 3D culture assays using Sca-1-GFP aortic rings. Secondly, Dkk3 physically interacts with CXCR7 as indicated by co-immunoprecipitation and His-tag pull-down analysis, ligand-receptor affinity binding assays, artificial CXCR7-expressing system chemotaxis assay and receptor blocking assays. Thirdly, downstream signalling pathways mediated by Dkk3-CXCR7 binding were identified and confirmed using chemical inhibition and functional migration assays. Finally, Dkk3-loaded engineered vessel grafts displayed enhanced cellularization *in vivo* that could be blocked by application of anti-CXCR7 antibodies. Thus, the discovery of a new functional role for Dkk3 and the identification of its functional receptor could provide the basic information for the development of new drugs for vascular disease.

Chemokines are a class of cytokines that direct cell migration by binding to their corresponding chemokine receptor present on target cell types. The most important question is whether the chemotaxis of stem/progenitor cells is activated via binding of Dkk3 to a chemokine receptor. The chemokine receptors profile on Sca-1+ cells has been previously investigated through a systematic PCR array analysis, and the result showed that CXCR7

expression was remarkably higher than the expression of all the other receptors[14]. Because CXCR7 is a receptor of Sdf-1α (a well-known chemokine with an established role in inducing chemotaxis of various cell types, including SMCs, ECs and Sca-1+ VPCs[28, 29, 42, 43]), we investigated whether CXCR7 could be the receptor of Dkk3 considering the similarity in the chemotactic ability between Dkk3 and Sdf-1α. In line with published data, our results confirmed that most Sca-1+ cells considerably expressed CXCR7, while the expression of the other receptors of Sdf-1α, such as CXCR4, was significantly low. Interestingly, as reported for Dkk3, CXCR7 expression is also upregulated in several cancers and tumour associated vasculature, which confers a pro-angiogenic role to CXCR7[44, 45]. Originally, CXCR7 was recognized as a mere scavenger receptor with the function to generate gradients of Sdf-1α[46, 47]. More recently, it is accepted as a functional receptor directly involved in cellular responses, including cell migration[33, 36, 48-50]. In this study, knockdown of CXCR7 abrogated Dkk3-driven migration of progenitors, whereas CXCR7 overexpression increased Dkk3-mediated cell migration. Our data revealed that CXCR4 inhibition had no effect in Dkk3- or Sdf-1α-mediated migration of Sca-1+ cells, which indicated that CXCR4 was not implicated in this process. These results were consistent with previous studies which showed that CXCR4 antagonism with AMD3100 did not always inhibit Sdf-1α-induced migration, as the chemokine acted via CXCR7[50, 51]. Collectively, our results demonstrate that CXCR7 is involved in Dkk3-driven migration of Sca-1+ cells.

Our data demonstrated that Dkk3 binds to CXCR7 with high affinity and in a specific, dose dependent and saturable manner. The strength of their binding affinity was comparable to the interactions between Sdf-1α and CXCR7 or CXCR4, which were consistent with the reported data[49, 52]. Remarkably, our results are also in accordance with the findings described by Mao et al, that, contrarily to Dkk1 and Dkk2, Dkk3 does not bind to Kremen1 and Kremen2[22, 24]. Our findings provide the direct evidence of physical binding of Dkk3 to CXCR7, which may serve as a Dkk3 receptor.

Among the multiple molecular mechanisms that regulate cell migration, we found that ERK1/2, PI3K/AKT, Rac1 and RhoA signalling cascades were involved in Sca-1+ VPCs migration induced by Dkk3. Dkk proteins are classically associated with the Wnt signalling pathway, which comprises the canonical (β-catenin dependent) and the non-canonical (β-Catenin independent) pathways[39, 40]. Unlike the other members of the Dickkopf family which are Wnt/β-Catenin pathway antagonists[39, 40], Dkk3, according to specific cell context, can act either as an agonist or inhibitor of the canonical Wnt signalling[25, 53], or even exerts its effect independently of the Wnt pathway[19, 24]. Our findings show that none of the Wnt signalling pathways is implicated in the migration mechanism of Sca-1+ cells stimulated by Dkk3. Noticeably, Sdf-1α induced the same migration molecular mechanisms. This result, allied with the fact that Dkk3 is the most divergent member of the Dicckopf family by several criteria[40, 54], further supported our hypothesis of the chemotactic role of Dkk3 in stem/progenitor cells through CXCR7 activation. Finally, the molecular mechanisms involved in Dkk3-driven Sca-1+ cell migration are summarized in Figure 8.

Encouraged by the chemotactic properties of Dkk3 demonstrated from *in vitro* and *ex vivo* assays, we further assessed the role of Dkk3 in the migration of Sca-1+ progenitor cells *in vivo* and evaluated its potential to harness endogenous vascular regeneration by using a tissue engineered vessel graft model. Dkk3-loaded tissue engineered grafts effectively promoted the infiltration of Sca-1+ progenitor cells into the grafts, which was abrogated after

the incorporation of CXCR7 antibody. Consistent with our results, studies have reported that Sdf-1α-loaded vascular grafts display an increased recruitment of endogenous progenitor cells and accelerate vascular regeneration. Additionally, these cells expressed CXCR7 and appeared to be progenitors of endothelial and smooth muscle cells[2]. Our results also confirm the similarity between Dkk3 and Sdf-1α in terms of chemotactic function, and establish the Dkk3-CXCR7 axis as regulator of regeneration.

An intact endothelial cell monolayer and the presence of mature SMCs in the media is vital in preventing thrombogenesis and maintaining long-term patency of vessel grafts[55]. The ability of Dkk3 to promote re-endothelialisation has been demonstrated in different models[19, 56, 57]. Our study revealed that DKK3 promoted endothelialisation in TEVGs, which exhibited a more uniform and organized alignment of endothelial cells along the blood flow. Vascular cellularisation and remodelling, which relies on the generation of a functional tunica media, is a critical aspect in TEVGs. Previous studies have demonstrated that Sca-1+ stem/progenitor cells can differentiate into SMCs under the stimuli of various cytokines[13, 28, 29, 41, 58], including Dkk3[16, 17, 59]. In this study, Dkk3-loaded TEVGs exhibited a greater number of Sca-1+ cells, which co-expressed α-SMA. *In vivo* blocking of CXCR7 lead to a decreased number of SMCs present in the TEVGs, which may be due to the preceding reduction in the recruitment of VPCs into the TEVGs, that have the ability to migrate and differentiate into SMCs promoted by Dkk3 stimulation. More importantly, the SMC phenotype in Dkk3-TEVGs switched to a more mature and contractile phenotype in comparison to control TEVGs. Recently, we published a study proving the ability of Dkk3 to induce the differentiation of Sca-1+ VPCs into SMCs[59].The neo-vessel of Dkk3-loaded TEVGs exhibited a better tissue organization in structure with several SMC layers aligned beneath the endothelium, thus resembling that of native arteries. Therefore, our data show that Dkk3, through CXCR7, promotes better restructuration of the TEVGs and communication between the endothelial and SMC layers, which potentially contributes to a better endothelization of the vessel. Additionally, Dkk3 release over time increased the production of extracellular matrix, which is essential to provide the required mechanical strength to withstand the arterial pressure. The deposition of elastin and collagen was associated with the maturation of the smooth muscle layer, which supported the role of Dkk3 in promoting vascular graft arterialisation. These findings are consistent with a recent study which reports that Dkk3, not only induces the differentiation of Sca-1+ VPCs into SMCs, but also increases the content in SMCs and extracellular matrix deposition in atherosclerotic plaques, thus having a plaque stabilizing effect[59]. The functionality of the TEVGs has been assessed previously[60]. After 12 months implantation, our TEVGs grafts contracted and relaxed appropriately in response to the respective stimulants, thus proving that regenerated contractile SMCs were present in the grafts, which displayed a good vasoactivity, although still not at the level of the native vessel because of the slow degradation of the graft material. Furthermore, at 12-months implantation, all the grafts were patent, with absence of calcification, aneurism, stenosis, or harmful remodeling, and exhibited rapid endothelization. Considering the ability of Dkk3 to induce an increase in the mature SMC pool and extracellular matrix deposition in the grafts when compared to control, we would speculate that the functionality and regeneration of the Dkk3-TEVGs would be at least similar to or better than the control-TEVGs. Nevertheless, further investigation is required to clarify this issue.

The precise origin of the Sca-1+ progenitor cells present in the Dkk3-TEVGs is still under investigation. Published studies indicate that the Sca1+ cells could originate from two sources, which are local resident and circulating progenitor cells[8, 13, 41, 61, 62]. For the current study, we speculate that both sources may contribute to the pool of Sca-1+ cells in the Dkk3-TEVGs. We have shown in another study that a large number of cells infiltrating our TEVGs derives from the surrounding native tissue, as the infiltrating cells migrate from the outer layers of the TEVGs to the inner layers[3]. The use of Sca-1 knock-in inducible Cre animal models would help us clarify the fate and progeny of Sca-1+ VPCs. Rigorous lineage-tracing methodology may further elucidate the role of Dkk3 in the migration, proliferation and differentiation of Sca-1+ progenitor cells.

In summary, our work shows for the first time that Dkk3 plays a chemotactic role in vascular progenitor cells through binding to the chemokine receptor CXCR7 and activating relevant downstream molecular mechanisms. Furthermore, the *in vivo* chemotactic property of Dkk3 as well as the potential to promote *in vivo* vascular graft arterialisation have been verified in an animal model. Our findings herein presented provide evidence of the potential of Dkk3 and its receptor CXCR7 as targets for vascular drug targeted therapy strategies and regenerative medicine.

