The Wnt Antagonist Dickkopf-1 Increases Endothelial Progenitor Cell Angiogenic Potential


Objective—To determine the role of Wnt antagonist Dickkopf (DKK) 1 in human endothelial colony-forming cells (ECFCs) in view of the emerging importance of Wnt pathways in vascular biology.

Methods and Results—Endothelial progenitor cells have been proposed to be crucial in tumor neovascularization. DKK1 enhances ECFC proliferation and the capacity of ECFCs to form pseudotubes in Matrigel. These effects have been attributed to enhancement of vascular endothelial growth factor receptor 2, SDF-1, and CXCR4. DKK1 gene silencing has been realized on ECFCs and mesenchymal stem cells, and we found that DKK1 silencing in the 2 cell types decreased their angiogenic potential. We then examined the possible role of DKK1 in tumor neovascularogenesis and found that blood vessels of breast cancer tissues expressed DKK1 far more strongly in human breast tumors than in normal breast tissues. By studying 62 human breast tumors, we found a significant positive correlation between DKK1 expression and von Willebrand factor. In vivo, DKK1 strongly enhanced the vascularization of Matrigel plugs and increased tumor size in a xenograft model of human breast carcinoma in nude mice.

Conclusion—DKK1 enhances angiogenic properties of ECFCs in vitro and is required for ECFC and mesenchymal stem cell angiogenic phenotypes in vivo. DKK1 also increases tumoral angiogenesis. Thus, we demonstrated a major role of DKK1 in angiogenic processes. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: vascular biology ■ endothelial progenitor cells

Wnts are powerful regulators of cell proliferation and differentiation, and their signaling pathway involves proteins that participate directly in both gene transcription and cell adhesion. The major signaling pathway of Wnt is the canonical pathway that results from Wnt binding to the frizzled and LRP families on the cell surface. Complex formation induces β-catenin nuclear entry and forms a complex with TCF to activate transcription of Wnt target genes. The Wnt pathway is modulated by several Wnt antagonists, including Dickkops (DKKs). The DKK family encodes secreted proteins of 255 to 350 amino acids and comprises 4 main members in vertebrates (DKK1 to DKK4). DKK1, the most widely studied member of this family, has been implicated in various physiological and pathological processes in human adults. DKK1 mediates inflammation in atherosclerotic lesions and mobilizes progenitor cells by activating the bone marrow endosteal stem cell niche. Recently, serum levels of DKK1 correlated with the extent of bone disease in patients with multiple malignant neoplasms, such as breast cancer. In tumoral angiogenesis, endothelial cells have had distinct gene expression profiles when compared with normal endothelial cells. Notably, they express high levels of DKK3. However, the role of DKK1 in tumoral angiogenesis and postnatal vasculogenesis by endothelial progenitor cells (EPCs) has not yet been studied. Bone marrow–derived cells have contributed to tumor neovascularization. The active cell population in bone marrow–derived cells has been proposed to be EPCs. EPCs are a crucial interface in tumor neovascularization and dissemination and have been considered as a breast tumor biomarker. At least 2 populations of EPCs have been described. “Early” EPCs, also called “circulating angiogenic cells” (CACs), are spindle shaped and express both endothelial and leukocyte markers. “Late” EPCs, also called endothelial colony-forming cells (ECFCs), develop after 2 to 3 weeks of culture and have the characteristics of precursor cells committed to the endothelial lineage. CACs and ECFCs have therapeutic potential; however, in vivo, cells that merge into new vessels have an ECFC phenotype. To determine the potential of ECFCs as a cell therapy product.

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and/or as an angiogenic cell type responsive of tumoral angiogenesis, a better understanding of the mechanisms underlying their angiogenic properties is needed.

Herein, we report that the Wnt canonical pathway antagonist DKK1 enhances the angiogenic properties of ECFCs. Because increased DKK1 levels are in the serum of patients with breast cancer,\(^5\) we suspected that DKK1 might correlate with breast tumor vascularization. We further investigated how DKK1 secreted from ECFCs, and from another proposed angiogenic cell type (mesenchymal stem cells [MSCs]), could interfere with cell angiogenic properties.

Methods

Experiments, including in vitro angiogenic assays, reverse transcription–quantitative polymerase chain reaction (RT-qPCR), and in vivo Matrigel plug, have been described elsewhere and in detail in supplemental files (available online at http://atvb.ahajournals.org).

A human breast carcinoma HBCx-12 xenograft was previously described.\(^6\) HBCx-12 xenograft fragments of 30 mm\(^3\) were subcutaneously grafted into the interscapular fat pad of 4- to 6-week-old female athymic nude mice (Janvier, Le Genest St Isle, France).