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Disclosures

None.

References

1. L'Heureux N, Dusserre N, Marini A, Garrido S, de la Fuente L, McAllister T. Technology insight: the evolution of tissue-engineered vascular grafts--from research to clinical practice. *Nat Clin Pract Cardiovasc Med.* 2007;4:389-395.
2. Yu J, Wang A, Tang Z, Henry J, Li-Ping Lee B, Zhu Y, Yuan F, Huang F, Li S. The effect of stromal cell-derived factor-1alpha/heparin coating of biodegradable vascular grafts on the recruitment of both endothelial and smooth muscle progenitor cells for accelerated regeneration. *Biomaterials.* 2012;33:8062-8074.
3. Wang Z, Cui Y, Wang J, Yang X, Wu Y, Wang K, Gao X, Li D, Li Y, Zheng XL, Zhu Y, Kong D, Zhao Q. The effect of thick fibers and large pores of electrospun poly(epsilon-caprolactone) vascular grafts on macrophage polarization and arterial regeneration. *Biomaterials.* 2014;35:5700-5710.
4. Ravi S, Chaikof EL. Biomaterials for vascular tissue engineering. *Regen Med.* 2010;5:107-120.
5. Thottappillil N, Nair PD. Scaffolds in vascular regeneration: current status. *Vasc Health Risk Manag.* 2015;11:79-91.
6. Briasoulis A, Tousoulis D, Antoniades C, Papageorgiou N, Stefanadis C. The role of endothelial progenitor cells in vascular repair after arterial injury and atherosclerotic plaque development. *Cardiovasc Ther.* 2011;29:125-139.
7. Xu Q, Zhang Z, Davison F, Hu Y. Circulating progenitor cells regenerate endothelium of vein graft atherosclerosis, which is diminished in ApoE-deficient mice. *Circ Res.* 2003;93:e76-86.
8. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med.* 2002;8:403-409.
9. Saiura A, Sata M, Hirata Y, Nagai R, Makuuchi M. Circulating smooth muscle progenitor cells contribute to atherosclerosis. *Nat Med.* 2001;7:382-383.
10. Torsney E, Xu Q. Resident vascular progenitor cells. *Journal of Molecular and Cellular Cardiology.* 2011;50:304-311.
11. Majesky MW, Dong XR, Hoglund V, Mahoney WM, Jr., Daum G. The adventitia: a dynamic interface containing resident progenitor cells. *Arterioscler Thromb Vasc Biol.* 2011;31:1530-1539.
12. Xie Y, Fan Y, Xu Q. Vascular Regeneration by Stem/Progenitor Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 2016;36:e33-e40.
13. 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. *Journal of Clinical Investigation.* 2004;113:1258-1265.
14. Yu B, Wong MM, Potter CM, Simpson RM, Karamariti E, Zhang Z, Zeng L, Warren D, Hu Y, Wang W, Xu Q. Vascular Stem/Progenitor Cell Migration Induced by Smooth Muscle Cell-Derived Chemokine (C-C Motif) Ligand 2 and Chemokine (C-X-C motif) Ligand 1 Contributes to Neointima Formation. *Stem Cells.* 2016;34:2368-2380.
15. Majesky MW, Horita H, Ostriker A, Lu S, Regan JN, Bagchi AK, Dong XR, Poczobutt JM, Nemenoff RA, Weiser-Evans MC. Differentiated Smooth Muscle Cells Generate a Subpopulation of Resident Vascular Progenitor Cells in the Adventitia Regulated by KLF4. *Circulation Research.* 2016.
16. 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.
17. 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.

18. Medinger M, Tzankov A, Kern J, Pircher A, Hermann M, Ott HW, Gastl G, Untergasser G, Gunsilius E. Increased Dkk3 protein expression in platelets and megakaryocytes of patients with myeloproliferative neoplasms. *Thromb Haemost.* 2011;105:72-80.
19. Yu B, Kiechl S, Qi D, et al. A Cytokine-like Protein DKK3 Is Atheroprotective. *Circulation.* 2017;136:1022-1036.
20. Zhang Y, Liu Y, Zhu XH, et al. Dickkopf-3 attenuates pressure overload-induced cardiac remodelling. *Cardiovasc Res.* 2014;102:35-45.
21. Bao MW, Cai Z, Zhang XJ, Li L, Liu X, Wan N, Hu G, Wan F, Zhang R, Zhu X, Xia H, Li H. Dickkopf-3 protects against cardiac dysfunction and ventricular remodelling following myocardial infarction. *Basic Res Cardiol.* 2015;110:25.
22. Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stannek P, Walter C, Glinka A, Niehrs C. Kremen proteins are Dickkopf receptors that regulate Wnt/[beta]-catenin signalling. *Nature.* 2002;417:664-667.
23. Mao B, Wu W, Li Y, Hoppe D, Stannek P, Glinka A, Niehrs C. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature.* 2001;411:321-325.
24. Mao B, Niehrs C. Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. *Gene.* 2003;302:179-183.
25. Nakamura RE, Hackam AS. Analysis of Dickkopf3 interactions with Wnt signaling receptors. *Growth factors.* 2010;28:232-242.
26. Fujii Y, Hoshino T, Kumon H. Molecular simulation analysis of the structure complex of C2 domains of DKK family members and beta-propeller domains of LRP5/6: explaining why DKK3 does not bind to LRP5/6. *Acta Med Okayama.* 2014;68:63-78.
27. Poorebrahim M, Sadeghi S, Rahimi H, Karimipoor M, Azadmanesh K, Mazlomi MA, Teimoori-Toolabi L. Rational design of DKK3 structure-based small peptides as antagonists of Wnt signaling pathway and in silico evaluation of their efficiency. *PLoS One.* 2017;12:e0172217.
28. Wong MM, Winkler B, Karamariti E, Wang X, Yu B, Simpson R, Chen T, Margariti A, Xu Q. Sirolimus stimulates vascular stem/progenitor cell migration and differentiation into smooth muscle cells via epidermal growth factor receptor/extracellular signal-regulated kinase/beta-catenin signaling pathway. *Arterioscler Thromb Vasc Biol.* 2013;33:2397-2406.
29. Chen Y, Wong MM, Campagnolo P, Simpson R, Winkler B, Margariti A, Hu Y, Xu Q. Adventitial stem cells in vein grafts display multilineage potential that contributes to neointimal formation. *Arterioscler Thromb Vasc Biol.* 2013;33:1844-1851.
30. Kokkinopoulos I, Wong MM, Potter CMF, et al. Adventitial SCA-1+ Progenitor Cell Gene Sequencing Reveals the Mechanisms of Cell Migration in Response to Hyperlipidemia. *Stem Cell Reports.* 2017;9:681-696.
31. Dai X, Tan Y, Cai S, Xiong X, Wang L, Ye Q, Yan X, Ma K, Cai L. The role of CXCR7 on the adhesion, proliferation and angiogenesis of endothelial progenitor cells. *J Cell Mol Med.* 2011;15:1299-1309.
32. Zhang XY, Su C, Cao Z, Xu SY, Xia WH, Xie WL, Chen L, Yu BB, Zhang B, Wang Y, Tao J. CXCR7 upregulation is required for early endothelial progenitor cell-mediated endothelial repair in patients with hypertension. *Hypertension.* 2014;63:383-389.
33. Dai X, Yan X, Zeng J, et al. Elevating CXCR7 Improves Angiogenic Function of EPCs via Akt/GSK-3β/Fyn-Mediated Nrf2 Activation in Diabetic Limb Ischemia. *Circulation Research.* 2017;120:e7-e23.
34. Curnock AP, Logan MK, Ward SG. Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases. *Immunology.* 2002;105:125-136.
35. Huang C, Jacobson K, Schaller MD. MAP kinases and cell migration. *J Cell Sci.* 2004;117:4619-4628.
36. Chen D, Xia Y, Zuo K, Wang Y, Zhang S, Kuang D, Duan Y, Zhao X, Wang G. Crosstalk between SDF-1/CXCR4 and SDF-1/CXCR7 in cardiac stem cell migration. *Sci Rep.* 2015;5:16813.