When the tumors reached a volume of approximately 200 mm\(^3\), animals were ranked according to the tumor volume and divided into 3 groups (6 animals per group) such that the mean and median of tumor volume of the 3 groups were closely matched. Treatment was started on day 1 and given over 3 weeks. Mice were injected twice a week peritumorally with recombinant mouse DKK1 at a dosage of 100 or 500 ng per animal or with PBS containing 0.1% bovine serum albumin as a vehicle control, in a total volume of 100 \(\mu\)L.

The study was approved by the local ethics committee of Hôpital des Instructions et des Armées de Begin in France (201008043234797), and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Results

Differential Expression of the Wnt Canonical Pathway in CACs and ECFCs

The starting material used to isolate endothelial populations consisted of mononuclear cells (MNCs) from either adult peripheral blood or umbilical cord blood. On day 5, CAC colonies were identified as elongated sprouting cells that express leukocyte, monocyte, and endothelial markers (supplemental Figure I). ECFCs have a cobblestone appearance typical of the endothelial lineage and express only endothelial markers (supplemental Figure I). To identify the Wnt canonical pathway components expressed in human ECFCs and CACs, gene expression of LRP5, LRP6, and TCF/LEF transcription cofactors was quantified by RT-PCR. Total RNA was isolated from adult peripheral blood and cord blood ECFCs between 30 and 40 days of culture and from CACs after 5 days of culture. LRP5/LRP6 were strongly expressed by ECFCs from both sources; they were 100-fold lower expressed by CACs (supplemental Figure II). Because these genes encode Wnt coreceptors specific for the canonical pathway, this finding suggested that the \(\beta\)-catenin pathway is constitutively expressed in ECFCs but not in CACs. We confirmed the presence of \(\beta\)-catenin protein in ECFCs by Western blot and its absence from CACs (supplemental Figure II). We detected \(\beta\)-catenin mRNA in CACs, at a level comparable to that found in total MNCs and whole blood (supplemental Figure III) and significantly lower than that found in ECFCs from adult or cord blood. Moreover, we found strong expression of Wnt ligands and FZD receptors in CACs (supplemental Figure IV), strongly suggesting that the Wnt pathway is expressed in CACs, even if \(\beta\)-catenin is not detected at the protein level. TCF/LEF gene expression also differed between the 2 types of EPC (supplemental Figure II). Indeed, although TCFL1 and TCFL2 (TCF4) were strongly expressed in ECFCs, LEF1 and TCFL7 were expressed at high levels in CACs but not in ECFCs.

Wnt Antagonist DKK1 Enhances Angiogenesis Mediated by ECFCs in Vitro

Based on our finding that the \(\beta\)-catenin pathway is present in ECFCs, and on recent data showing a role of Wnt signaling in endothelial commitment of embryonic stem cells,\(^19\) we first investigated the effect of the Wnt canonical pathway antagonist DKK1 on the emergence of ECFC colonies. As shown in Figure 1A, adding DKK1 from the first week of culture did not modify the number of ECFC colonies obtained with MNCs from either cord or adult blood (\(P = 0.31\) and \(P = 0.23\), respectively). Moreover, DKK1 did not modify the length of this period (\(P = 0.22\) for cord blood, and \(P = 0.92\) for adult blood) (Figure 1B). We also observed a more important size of colonies observed at day 14 of culture (supplemental Figure V).

We further studied the angiogenic implication of DKK1 in cord blood–derived ECFCs. Thus, to examine the effects of DKK1 on proliferation, ECFCs were treated with recombinant DKK1 for 72 hours. As shown in Figure 1C and 1D, 100 and 1000 ng/mL of DKK1 significantly promoted ECFC proliferation, which was measured with 2 different methods (pNPP release in Figure 1C and \(^3\)H thymidine incorporation in Figure 1D), with no dose effect between 100 and 1000 ng/mL. Lower doses, ranging between 1 and 10 ng/mL, had no proliferative effect on ECFCs (data not shown). To determine whether DKK1 is involved in progenitor proliferation or endothelial cell proliferation in general, we explored its effect on human umbilical vein endothelial cells and found that, in this mature cell type, DKK1 had no proliferative effects (supplemental Figure VI). We used a Matrigel model to examine the capacity of DKK1-activated ECFCs to differentiate into capillarylike structures. Treatment with DKK1 induced a significant increase in ECFC organization into branched structures and pseudotubes with enclosed areas (Figure 1F). Overall, DKK1 did not modify the commitment of MNCs to ECFCs; instead, it stimulated their angiogenic properties in vitro.

CXCR4 and Vascular Endothelial Growth Factor Receptor 2 Pathway Blockade Inhibits ECFC Proliferation and Tube Formation Induced by DKK1 Activation

To decipher the specific role of DKK1 in ECFCs, we quantified DKK1-induced transcriptional modifications by real-time RT-qPCR. We focused on angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietins, and SDF-1, and their receptors. No change in angiopoietin gene expression was observed. In contrast, we found that VEGF receptor 2 (VEGFR2), SDF-1, and CXCR4 were slightly increased after 8 and 12 hours; a strong increase was
observed after 24 hours (supplemental Table). CXCR4 and VEGFR2 protein expression, quantified by flow cytometry, was increased at 48 hours (supplemental Figure VII). Given the role of DKK1 in VEGFR2 and CXCR4/SDF-1 gene expression, we examined the effect of anti-CXCR4– and anti-VEGFR2–blocking antibodies on DKK1-induced proliferation and tubule morphogenesis. The proliferative effect of DKK1 was abrogated by anti-VEGFR2 but not by anti-CXCR4 (Figure 1E). Conversely, the DKK1-induced increase in tube formation in Matrigel was blocked by anti-CXCR4 but not by anti-VEGFR2 (Figure 1F) or by the irrelevant isotype-matched antibody (supplemental Figure VIII and supplemental Figure IX).

**Comparative DKK1, VEGFR2, and SDF-1 Pathway Gene Expression in Human Breast Tumors**

To explore the role of DKK1 in breast tumor vascularization, we quantified the expression of DKK1, VEGFR2, CXCR4, and SDF-1 by real-time RT-qPCR and the degree of neovascularization, quantified by von Willebrand factor (vWF) expression, in a well-characterized series of 62 estrogen receptor α–positive human breast tumors. We found that DKK1 expression was increased in breast cancer tissue compared with normal breast tissue (data not shown) and that DKK1 correlated positively with vWF content and with VEGFR2 and SDF-1 transcript levels (Spearman rank correlation test, Table).
The association between DKK1 expression and tumor vascularization was confirmed at the protein level by comparative immunohistochemical analysis of tissue sections from human breast malignant tissues (n=5) and normal breast tissues (n=5). Vessels present in the tumor specimens and healthy breast tissues stained positive for CD31, whereas only tumor vessels stained positive for DKK1 (Figure 2A through 2D). Moreover, we quantified, by immunofluorescence analysis, the expression of CD31 (red, Figure 2E) and DKK1 (green, Figure 2F) and show a colocalization of CD31 and DKK1 in endothelial cells of human breast cancer (merge, supplemental Figure X).

### Table. Relationship Between Expression of DKK1 and Identified DKK1-Inducible Genes in 62 Primary Breast Tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Spearman Correlation Coefficient</th>
<th>$P$ Value, Spearman Rank Correlation Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF</td>
<td>0.319</td>
<td>0.011</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>0.282</td>
<td>0.024</td>
</tr>
<tr>
<td>CXCR4</td>
<td>0.028</td>
<td>NS</td>
</tr>
<tr>
<td>SDF-1</td>
<td>0.437</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

NS indicates not significant.

**DKK1 Silencing in Cord Blood ECFCs Decreases Their Angiogenic Phenotype**

EPCs are involved in cancer development and neovascularization, and the level of angiogenic activity in breast tumors is a prognostic factor. Newly formed vessels that appear after treatment with BM-MNCs in patients with critical leg ischemia have an ECFC phenotype, confirming preclinical data from Yoder et al. HEREIN, we found that ECFCs expressed DKK1 transcripts, contrary to CACs (Figure 3A). We then explored the effect of DKK1 gene inhibition by RNA interference on ECFC angiogenic potential. We verified that DKK1 mRNA expression remained strongly inhibited by the specific small and interfering RNA (siRNA) during the days after transfection (Figure 3B) and confirmed that siRNA induces a strong decrease in DKK1 levels in ECFC supernatants ($P=0.0002$, Figure 3C). We then explored the effect of DKK1 gene inhibition on the angiogenic properties of ECFCs in vivo. DKK1 inhibition resulted in decreased expression of SDF-1, CXCR4, and VEGFR2 (Figure 3D). These findings are in line with an autocrine effect of DKK1 on ECFCs, which partially reproduces the effect of exogenous DKK1. We then studied the effect of DKK1 inhibition on ECFC angiogenic properties and found that DKK1-siRNA induced a strong decrease in proliferation and pseudotube formation (Figure 3E and 3F). Finally, we tested DKK1-inhibited ECFCs in a nude mouse...
model of hind limb ischemia and found a significant decrease of revascularization of DKK1-inhibited ECFCs compared with control ECFCs \( (P<0.05, \text{Figure } 3G) \).