37. Zheng H, Fu G, Dai T, Huang H. Migration of endothelial progenitor cells mediated by stromal cell-derived factor-1alpha/CXCR4 via PI3K/Akt/eNOS signal transduction pathway. *J Cardiovasc Pharmacol.* 2007;50:274-280.
38. Ryu CH, Park SA, Kim SM, Lim JY, Jeong CH, Jun JA, Oh JH, Park SH, Oh WI, Jeun SS. Migration of human umbilical cord blood mesenchymal stem cells mediated by stromal cell-derived factor-1/CXCR4 axis via Akt, ERK, and p38 signal transduction pathways. *Biochem Biophys Res Commun.* 2010;398:105-110.
39. Bao J, Zheng JJ, Wu D. The Structural Basis of DKK-Mediated Inhibition of Wnt/LRP Signaling. *Science Signaling.* 2012;5:pe22.
40. Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene.* 2006;25:7469-7481.
41. Passman JN, Dong XR, Wu SP, Maguire CT, Hogan KA, Bautch VL, Majesky MW. A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. *Proc Natl Acad Sci U S A.* 2008;105:9349-9354.
42. Thomas MN, Kalnins A, Andrassy M, et al. SDF-1/CXCR4/CXCR7 is pivotal for vascular smooth muscle cell proliferation and chronic allograft vasculopathy. *Transpl Int.* 2015;28:1426-1435.
43. Ho TK, Shiwen X, Abraham D, Tsui J, Baker D. Stromal-Cell-Derived Factor-1 (SDF-1)/CXCL12 as Potential Target of Therapeutic Angiogenesis in Critical Leg Ischaemia. *Cardiology Research and Practice.* 2012;2012:7.
44. Ma D-M, Luo D-X, Zhang J. SDF-1/CXCR7 axis regulates the proliferation, invasion, adhesion, and angiogenesis of gastric cancer cells. *World Journal of Surgical Oncology.* 2016;14:256.
45. Yamada K, Maishi N, Akiyama K, Towfik Alam M, Ohga N, Kawamoto T, Shindoh M, Takahashi N, Kamiyama T, Hida Y, Taketomi A, Hida K. CXCL12-CXCR7 axis is important for tumor endothelial cell angiogenic property. *Int J Cancer.* 2015;137:2825-2836.
46. Graham GJ, Locati M, Mantovani A, Rot A, Thelen M. The biochemistry and biology of the atypical chemokine receptors. *Immunol Lett.* 2012;145:30-38.
47. Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, Gerard C, Lefkowitz RJ. Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci U S A.* 2010;107:628-632.
48. Ma W, Liu Y, Wang C, Zhang L, Crocker L, Shen J. Atorvastatin inhibits CXCR7 induction to reduce macrophage migration. *Biochem Pharmacol.* 2014;89:99-108.
49. Balabanian K, Lagane B, Infantino S, Chow KY, Harriague J, Moepps B, Arenzana-Seisdedos F, Thelen M, Bachelerie F. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem.* 2005;280:35760-35766.
50. Zabel BA, Lewen S, Berahovich RD, Jaen JC, Schall TJ. The novel chemokine receptor CXCR7 regulates trans-endothelial migration of cancer cells. *Mol Cancer.* 2011;10:73.
51. Grymula K, Tarnowski M, Wysoczynski M, Drukala J, Barr FG, Ratajczak J, Kucia M, Ratajczak MZ. Overlapping and Distinct Role of CXCR7-SDF-1/ITAC and CXCR4-SDF-1 Axes in Regulating Metastatic Behavior of Human Rhabdomyosarcomas. *International journal of cancer Journal international du cancer.* 2010;127:2554-2568.
52. Crump MP, Gong JH, Loetscher P, Rajarathnam K, Amara A, Arenzana-Seisdedos F, Virelizier JL, Baggiolini M, Sykes BD, Clark-Lewis I. Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *Embo j.* 1997;16:6996-7007.
53. Lin CH, Guo Y, Ghaffar S, McQueen P, Pourmorady J, Christ A, Rooney K, Ji T, Eskander R, Zi X, Hoang BH. Dkk-3, a secreted wnt antagonist, suppresses tumorigenic potential and pulmonary metastasis in osteosarcoma. *Sarcoma.* 2013;2013:147541.
54. Krupnik VE, Sharp JD, Jiang C, et al. Functional and structural diversity of the human Dickkopf gene family. *Gene.* 1999;238:301-313.

55. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol.* 2003;23:168-175.
56. 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.
57. Zitt M, Untergasser G, Amberger A, Moser P, Stadlmann S, Zitt M, Muller HM, Muhlmann G, Perathoner A, Margreiter R, Gunsilius E, Ofner D. Dickkopf-3 as a new potential marker for neoangiogenesis in colorectal cancer: expression in cancer tissue and adjacent non-cancerous tissue. *Dis Markers.* 2008;24:101-109.
58. Xiao Q, Zeng L, Zhang Z, Hu Y, Xu Q. Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways. *Am J Physiol Cell Physiol.* 2007;292:C342-352.
59. Karamariti E, Zhai C, Yu B, et al. DKK3 (Dickkopf 3) Alters Atherosclerotic Plaque Phenotype Involving Vascular Progenitor and Fibroblast Differentiation Into Smooth Muscle Cells. *Arterioscler Thromb Vasc Biol.* 2017.
60. Wu Y, Qin Y, Wang Z, Wang J, Zhang C, Li C, Kong D. The regeneration of macro-porous electrospun poly(varepsilon-caprolactone) vascular graft during long-term in situ implantation. *J Biomed Mater Res B Appl Biomater.* 2018;106:1618-1627.
61. Zernecke A, Schober A, Bot I, von Hundelshausen P, Liehn EA, Mopps B, Mericskay M, Gierschik P, Biessen EA, Weber C. SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. *Circ Res.* 2005;96:784-791.
62. Sainz J, Al Haj Zen A, Caligiuri G, Demerens C, Urbain D, Lemitre M, Lafont A. Isolation of "side population" progenitor cells from healthy arteries of adult mice. *Arterioscler Thromb Vasc Biol.* 2006;26:281-286.

Figure Legends

Figure 1. Dkk3 induces migration of resident Sca-1+ VPCs *in vitro* and *ex vivo*.
(a) Representative images of migrated Sca-1+ cells induced by increasing concentration of either Dkk3 or Sdf-1α (0, 10, 25, 50 and 100 ng/mL), in a transwell migration assay performed during 12 hours (20x magnification). (b, c) Quantification of the transwell assay with Dkk3 and Sdf-1α stimulation, respectively (n=5). (d) Representative images of migrated Sca-1+ cells, 16 hours after the initiation of wound healing assay, at increasing concentration of either Dkk3 or Sdf-1α (0, 10, 25, 50 and 100 ng/mL). Blue solid lines denote the margins of the wound (20x magnification). (e, f) Quantification of cells that migrated to close the wounded area in response to Dkk3 and Sdf-1α, respectively (n=5). Both migration assays showed that Dkk3 induces Sca-1+ cell migration and that the chemotactic effect of Dkk3 is similar to the observed with Sdf-1α stimulation. (g) Representative images of cell outgrowth from aortic rings from wild type and ApoE$^{-/-}$ mice induced by either Dkk3 (25 ng/mL) or Sdf-1α (25 ng/mL) treatment. (h, i) Quantitative analysis of cell outgrowth from wild type and ApoE$^{-/-}$ mice aortic rings, respectively (n=4). Dkk3 induces cell outgrowth from ApoE$^{-/-}$ aortic rings, whereas Sdf-1α induces cell outgrowth from both wild type and ApoE$^{-/-}$ explants. (j) Representative immunofluorescence images of aortic rings from transgenic Sca-1-GFP ApoE$^{-/-}$ mice treated with either Dkk3 (25 ng/mL) or Sdf-1α (25 ng/mL). DAPI was included to counterstain the nuclei (20x magnification). (k) Quantification of the number of Sca-1+-GFP cells that outgrew from the aortic rings (n=3). (l) Quantification of the percentage of Sca-1+-GFP cells against the total number of cells present in the outgrowth (n=3). Dkk3 promotes Sca-1+ cell outgrowth from ApoE$^{-/-}$ aortic rings in a similar manner as Sdf-1α does. The data are expressed as the mean±s.e.m. of 5 independent experiments for the *in vitro* migration assays (a-f) and of 3 to 4 independent experiments for the *ex vivo* migration assays (g-l). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with control group (0 ng/mL) (One-way ANOVA followed by Bonferroni's post-hoc test).

Figure 2. CXCR7 is involved in Dkk3-driven migration of Sca-1+ cells.
(**a**) Representative flow cytometry dot plots of CXCR4 and CXCR7 surface expression in Sca-1+ cells. (**b**) Quantification of Sca-1+/CXCR4+, Sca-1+/CXCR7+ and Sca-1+/CXCR7+/CXCR4+ cells by flow cytometry analysis (n=3, one-way ANOVA followed by Bonferroni's post-hoc test). CXCR7 is expressed in 78% (77.9%±6.2%) of Sca-1+ cells whereas CXCR4 is expressed in only 1% (0.9%±0.2) of the cells. (**c**) Western blot analysis of CXCR4 and CXCR7 expression in murine endothelial cells (EC), Sca-1+ cells (VPC) and smooth muscle cells (SMC). CXCR7 is highly expressed in Sca-1+ cells and SMCs. CXCR4 expression in Sca-1+ cells is low in comparison to its expression in ECs and SMCs. (**d**). Quantitative RT-qPCR analysis of CXCR7 expression in Sca-1+ cells transfected with siRNA targeting CXCR7. Expression levels were normalized against GAPDH (n=5, two-tailed Student's t-test). (**e**) Western blot analysis of CXCR7 knockdown in Sca-1+ cells transfected with CXCR7 siRNA. (**f, h**) Representative images of transwell migration assay of Sca-1+ cells transfected with CXCR7 siRNA in response to Dkk3 (25 ng/mL) and Sdf-1α (25 ng/mL) treatment, respectively. (**g, i**) Quantitative analysis of the migrated cells in response to Dkk3 or Sdf-1α treatment (n=4, Two-way ANOVA followed by Bonferroni's test). CXCR7 knockdown in Sca-1-VPCs supresses Dkk3-mediated migration, similarly to the observed with Sdf-1α treatment. The data are expressed as the mean±s.e.m. of 3 to 5 independent experiments. **P<0.01, ***P<0.001, ****P<0.0001, compared with control group (0 ng/mL).