**Effect of DKK1 on Angiogenesis In Vivo**

We used the Matrigel plug assay as a model of in vivo angiogenesis. Basic fibroblast growth factor–containing plugs, with and without DKK1 (100 or 1000 ng per plug), were implanted subcutaneously in C57/B16J mice. The plugs were recovered 14 days later, and their blood vessel content was analyzed. DKK1 enhanced blood vessel formation in the plugs macroscopically (Figure 4A and 4B), as confirmed by measuring the hemoglobin content (Figure 4C and supplemental Figure XI) and the sVEGFR2 content (Figure 4D).

**Figure 3.** Inhibition of DKK1 in ECFC decreases their angiogenic potential in vivo. A, The DKK1 gene is expressed by ECFCs, contrary to CACs. B, Quantitative analysis of DKK1 mRNA by RT-qPCR, after 24, 48, and 72 hours of transfection with control-siRNA and DKK1-siRNA. The mean±SEM of 3 experiments is shown. \( P<0.05 \). C, Quantitative analysis of DKK1 release over 24 hours in conditioned medium of ECFCs after 48 hours of transfection. The mean±SEM of 3 experiments is shown. \( P<0.05 \). D, Inhibition of DKK1 in ECFCs decreases expression of VEGFR2, SDF-1, and CXCR4 (RT-qPCR). Values are given as mean±SEM \((n=3)\). E, Inhibition of DKK1 in ECFCs decreases proliferation potential of ECFCs, measured by pNPP release. Values are given as mean±SEM \((n=3)\). F, Inhibition of DKK1 in ECFCs decreases pseudotube formation by ECFCs. Values are given as mean±SEM \((n=3)\). G, Inhibition of DKK1 in ECFCs decreases their proangiogenic potential in hind limb ischemia. Values are given mean±SEM \((n=10 \text{ mice per group})\). AB indicates adult peripheral blood; CB, cord blood.
To confirm in a second model the implication of DKK1 in neovascularization, we used a model of breast cancer xenograft in nude mice. When the tumor reached a volume of approximately 200 mm$^3$, mice were injected peritumorally with 100 or 500 ng of DKK1 every 3 days over 14 days. Tumor development was monitored every 3 to 4 days using a caliper. As shown in Figure 4E, a significant tumor growth increase was obtained by the administration of 100 ng per mouse ($p<0.05$). Similar results were obtained with 500 ng per animal (data not shown). Because these data suggested that neovascularization could have promoted tumor development, mice were euthanized at day 18 after the beginning of the treatment and tumors were collected for immunofluorescent assays. Anti-CD31 staining clearly revealed that xenografts treated with DKK1 displayed more blood vessels (Figure 4F, right panel) than those injected only with the vehicle (Figure 4F, left panel).

DKK1 Silencing in Mesenchymal Stem Cells Decreases Their Angiogenic Potential

MSCs have been described in the tumor angiogenic process$^{21}$ and are a potential source of DKK1.$^{22}$ We used MSCs isolated from the bone marrow of patients with critical leg ischemia (Figure 5A) and inhibited DKK1 secretion by RNA interference (Figure 5B). Decreased DKK1 expression was associated with a decrease in MSC proliferation (Figure 5C). The conditioned medium of MSCs transfected with DKK1-siRNA or control-siRNA was used to activate ECFCs. We found that MSC–conditioned medium from DKK1-siRNA–transfected MSCs had a smaller effect on ECFC proliferation (Figure 5D) and pseudotube formation (Figure 5E) compared with control siRNA-transfected MSCs. Finally, MSCs transfected with DKK1-siRNA had lower angiogenic potential in ischemic nude mice (Figure 5F). These results suggest that MSCs have angiogenic potential in vitro on ECFCs and in vivo, probably, at least in part, because of their secretion of DKK1.

Discussion

Recent studies$^{1,19}$ have suggested an important role of Wnt signaling in vascular development and cancer. Herein, we show that the Wnt antagonist DKK1 is an angiogenic factor for ECFCs, which participate in neovessel formation in human and preclinical models of lower-limb ischemia.$^{11,12}$ As
A role of the Wnt canonical inhibitor, DKK1, has been implicated in malignant neoplasms, a situation in which neoangiogenesis is strongly enhanced; we chose to explore the function of DKK1 in ECFCs. Physiologically, DKK1 mobilizes progenitor cells from the bone marrow endosteal stem cell niche. In breast cancer, the DKK1 expression level correlates with the rate of progression. In atherosclerosis, DKK1 is overexpressed in plaque and enhances the inflammatory interaction between platelets and endothelial cells. However, little is known about the role of DKK1 in adult angiogenesis mediated by ECFCs.