Figure 3. CXCR7 is a high-affinity binding receptor of Dkk3.
(a) Co-Immunoprecipitation of Dkk3 with CXCR7. Dkk3 co-immunoprecipitates with CXCR7 from Sca-1+ cells. (b) Immunoblotting of the receptors pulled-down from Dkk3-His tagged, using Nickel magnetic beads. Sca-1+ cells were treated with Dkk3-His tagged. CXCR7, but not CXCR4, is pulled-down with Dkk3-His. (c) Quantitative RT-PCR analysis of CXCR7 mRNA expression in HEK 293T cells transfected with CXCR7 expression plasmid. Expression levels were normalized against GAPDH (n=4, two-tailed unpaired Student's t-test). (d) Western blot analysis of CXCR7 overexpression in HEK 293T cells transfected with CXCR7 expression plasmid. (e, f) Representative binding curves and respective Scatchard analysis of Sdf-1α–AP binding to CXCR4 or CXCR7 overexpressed in HEK 293T cells and of Dkk3–AP binding to CXCR7, CXCR4, Kremen1 or Kremen2 overexpressed in HEK 293T cells, respectively. The dissociation constants (Kd) are represented for each receptor (n=3). Dkk3-AP does not bind to CXCR4, Kremen1 and Kremen2, but it does bind with high affinity to CXCR7, as represented by the characteristic hyperbolic binding curve. CXCR7 is also a high-affinity binding receptor of Sdf-1α, alongside its cognate receptor CXCR4. AP alone does not bind to CXCR7, as depicted in blue. (g, i) Representative images of transwell migration assay of HEK 293T cells overexpressing CXCR7 in response to Dkk3 and Sdf-1α stimulation, respectively. (h, j) Quantitative analysis of the transwell migration assays. Dkk3 induces migration of CXCR7 overexpressing HEK 293T cells, analogously to Sdf-1α. (n=5, Two-way ANOVA followed by Bonferroni's post-hoc test). The data are expressed as the mean±s.e.m. of 3 to 5 independent experiments. *P<0.05, **P<0.01, compared with control group.

Figure 4. ERK1/2 and PI3K/AKT signalling pathways act downstream of Sca-1+ cell migration mediated by Dkk3 binding to CXCR7.
(**a, b**) Western blot analysis of phosphorylated and total ERK1/2 and AKT proteins in Sca-1+ cells stimulated with Dkk3 at the indicated time points. ERK1/2 and AKT phosphorylation increases in response to Dkk3. (**c, d**) Western blot analysis of Dkk3-induced ERK1/2 and AKT activation in Sca-1+ cells after CXCR7 knockdown by siRNA transfection. Downregulation of CXCR7 abolishes Dkk3-triggered phosphorylation of ERK1/2 and AKT. (**e, f**) Western blot analysis of ERK1/2 and AKT activation in Sca-1+ cells stimulated with Dkk3 for 5 minutes and pre-treated with, respectively, PD98059 and LY294002. ERK1/2 and AKT activation is supressed upon treatment with the inhibitors. (**g, h, i**) Quantification of Dkk3-driven Sca-1+ cell migration upon treatment with, respectively, PD98059 (10 μM, n=5), LY294002 (10 μM, n=3) and AKT inhibitor (2.5 μM, n=4). Dkk3-driven cell migration is abrogated in response to all inhibitors. Western blot images are representative of three independent experiments. The data are expressed as the mean±s.e.m. of 3 or 5 independent experiments. *P<0.05, **P<0.01, ***P<0.001, compared with control group (0 ng/mL) (Two-way ANOVA followed by Bonferroni's post-hoc test).

Figure 5: Rac1 and RhoA GTPases activation is required for Sca-1+ cell migration induced by Dkk3 binding to CXCR7.
(**a, e**) Quantification of Rac1-GTP and RhoA-GTP in Sca-1+ cells treated with Dkk3 at the time points indicated. Dkk3 induces Rac1 and RhoA activation. (**b**, **f**) Quantification of Dkk3-triggered activation of Rac1 and RhoA after CXCR7 knockdown by siRNA transfection. Downregulation of CXCR7 abolishes Dkk3-triggered activation of Rac1 and RhoA (n=3). (**c**) Quantification of Dkk3-induced Rac1 activation upon treatment with NSC23766 (10 μM,

n=3). Dkk3-induced increase of Rac1-GTP level at 10 and 15 minutes is repressed by the inhibitor NSC23766. (**d**) Quantification of Dkk3-driven Sca-1+ cell migration upon NSC23766 treatment. NSC23766 inhibitor abolishes Sca-1+ cell migration promoted by Dkk3 treatment (n=4). (**g**) Quantification of Dkk3-induced RhoA activation upon Rhosin (10 μM, n=3) treatment. Dkk3-induced increase of RhoA-GTP level is repressed by the inhibitor Rhosin, at the time points indicated. (**h**) Quantification of Dkk3-driven Sca-1+ cell migration upon Rhosin treatment (n=3). Rhosin abolishes Sca-1+ cell migration promoted by Dkk3 treatment. (**i**) Western blot analysis of MLC phosphorylation in response to Dkk3 stimulation. MLC phosphorylation is induced by Dkk3 at the time points indicated. (**j**) Western blot analysis of Dkk3-driven MLC phosphorylation upon pre-treatment with Rhosin or Y27632, at the time point indicated. Dkk3-induced MLC phosphorylation is repressed in response to the inhibitors. (**k**) Quantification of Dkk3-driven Sca-1+ cell migration upon Y27632 treatment. Sca-1+ cell migration promoted by Dkk3 treatment is inhibited by Y27632 (5 μM, n=4) treatment. The western blot images are representative of 3 independent experiments. The data are expressed as the mean±s.e.m. of 3 to 4 independent experiments. *P<0.05, **P<0.01, ****P<0.0001, compared with control group (0 ng/mL) (One-way ANOVA for **a** and **e** and Two-way ANOVA for **b**, **c**, **d**, **f**, **g**, **h** and **k**, followed by Bonferroni's post-hoc test).

Figure 6. Delivery of Dkk3 in tissue engineered vessel grafts (TEVGs) enhances vascular regeneration.

Dkk3-loaded TEVGs were implanted into rats to replace the abdominal artery and then harvested at 2 weeks, 1 and 3 months post-implantation. (**a**) H&E staining on cross-sections of control and Dkk3-loaded TEVGs 3 months post-implantation. Both grafts are fully cellularized and no evident intimal hyperplasia is observed. (**b**) Quantification of the inner diameter of the TEVGs at 2 weeks, 1 and 3 months. The inner diameters of the grafts remain unchanged up to three months post-implantation. (**c**). Representative images of the luminal surface of the explanted TEVGs, 3 months post-implantation, obtained by scanning electronic microscopy. Three locations were selected continuously from the anastomotic sites to the middle of the grafts and the respective magnified images are shown. The surface of Dkk3-TEVGs throughout the graft is more continuous and homogeneous in comparison with the control group. (**d**) Representative immunofluorescence images of control- and Dkk3-TEVGs showing SMC recruitment by staining the sections with anti-α-SMA and anti-SMMHC antibodies. DAPI was used to counterstain the nuclei. (**e**, **f, g**) Quantification of smooth muscle regeneration by determination of the total area of α-SMA and of SM-MHC positive cells in the luminal region (delineated by the white dashed lines) at different time points. 2 weeks after implantation, Dkk3-loaded TEVGs display an increase of α-SMA+ cells, compared to control. Over time, the smooth muscle becomes more mature in Dkk3-TEVGs than in control grafts, as the area of SM-MHC+ cells is also increased. The images are representative of 4 grafts for each group. The data are expressed as the mean±s.e.m. of 4 independent experiments. *P<0.05, compared with control group (Two-way ANOVA followed by Bonferroni's post-hoc test for **b, e, f** and **g**).

Figure 7. Dkk3 increases Sca1+ progenitor migration and smooth muscle differentiation.

Schematic illustration of the three types of TEVGs. Control TEVGs composed of PCL and collagen fibers; Dkk3-TEVG composed of PCL and Dkk3-collagen fibers; Dkk3-CXCR7 ab-TEVG composed of PCL, Dkk3-collagen and CXCR7 antibody-collagen fibers. (**b**)

Representative immunofluorescence images of the cross-sections of TEVGs displaying Sca1+ progenitor cells recruitment into the grafts detected with anti-Sca-1 antibody (green), at the time points indicated. (**c**) Quantification of Sca-1+ progenitor cell infiltration into the three types of TEVGs. DKK3 delivery significantly promotes the infiltration of Sca1+ progenitor cells into the grafts. The incorporation of CXCR7 antibody in the grafts markedly inhibits infiltration of Sca1+ progenitor cells into the grafts. (**d**) Representative immunofluorescence images of the cross-sections of TEVGs exhibiting double immunostaining with anti-α-SMA (green) and anti-Sca-1 (red) antibodies, at the time points indicated. The increased contribution of double positive cells (Sca-1+/α-SMA+) to the smooth muscle regeneration in Dkk3-TEVGs, compared to control group, seems to decrease in Dkk3-CXCR7 ab-TEVGs. DAPI was included to counterstain the nuclei. The data presented are representative images of four grafts for each group and expressed as the mean±s.e.m. of 4 independent experiments. **$P<0.01$, compared with control group (Two-way ANOVA, followed by Bonferroni's post-hoc test).