Aicher et al showed that DKK1 suppresses canonical Wnt signaling in bone marrow endosteal cells, suggesting a regulatory role for DKK1 in the bone marrow stem cell niche.
Intriguingly, DKK1 mobilized vasculogenic progenitor cells without concomitant release of inflammatory cells. CACs are committed to the endothelial lineage but retain monocyte markers. These cells could be considered as inflammatory cells because they are mobilized in this context. One possible explanation for the lack of mobilization could be inadequate LRP5/LRP6 levels and β-catenin pathway activity in inflammatory cells, as observed in CACs.

Wnt signaling modulates the stem cell niche and directly modulates stem cell proliferation, expansion, and differentiation. Moreover, Wnt was recently shown to induce embryonic stem cell commitment to the endothelial lineage. Therefore, DKK1 might diminish, rather than enhance, ECFC proliferation. The observed stimulation of ECFC proliferation by DKK1 may be explained by the fact that DKK1 can only affect progenitor cells with active Wnt signaling. Indeed, our experiments involved homogeneous populations raised from single EPCs. These findings suggest that DKK1 might have the potential to enhance vasculogenic cell therapy approaches.

Herein, we show that DKK1 added to the plugs enhances vasculogenesis in situ. In the experiments conducted by Aicher et al., DKK1 systemically administered induced a mobilization of stem cells from the bone marrow niche into the plugs. One limitation of our study is the difficulty in demonstrating that Wnt signaling is blocked in the presence of DKK1 in the vessels within the Matrigel plugs in vivo. Moreover, we cannot exclude any effect of DKK1 on a wide variety of cells (eg, leukocytes, which may be recruited [or prevented from being recruited] into the Matrigel plugs). These cells might produce angiogenic factors and could directly affect local endothelium. However, we explored DKK1 effect on human umbilical vein endothelial cells and found that, in this mature cell type, DKK1 had no influence on proliferation.

DKK1 was recently reported to be involved in cardiovascular lineage commitment. However, therapies based on DKK1 may result in increased mobilization of vasculogenic progenitor cells and, subsequently, in increased local neovascularization processes that might drive tumor progression. In atherosclerosis, a recent study showed that platelets are an important source of DKK1 and that DKK1 influences platelet-mediated endothelial cell activation and atherosclerotic lesions. The given importance of platelets as a source of both angiogenic factors, such as VEGF, and DKK1, modulation of platelet secretion might represent a further means of controlling angiogenesis, such as MSCs. Because MSCs have been described in the tumor angiogenic process and are a potential source of DKK1, we inhibited DKK1 in MSCs and showed, such as previously described for another Wnt inhibitor (ie, FRP1), that secreted DKK1 had an angiogenic effect on ECFCs. Moreover, DKK1-inhibited MSCs had a smaller therapeutic angiogenic effect in vivo.

In conclusion, we present evidence that the Wnt inhibitor, DKK1, enhances ECFC angiogenic in vitro and in vivo properties. DKK1 could exert its effects via the VEGFR2 and SDF-1 signaling pathways. The positive correlation between DKK1 expression and vWF, VEGFR2, and SDF-1 expression in breast tumors suggests that dysregulated or DKK1-stimulated EPCs might play an important role in breast cancer and neovascularization. As a specific angiogenic growth factor, DKK1 might serve to optimize regenerative cell therapy and as a therapeutic target for inhibiting neovascularization.

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We thank Chantal Martin and all technicians from the animal facility of IMTCE; and the maternity department of Begin hospitals for providing cord blood. Umbilical cord blood samples were collected from consenting mothers.

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Disclosures
None.

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The Wnt antagonist Dickkopf-1 increases endothelial progenitor cell angiogenic potential

David M. Smadja et al. Supplementary data

Materials and methods

Preparation of mononuclear cells (MNC)

Cord or adult blood was diluted 1:1 with PBS, EDTA 0.2 M and overlaid on Histopaque-1077 (Sigma-Aldrich, Saint-Quentin Fallavier, France). Cells were centrifuged at 1200 g for 20 min. MNC were collected and washed three times in PBS, EDTA 0.2 M.

EPC culture and transfection

CAC and ECFC were cultured and characterized as described in detail elsewhere. Recombinant DKK1 from R&D Systems (Lille, France) was added to ECFC on the first day of culture and was present in all freshly added media. DKK1 has been tested on ECFC commitment in adult and cord blood, while angiogenic effect has been studied only in cord blood derived ECFC.

SiRNA silencing DKK1 (sc-37082, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Id2 (sc-38000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was mixed with the Primefect reagent (LONZA) at 10 µM to obtain transfection complexes, which were added to 2x10^5 cord blood (CB) late EPC in 6-well plates containing complete EGM2 medium. Scrambled siRNA at 10 µM (Allstars Neg. control siRNA, Qiagen, Cambridge, MA, USA) was used as control.
Real-time quantitative RT-PCR

The theoretical and practical aspects of real-time quantitative RT-PCR on the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems Courtaboeuf, France) are described in detail elsewhere.\(^6\) Primers for TBP and the target genes (nucleotide sequences available on request) were chosen with the assistance of Oligo 5.0 software (National Biosciences, Plymouth, MN, USA).

Cell proliferation assay

The effect of DKK1 and siRNA Id2 on ECFC proliferation was examined by measuring cell phosphatase activity based on the release of paranitrophenol (pNPP) (Sigma) measured at OD 405 nm (Fluostar optima; BMG labtech, Champigny-sur-Marne, France) after 72 h of incubation. EPC were activated in 5% FBS-EBM2 medium containing recombinant DKK1 (100 ng/mL, R&D systems).

In vitro Matrigel tube formation assay

ECFC (3x10\(^4\) cells/well) treated with 100 ng/ml DKK1 in 5% FBS-EBM2 medium were seeded on growth-factor-reduced Matrigel (200 µl) (BD Biosciences) and cultured for 18 h at 37°C with 5% CO2. Capillary-like structures were observed by phase-contrast microscopy and networks formed by ECFC were quantified with Videomet software version 5.4.0.

In vivo Matrigel-plug assay

As previously described\(^7\), Matrigel-plugs were prepared on ice by mixing Matrigel (BD Matrigel Matrix) with recombinant mouse bFGF (750 ng/mL, R&D systems) in the presence or absence of recombinant mouse DKK1 (200 ng per mL of Matrigel). The haemoglobin concentration was measured in the supernatants with Drabkin’s reagent (Sigma, Saint Louis,
Mo, USA). The mouse sVEGFR2 concentration in the supernatant was determined with an ELISA method (R&D Systems), following the manufacturer’s instructions.

**Human breast carcinoma xenografts in mice**

Human breast carcinoma HBCx-12 xenograft has been previously described. HBCx-12 xenograft fragments of 30 mm$^3$ were subcutaneously grafted into the interscapular fat pad of 4- to 6-week-old female athymic nude mice (Janvier, Le Genest St Isle, France).

When the tumors reached a volume of approximately 200 mm$^3$, animals were ranked according to the tumor volume and divided into three groups (6 animals per group) such that the mean and median of tumor volume of the three groups were closely matched. Treatment was started on day 1 and given during 3 weeks. Mice were injected twice a week peritumorally with recombinant mouse DKK1 at a dosage of 100 ng or 500 ng /animal, or with PBS containing 0.1% Bovine Serum Albumine (MP Biomedicals, Illkirch, France) as vehicle control, in a total volume of 100 µL. Tumors were measured thrice per week with calipers and each volume ($V$) was calculated according to the following formula: $V = a \times b^2 / 2$, where $a$ and $b$ are the largest and smallest perpendicular tumor diameters. Relative tumor volumes (RTV) were calculated from the following formula: $\text{RTV} = (V_x / V_1)$, where $V_x$ is the tumor volume on day $x$ and $V_1$ is the tumor volume at initiation of treatment (day 1).

Statistical significance of differences observed between the individual RTVs corresponding to the treated mice and control groups was calculated by the paired Student's $t$ test.

**Tissue processing and immunofluorescence**

For confocal microscopy, HBCx-12 tumors were fixed overnight in 4% paraformaldehyde at 4°C, cryoprotected in 15% sucrose before freezing in cryomatrix (Thermo Shandon, Cergy Pontoise, France) with liquid nitrogen-cooled isopentane. Cryosections of embedded tumors
were cut at 10-μm thickness, air-dried, and treated with 50 mmol/L NH₄Cl in PBS for 15 minutes. Sections were then solubilized with 0.2% Triton X-100 for 15 minutes and blocked with PBS containing 1% BSA and 10% goat serum for 1h. Tumors sections were stained at room temperature for 1h with anti-human EpCAM (clone HEA-125) fluorescein isothiocyanate (Miltenyi-Biotec SAS, Paris, France) and anti-mouse CD31 (BD Pharmingen, Evry, France) in PBS followed by an incubation with anti-rat Alexa-Fluor®555 secondary antibody (Invitrogen-Molecular Probes, Cergy Pontoise, France) for 30 minutes. The DNA marker, TOPRO-3 (Invitrogen-Molecular Probes) was then applied for 10 min at room temperature. Sections were mounted in glycerol/PBS (90/10: v/v) and images were recorded on a Leica TCS SP2 confocal microscope.