Figure 8. Schematic illustration of Dkk3/CXCR7 mediated Sca1+ progenitor migration. The figure depicts the molecular mechanism involved in Sca-1+ progenitor migration induced by Dkk3 binding to CXCR7. Dkk3 present in the cell environment binds to CXCR7 expressed on the surface of Sca-1+ progenitors. Upon CXCR7 activation, ERK1/2 signalling pathway is activated. Phosphorylated ERK1/2 either activates Rac1 or directly modulates cytoskeleton rearrangement to promote cell migration. Rac1 activation also results from Dkk3 binding to CXCR7, which in turn directly regulates cell migration and/or stimulates ERK phosphorylation. Rac1 and ERK1/2 seem to work in a feedback manner. The Dkk3/CXCR7 axis promotes AKT phosphorylation via PI3K, which leads to cytoskeleton rearrangement. RhoA activation level is increased in response to Dkk3 binding to CXCR7, leading to MLC phosphorylation either directly or via ROCK activation. MLC activation results in actomyosin contraction required for cell migration.

NOVELTY AND SIGNIFICANCE

What Is Known?

*Dickkopf-3 (Dkk3) is involved in vascular remodelling, e.g. atherosclerosis, vascular injury-induced stenosis and plaque stability.

*Tissue-engineered vessel grafts constitute an effective alternative to the limited availability of autografts used for blood vessel replacement.

*Vascular progenitor cells play an active role in vascular remodelling and regeneration.

What New Information Does This Article Contribute?

*Dkk3 can specifically bind to a chemokine receptor CXCR7.

*Dkk3-CXCR7 axis is crucial for vascular stem cell migration.

*Tissue-engineered vessel grafts containing Dkk3 showed better cellularization and regeneration.

Vascular resident stem/progenitor cells have an ability to regenerate damaged tissues. A cytokine-like protein Dkk3 is able to bind to a chemokine receptor CXCR7 resulting cell signalling and cytoskeleton rearrangement, which led to stem cell migration. When Dkk3 was used to generate a degradable vessel grafts, it has a role to attract stem cells into the grafts in animal models. This tissue-engineered grafts displayed a better regeneration and potentially have a longer patency in vivo. Thus, Dkk3 induces vessel graft regeneration by recruiting host vascular stem cells via CXCR7 activation.

Figure 1.

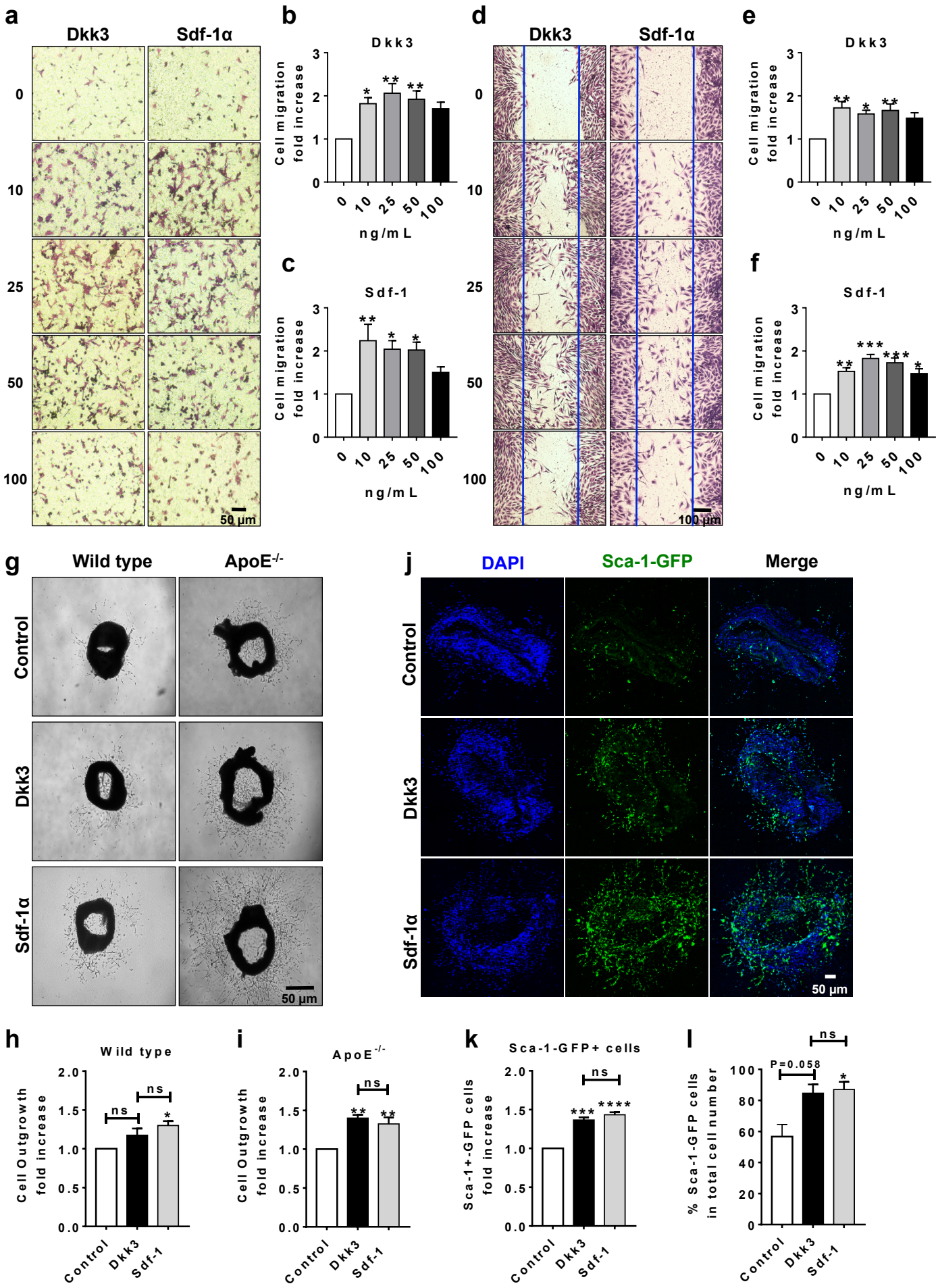

a
Dkk3
Sdf-1α
0
10
25
50
100
50 µm
b
Dkk3
Cell migration fold increase
ng/mL
c
Sdf-1
d
e
f
g
Wild type
ApoE-/-
Control
Dkk3
Sdf-1α
50 µm
j
DAPI
Sca-1-GFP
Merge
h
Wild type
Cell Outgrowth fold increase
Control
Dkk3
Sdf-1
i
ApoE-/-
k
Sca-1-GFP+ cells
Sca-1+-GFP cells fold increase
l
% Sca-1-GFP cells in total cell number
P=0.058
ns

Figure 2.

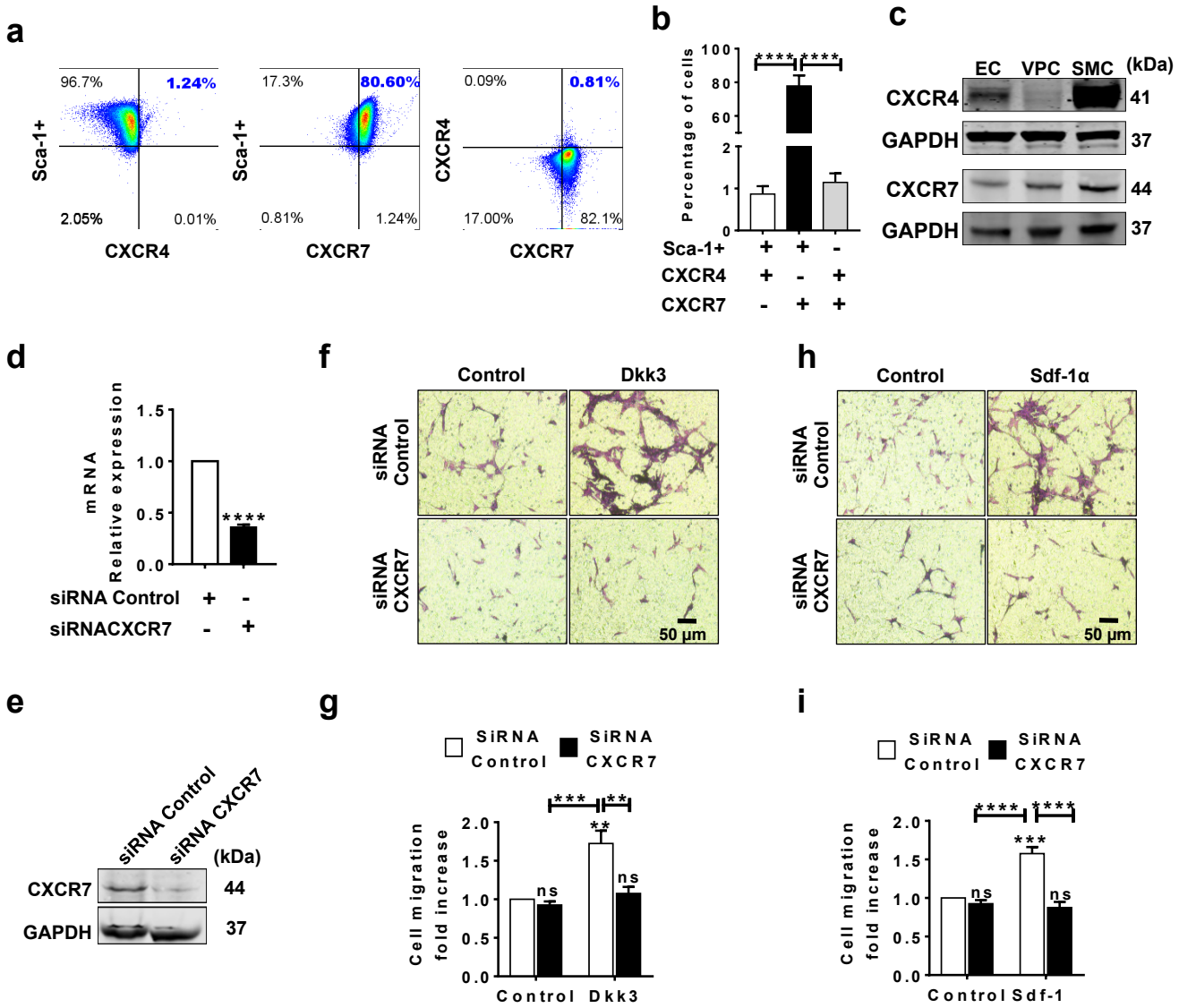

a
96.7%
1.24%
2.05%
0.01%
Sca-1+
CXCR4
17.3%
80.60%
0.81%
1.24%
Sca-1+
CXCR7
0.09%
0.81%
17.00%
82.1%
CXCR4
CXCR7
b
Percentage of cells
Sca-1+ + + -
CXCR4 + - +
CXCR7 - + +
c
EC VPC SMC (kDa)
CXCR4 41
GAPDH 37
CXCR7 44
GAPDH 37
d
mRNA Relative expression
siRNA Control + -
siRNACXCR7 - +
f
Control
Dkk3
siRNA Control
siRNA CXCR7
50 μm
h
Control
Sdf-1α
siRNA Control
siRNA CXCR7
50 μm
e
siRNA Control
siRNA CXCR7
(kDa)
CXCR7 44
GAPDH 37
g
SiRNA Control
SiRNA CXCR7
Cell migration fold increase
Control Dkk3
i
SiRNA Control
SiRNA CXCR7
Cell migration fold increase
Control Sdf-1

Figure 3.

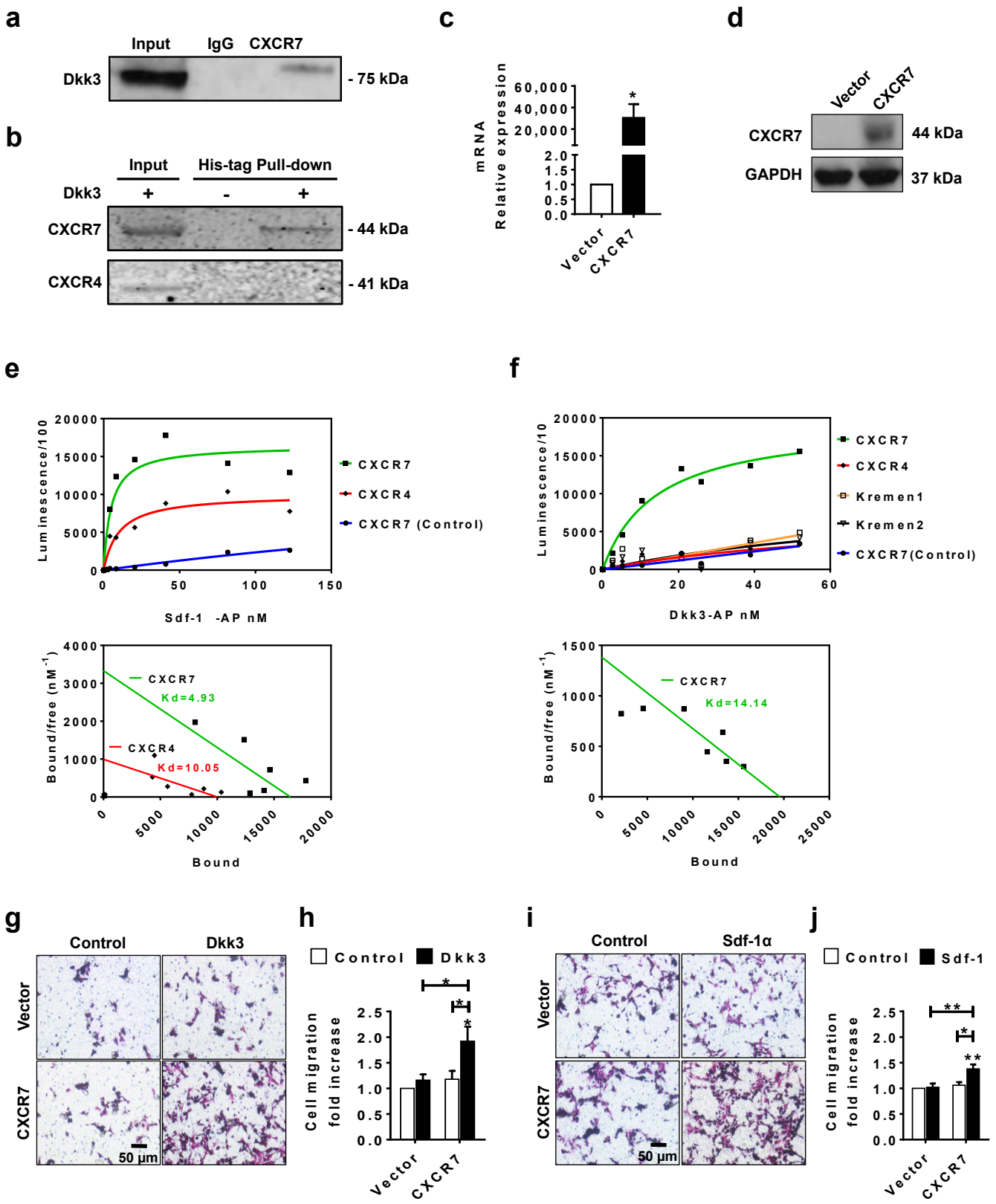

a
Input
IgG
CXCR7
Dkk3
- 75 kDa
b
Input
His-tag Pull-down
Dkk3
+
-
+
CXCR7
- 44 kDa
CXCR4
- 41 kDa
c
mRNA Relative expression
60,000
40,000
20,000
2.0
1.5
1.0
0.5
0.0
*
Vector
CXCR7
d
Vector
CXCR7
CXCR7
44 kDa
GAPDH
37 kDa
e
Luminescence/100
20000
15000
10000
5000
0
0
50
100
150
Sdf-1 -AP nM
CXCR7
CXCR4
CXCR7 (Control)
Bound/free (nM-1)
4000
3000
2000
1000
0
CXCR7
Kd=4.93
CXCR4
Kd=10.05
0
5000
10000
15000
20000
Bound
f
Luminescence/10
20000
15000
10000
5000
0
0
20
40
60
Dkk3-AP nM
CXCR7
CXCR4
Kremen1
Kremen2
CXCR7(Control)
Bound/free (nM-1)
1500
1000
500
0
CXCR7
Kd=14.14
0
5000
10000
15000
20000
25000
Bound
g
Control
Dkk3
Vector
CXCR7
50 μm
h
Control
Dkk3
Cell migration fold increase
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Vector
CXCR7
i
Control
Sdf-1α
Vector
CXCR7
50 μm
j
Control
Sdf-1
Cell migration fold increase
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
Vector
CXCR7

Figure 4.