Patients with Erα-positive breast tumors

To identify correlations between mRNA levels of the genes of interest in vivo, we analyzed a well-characterized serie of 62 ERα-positive breast tumors excised at René Huguenin Hospital (St Cloud, France). Immediately after surgery the tumor samples were placed in liquid nitrogen until RNA extraction. The patients met the following criteria: primary unilateral non metastatic breast carcinoma; complete clinical, histological and biological information available; no radiotherapy or chemotherapy before surgery; and full follow-up at this institution. Estrogen receptor alpha status was determined at the protein level by enzyme immunoassay and confirmed by ERα mRNA real-time quantitative RT-PCR assay.

Immunohistochemistry of human breast tissue

Paraffin-embedded tissue sections were deparaffinized and hydrated in xylene and graded alcohol series. Antigen retrieval was achieved by microwave treatment in citrate buffer (10 mM, pH 6.0), and endogenous peroxidase activity was blocked with 5% H₂O₂/methanol.
Sections were incubated in blocking solution containing 10% bovine calf serum (Dako Cytomation) for 45 min and then stained for 1 h with primary antiserum (rabbit anti-human DKK1 polyclonal, ab61034, Abcam®, Cambridge, UK). Serial sections were also incubated with a mouse anti-human CD31 monoclonal (1:40, Dako Cytomation). Immunohistochemistry (IHC) slide were performed the NexES System’s automated technology (Ventana®).

**Statistical analysis**

Data are shown as means ± SEM. Significant differences were identified by ANOVA followed by Fisher’s protected least-significant-difference test. Correlations between DKK1 and target tumoral gene expression (continuous variables) were tested with Spearman’s non parametric rank correlation test. Differences were considered significant at confidence levels greater than 95% (p < 0.05). The Stat View software package (SAS, Cary, NC, USA) was used for all statistical tests.
**Supplementary Results:**

**Effect of DKK1 on ECFC commitment**

*(Figure 1A, 1B and supplementary Figure V)*

As shown in figure 1A, adding DKK1 the first week of culture did not modify the number of ECFC colonies obtained with MNC from either cord blood or adult blood (p = 0.31 and p = 0.23 for cord and adult blood, respectively). These results were obtained with 10 cord blood samples and 8 adult blood samples. We also underline differences in ECFC numbers according to the origin of blood: counts were 25-fold higher in cord blood (median 2 colonies per 5x10^6 MNC) than in adult blood (0.08 colony). As previously described by Yoder’s group, ECFC emerged more rapidly from cord blood (median 7 days) than from adult blood (17.5 days), and DKK1 did not modify the length of this period (Figure 1B, p = 0.22 for cord blood, p = 0.92 for adult blood). Moreover, we observed a more important size of colonies observed at day 14 of culture (supplementary Figure V).

**Reversal of DKK1-induced cytokine expression and angiogenesis by Id2 silencing**

*(supplementary figure XII)*

To understand mechanism of DKK1 induced angiogenesis, we also focused our attention on two well-known Wnt-inducible genes, MSX2 and Id2. While no MSX-2 modification was observed, the level of Id2 mRNA increased rapidly (after 1 hour) after DKK1 treatment (100 ng/mL) of ECFC. Id2 mRNA level was inhibited by an average of 92%, 48 h after ECFC transfection with specific siRNA at 10 µM. Id2-siRNA-transfected ECFC were incubated with 100 ng/mL DKK1. Id2 silencing roughly halved VEGFR2 expression compared to cells transfected with the control-siRNA, and completely abolished basal CXCR4 and SDF-1
mRNA levels. DKK1 no longer exerted its stimulatory effect on VEGFR2, CXCR4 and SDF-1 expression.

To confirm the role of VEGFR2, CXCR4 and SDF-1 via Id2, we quantified proliferation and tube formation by Id2-siRNA-transfected cells in basal conditions and after DKK1 activation. DKK1 activation of Id2-siRNA-transfected ECFC induced no proliferation or tubule formation, confirming the importance of Id2 activation in DKK1-induced ECFC angiogenesis in vitro.