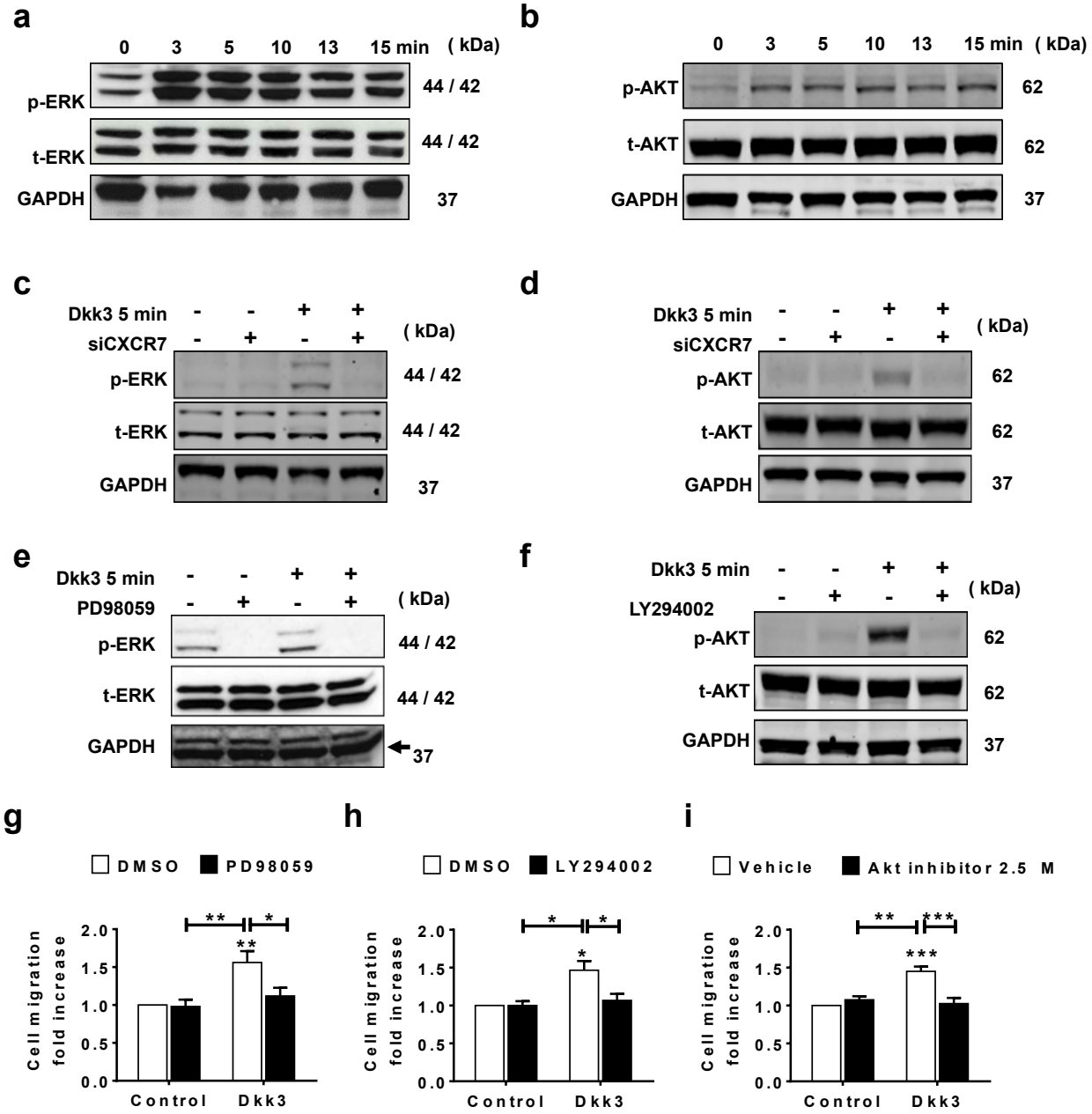

a
0 3 5 10 13 15 min (kDa)
p-ERK 44 / 42
t-ERK 44 / 42
GAPDH 37
b
0 3 5 10 13 15 min (kDa)
p-AKT 62
t-AKT 62
GAPDH 37
c
Dkk3 5 min - - + +
siCXCR7 - + - + (kDa)
p-ERK 44 / 42
t-ERK 44 / 42
GAPDH 37
d
Dkk3 5 min - - + +
siCXCR7 - + - + (kDa)
p-AKT 62
t-AKT 62
GAPDH 37
e
Dkk3 5 min - - + +
PD98059 - + - + (kDa)
p-ERK 44 / 42
t-ERK 44 / 42
GAPDH 37
f
Dkk3 5 min - - + +
LY294002 - + - + (kDa)
p-AKT 62
t-AKT 62
GAPDH 37
g
DMSO PD98059
Cell migration fold increase
2.0 1.5 1.0 0.5 0.0
** *
**
Control Dkk3
h
DMSO LY294002
Cell migration fold increase
2.0 1.5 1.0 0.5 0.0
* *
*
Control Dkk3
i
Vehicle Akt inhibitor 2.5 M
Cell migration fold increase
2.0 1.5 1.0 0.5 0.0
** ***
***
Control Dkk3

Figure 5.

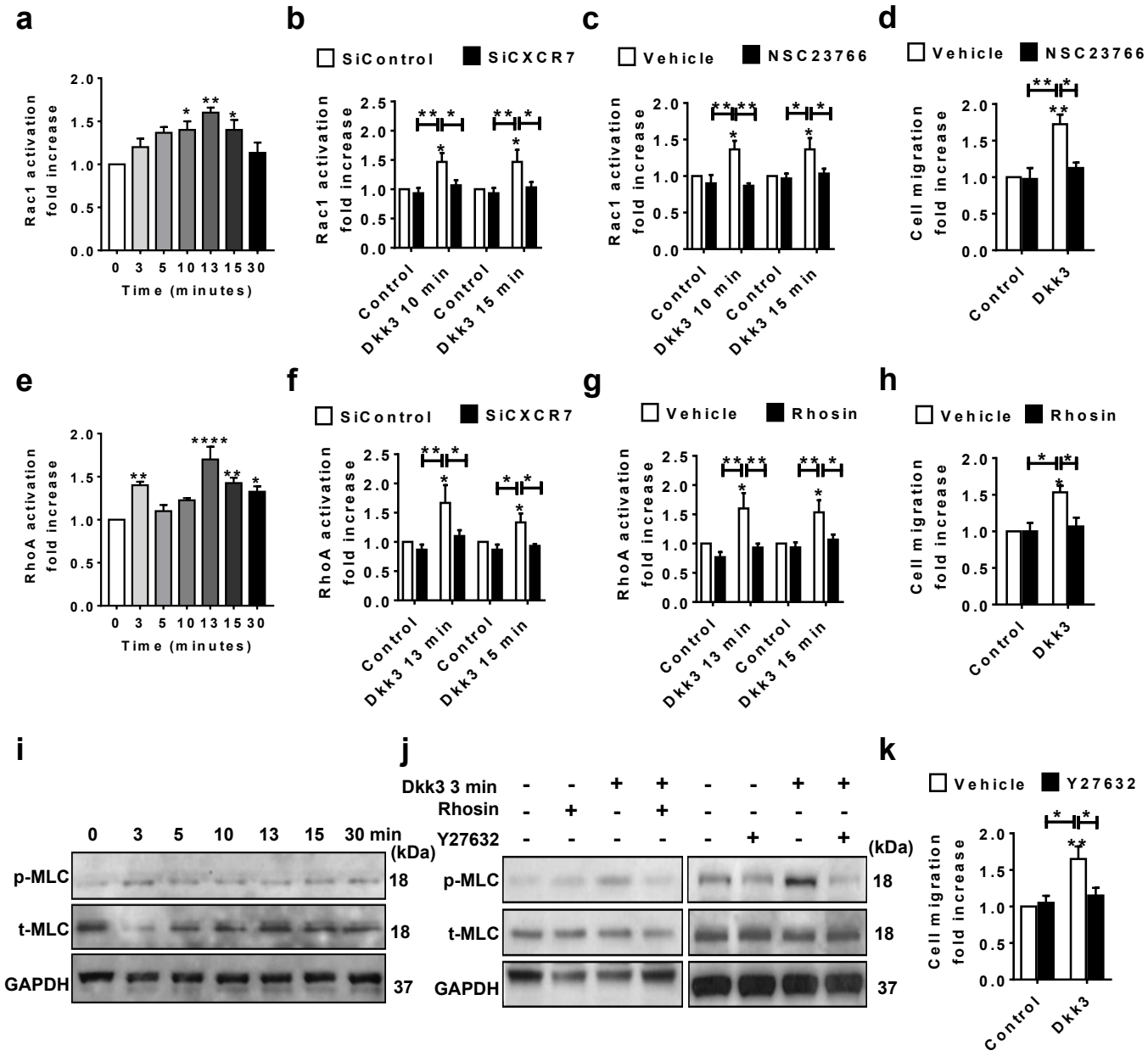

a
Rac1 activation fold increase
Time (minutes)
0 3 5 10 13 15 30
b
SiControl
SiCXCR7
Control
Dkk3 10 min
Dkk3 15 min
c
Vehicle
NSC23766
d
Cell migration fold increase
Control
Dkk3
e
RhoA activation fold increase
f
Dkk3 13 min
g
Rhosin
h
i
0 3 5 10 13 15 30 min
(kDa)
p-MLC
t-MLC
GAPDH
18
37
j
Dkk3 3 min
Rhosin
Y27632
k
Y27632

Figure 6.

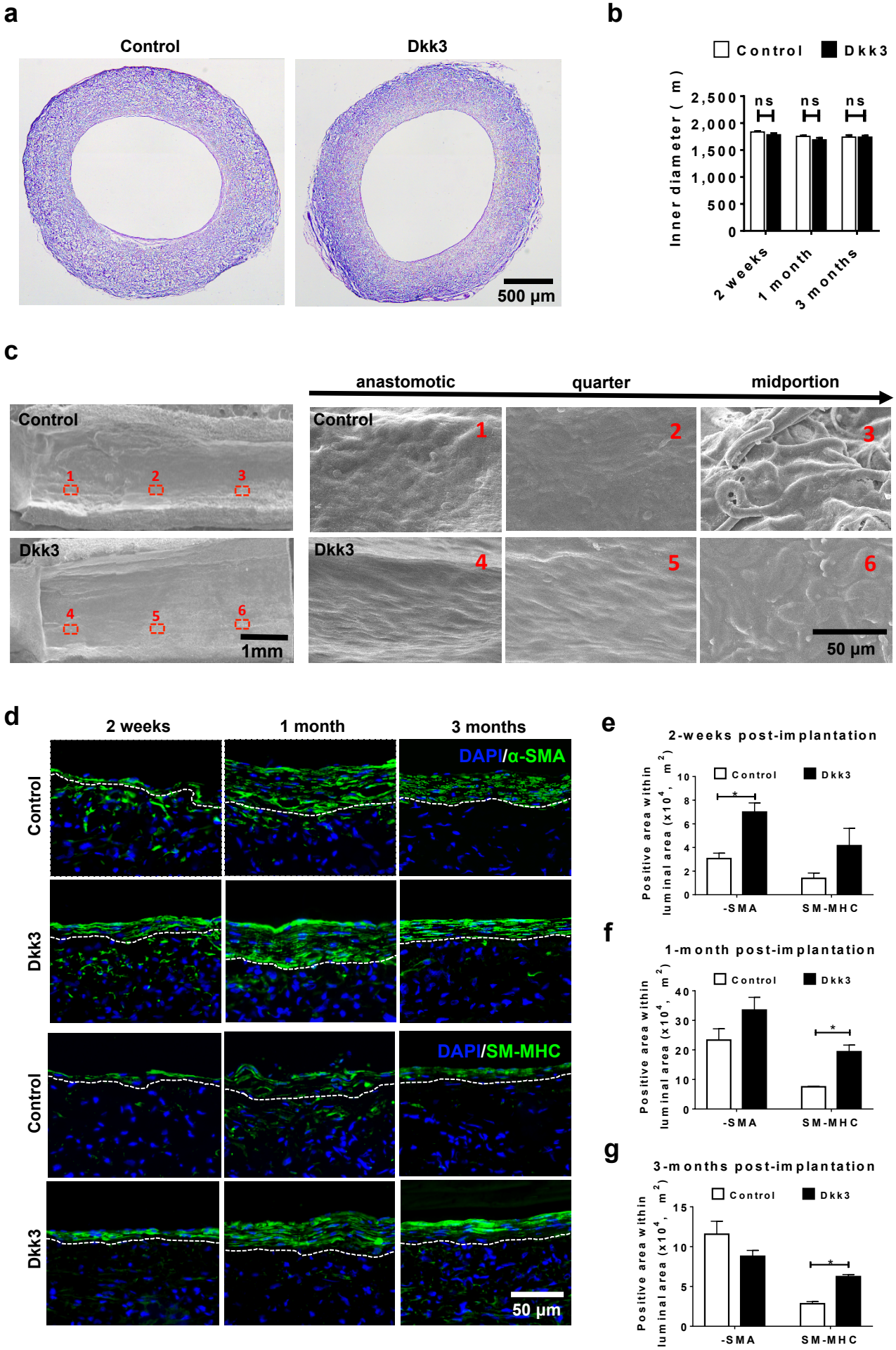

a
Control
Dkk3
500 μm
b
Control
Dkk3
Inner diameter (m)
2,500
2,000
1,500
1,000
500
0
ns
ns
ns
2 weeks
1 month
3 months
c
anastomotic
quarter
midportion
Control
Dkk3
1
2
3
4
5
6
1mm
50 μm
d
2 weeks
1 month
3 months
Control
Dkk3
DAPI/α-SMA
DAPI/SM-MHC
50 μm
e
2-weeks post-implantation
Positive area within luminal area (x10^4, m^2)
Control
Dkk3
-SMA
SM-MHC
f
1-month post-implantation
g
3-months post-implantation

Figure 7.

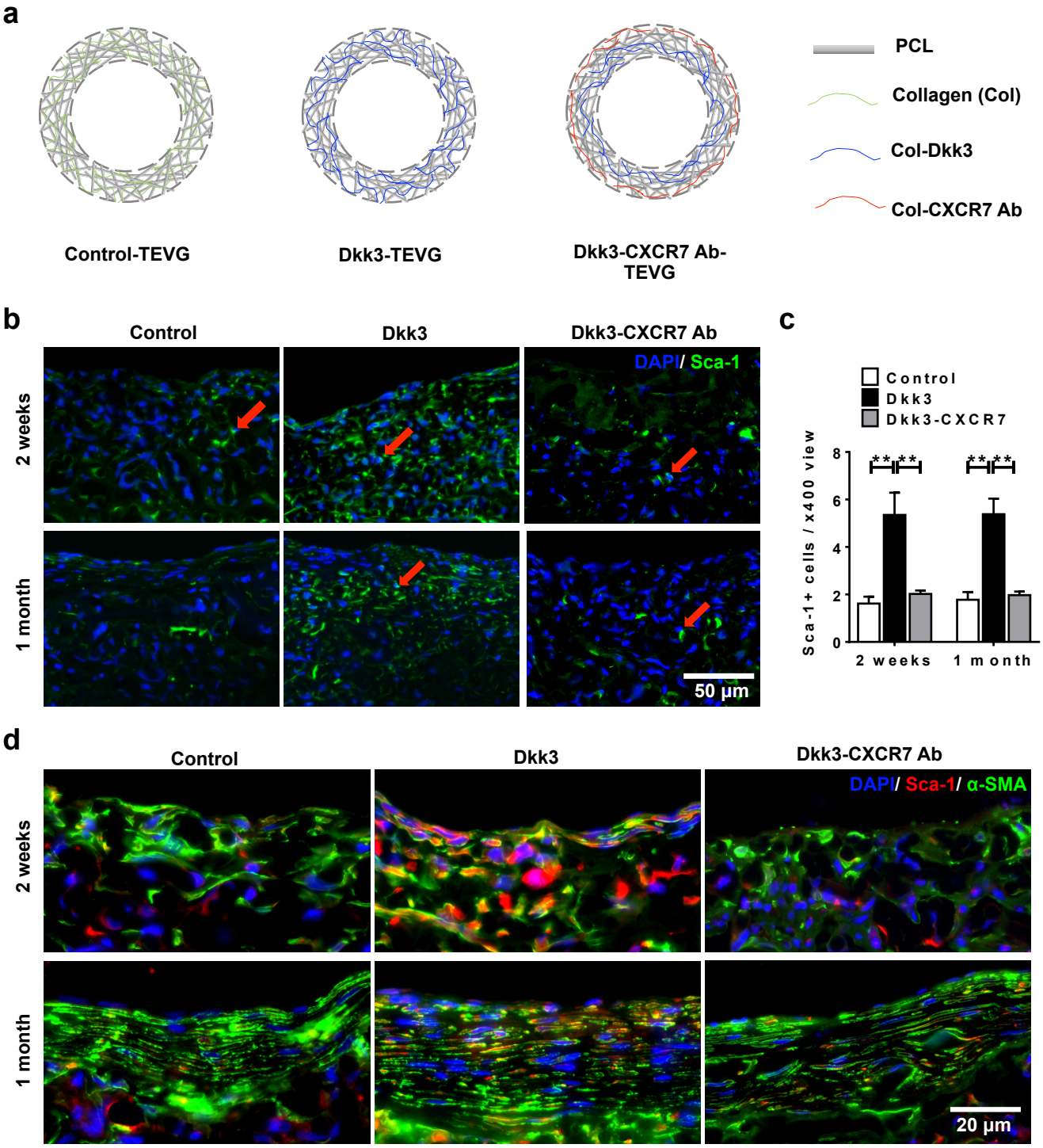
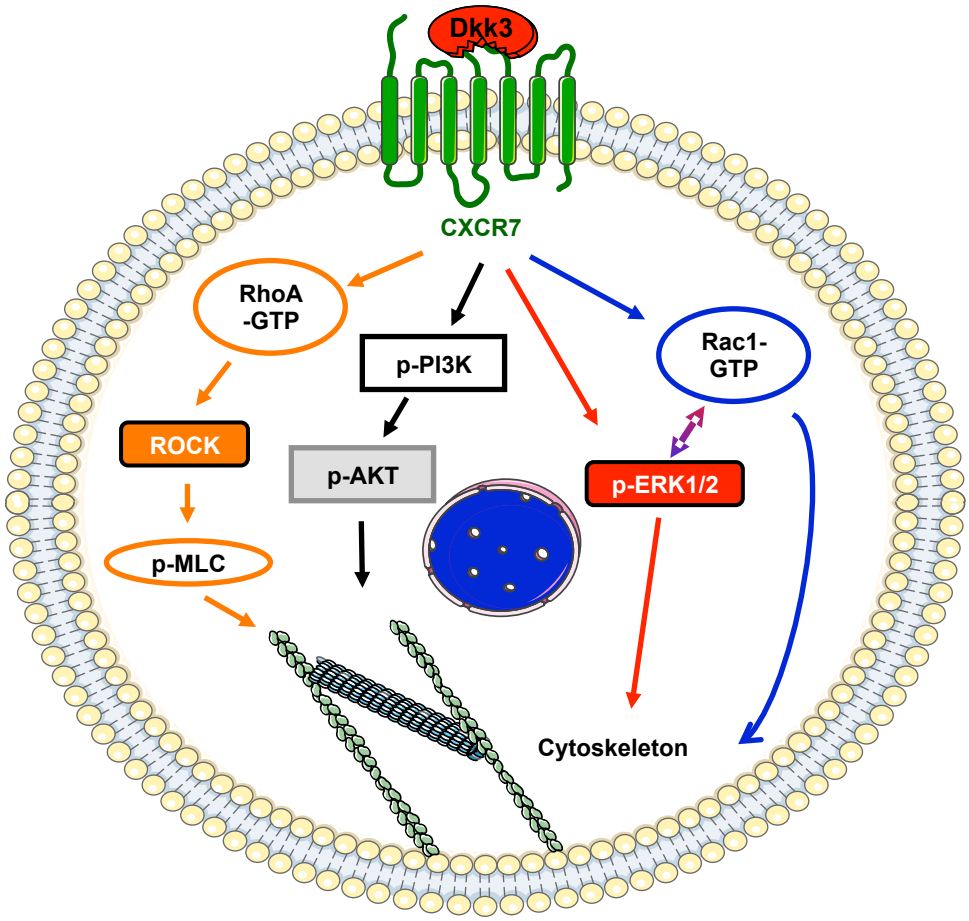

a
PCL
Collagen (Col)
Col-Dkk3
Col-CXCR7 Ab
Control-TEVG
Dkk3-TEVG
Dkk3-CXCR7 Ab-TEVG
b
Control
Dkk3
Dkk3-CXCR7 Ab
DAPI/ Sca-1
2 weeks
1 month
50 μm
c
Control
Dkk3
Dkk3-CXCR7
Sca-1+ cells / x400 view
0
2
4
6
8
**
**
**
**
2 weeks
1 month
d
Control
Dkk3
Dkk3-CXCR7 Ab
DAPI/ Sca-1/ α-SMA
2 weeks
1 month
20 μm

Figure 8.


Dkk3
CXCR7
RhoA
-GTP
p-PI3K
Rac1-
GTP
ROCK
p-AKT
p-ERK1/2
p-MLC
Cytoskeleton