DKK1 effects are at least partly mediated by Id2, which upregulates KDR/VEGFR2, SDF-1 and CXCR4 gene expression. Other teams have obtained similar results in different biological settings, indicating that VEGF pathway is a target of the Wnt signaling pathway in neoplasia. Id2 is a Wnt-inducible gene with an important role in angiogenesis during embryonic development and angiogenesis. The Id family consists of four members (Id1-4) that inhibit the activity of basic helix loop helix transcription factors by hindering their DNA binding. Loss of the Id1 gene in bone marrow leads to impaired tumor angiogenesis, through a failure of hematopoietic stem cells (Lin-) to differentiate towards the endothelial lineage. Furthermore, Id knock-out mice display vascular abnormalities in the forebrain and do not support the growth of xenografted tumors, probably owing to inadequate neovascularization. Sustained Id expression delays the onset of senescence in human endothelial cells and stimulates tube formation and migration by endothelial cells. Id2 induces the VEGF and SDF-1 pathways. We found that Id2 silencing abolished DKK1 activation of the VEGF and SDF-1 pathways. As VEGF and SDF-1 are two cytokines strongly involved in ECFC-induced neoangiogenesis, it is conceivable that neovascularization would be potently induced in ischemic disease by cell or gene therapy, locally stimulating Id production.
Supplementary Figure I. Characterization of CAC and ECFC.

A. CAC

B. ECFC
**Supplementary Figure II. Wnt canonical pathway in CAC and ECFC.**

**A/B.** Comparative LRP-5 and -6 and lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factor expression by AB-CAC and AB/CB-ECFC by RT-QPCR. mRNA levels were normalized to the *TBP* gene and to the lowest quantifiable level (i.e. 1 on the left ordinate, corresponding to a gene target Ct value of 35).

**A.**

**B.**

**C.** Western blots of β-catenin in AB-CAC, AB-ECFC and CB-ECFC.
Supplementary Figure III. β-catenin mRNA levels in CAC and ECFC.
Supplementary Figure IV. Expression of Wnt ligands and receptor in CAC

A.

B.
Supplementary Figure V. Photomicrograph of a representative colony of adult ECFC without DKK1 in culture at 14 days of culture.
Supplementary Figure VI. DKK1 did not modify HUVEC proliferation
Supplementary Figure VII. Flow cytometry analysis of CXCR4 and VEGFR2 expression

Expression of KDR (A) and CXCR4 (B) by flow cytometry is increased after a 48 hours of DKK1 stimulation.
Supplementary Figure VIII: Effect of control antibody on ECFC proliferation and pseudotubes formation with and without DKK1 stimulation.

A- Control IgG mAb does not modify ECFC proliferation.

B- Control IgG mAb does not modify ECFC pseudotube formation.
Supplementary Figure IX: Effect of specific blocking antibodies on ECFC proliferation and pseudotubes formation with and without DKK1 stimulation.

A- anti-VEGFR2 decrease ECFC proliferation in contrary to anti-CXCR4 in EBM, 5%SVF

B- anti-VEGFR2 and anti-CXCR4 do not modify ECFC pseudotubes formation in contrary to in EBM, 5%SVF.
Supplementary Figure X. DKK1 is expressed in ECFC and in blood vessels from human breast cancer.

Positivity of tumor blood vessels was confirmed by double labeling by immunofluorescence analysis (Merge, red: CD31, green: DKK1)
Supplementary Figure XI. Mouse DKK1 at 100 and 1000 ng/ml increases neovascularization in vivo
Supplementary Figure XII. Id2 gene silencing by siRNA in ECFC abolishes DKK1 enhancement of proliferation and differentiation.

A. Analysis of Id2 expression by RT-qPCR on ECFC, 48 hours after transfection with si scramble (All Star negative control, Qiagen®) and siId2 (Santacruz Biotechnologies®) using Primefect® (LONZA). *$P<0.05$

B. Inhibition of VEGFR2, SDF-1 and CXCR4 expression in siId2 transfected cells in basal conditions. The mean ± SEM of at least three experiments are shown. *$P<0.05$
C. Inhibition of Id2 in ECFC abolishes DKK1 activation of proliferation measured by pNPP release.

D. Inhibition of Id2 in ECFC abolishes DKK1 activation of pseudotube formation measured with Videomet software®.
Supplementary Table 1. Effect of DKK1 on mRNA levels of major growth factors involved in ECFC-induced angiogenesis.

ECFC were stimulated with DKK1 (100 ng/mL) for 15 min, 1, 4 and 24 hours after 16 hours of serum and growth-factor privation. mRNAs were measured by real-time quantitative RT-PCR, normalized to TBP mRNA and compared to unstimulated ECFC (= 1).

<table>
<thead>
<tr>
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<th>Fold increase after ECFC activation with DKK1</th>
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<tbody>
<tr>
<td></td>
<td>15 min</td>
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<tr>
<td>VEGF-A</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>1.8 ± 1.1</td>
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<tr>
<td>ANG-2</td>
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<tr>
<td>TIE-2</td>
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<tr>
<td>SDF-1</td>
<td>1.4 ± 0.3</td>
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<tr>
<td>CXCR4</td>
<td>1.0 ± 0.0</td>
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References