Biochemical and Biophysical Research Communications 418 (2012) 173–179

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of Notch signaling by Dll4-Fc promotes reperfusion of acutely ischemic tissues

Ren Liu [a,1], Alexandre Trindade [b,c,1], Zhanfeng Sun [d,1], Ram Kumar [e], Fred A. Weaver [e], Valery Krasnoperov [f], Kranthi Naga [f], Antonio Duarte [b,c], Parkash S. Gill [a,*]

[a] Department of Pathology, University of Southern California, Los Angeles, USA
[b] Centro Interdisciplinar de Investigação em Sanidade Animal (CIISA), Lisbon Technical University, Lisbon, Portugal
[c] Instituto Gulbenkian de Ciência, Oeiras, Portugal
[d] Department of Vascular Surgery, 2nd Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China
[e] Department of Surgery, University of Southern California, Los Angeles, USA
[f] Vasgene Therapeutics, Los Angeles, CA, USA

ARTICLE INFO

Article history:
Received 24 December 2011
Available online 9 January 2012

Keywords:
Dll4/Notch
Dll4-Fc
Angiogenesis
Perfusion
Ischemia

ABSTRACT

Notch pathway regulates vessel development and maturation. Dll4, a high-affinity ligand for Notch, is expressed predominantly in the arterial endothelium and is induced by hypoxia among other factors. Inhibition of Dll4 has paradoxical effects of reducing the maturation and perfusion in newly forming vessels while increasing the density of vessels. We hypothesized that partial and/or intermittent inhibition of Dll4 may lead to increased vascular response and still allow vascular maturation to occur. Thus tissue perfusion can be restored rapidly, allowing quicker recovery from ischemia or tissue injury. Our studies in two different models (hindlimb ischemia and skin flap) show that inhibition of Dll4 at low dose allows faster recovery from vascular and tissue injury. This opens a new possibility for Dll4 blockade's therapeutic application in promoting recovery from vascular injury and restoring blood supply to ischemic tissues.

1. Introduction

Vascular response to tissue damage is a well-organized physiological process [1]. Following loss of tissue perfusion or traumatic injury, angiogenesis is initiated by inflammatory response, release or induction of growth factors, and remodeling of cell-matrix [1]. New blood vessels grow via a cascade of well orchestrated events supporting tissue repair. This process is sustained until the terminal stages of healing, when angiogenesis is slowed by reduced levels of growth factors, resolution of inflammation, and stabilized tissue matrix [2]. Several pro-angiogenic factors have been investigated in order to accelerate wound repair, including recombinant human platelet-derived growth factor-BB (rhPDGF-BB) with success [3,4]. Other factors under investigation include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF), and transforming growth factor-beta (TGF-β) [5].

Notch signaling plays a central role in angiogenesis. Dll4 is an endothelium-specific Notch ligand [6]. Its expression is largely restricted to arteries, especially during embryonic development, while in adults it is expressed on small arteries and capillary networks [7,8]. *Dll4* haploinsufficiency in mice results in embryonic lethality at approximately E10.5 [7]. It is the only known gene with a haploinsufficient-lethal phenotype due to failure to form a functional vasculature besides VEGF, highlighting its critical role in angiogenesis.

Recently the blockade of Notch pathway with either a Dll4-selective neutralizing antibody or a soluble Dll4 fusion protein (Dll4-Fc) showed inhibition of tumor growth associated with an increase in tumor vascular density but poor vessel perfusion. It appears that excessive chaotic branching, lack of lumen formation and impaired vessel maturation are responsible for anti-tumor activity [9–11]. This can be explained by the fact that Dll4/Notch negatively regulates pro-angiogenic factors, such as VEGF, and positively regulates vascular maturation factors, like PDGFR-β and TGF-β [12,13]. Thus, anti-Dll4 therapy results in increased vascularization with decreased vascular maturation and, therefore, decreased vascular function [14]. We hypothesized that Dll4/Notch inhibition has a dose-dependent manner such that it may be possible to modulate vascular response to achieve pro-angiogenic outcome. Thus a modest increase in functional vascular proliferation can occur in which vessels grow orderly, form lumen and are able to carry blood flow. Here we provide

* Corresponding author. Address: University of Southern California, Norris Hospital, NOR 6332, 1441 Eastlake Avenue, Los Angeles, CA 90033, USA.
E-mail address: parkashg@usc.edu (P.S. Gill).
[1] These authors contribute equally to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2012.01.002

in vivo evidence supporting the use of anti-Dll4 therapy to increase vascularization without decreasing vascular function, and thus improve the recovery from acute vascular ischemia and tissue injury.

2. Materials and methods

2.1. Experimental animals

All procedures involving animals in this study were approved by the Institutional Animal Care and Use Committee of the University of Southern California or the Faculty of Veterinary Medicine of Lisbon Ethics and Animal Welfare Committee. The generation of *Dll4*$^{+/-}$ (*Dll4/LacZ*) mice on CD1 background has been reported previously [7]. *Dll4* conditional gain-of-function mice, *tetO7-Dll4* [12], were crossed with *Tie2-rtTA* mice. Doxycycline (4 mg/mL in drinking water starting from 5 days before surgery) was given to double transgenic offspring throughout the experiment, inducing endothelial-specific transgene expression. The control mice have the same *Dll4* gain-of-function genotype but were not induced with doxycycline.

2.2. Hindlimb ischemia model

Hindlimb ischemia was performed as previously described [15]. Briefly, 12-week old Swiss Webster mice were anesthetized by intraperitoneal (i.p.) administration of 100 mg/kg of ketamine and 10 mg/kg of xylazine. Both the proximal portion of the femoral artery and the distal portion of the saphenous artery in the right leg were ligated with 7–0 suture. Branches between the two sites were ligated and all arteries in between were excised. After ligation, the mice received i.p. administration of Dll4-Fc (0.75, 2.5, or 10 mg/kg) or PBS twice a week for 4 weeks. Dll4-Fc was produced in mammalian cells and purified as described previously [16]. There were four animals per group. Animals were taken for microbubble ultrasound study after one week and sacrificed for histological analysis after 4 weeks.

2.3. VEGFR2-targeted microbubble ultrasound

The VEGFR2-targeted microbubble ultrasonographic contrast agent (UCA) was purchased from VisualSonics (Toronto, Ontario, Canada) and prepared as instructed. Mice were anesthetized with 1.5% isoflurane and restrained on a heated stage during the examination. The ultrasound transducer transmits at a central frequency of 40 MHz with a focal area of interest of 9×9 mm. A surgically implanted 26-gauge jugular vein catheter was used for injections. Each mouse received a 50 μL bolus of an isotype control antibody conjugated microbubble UCA (containing 3.8×10^7 microbubbles) followed by a 50 μL saline flush. The contrast agent was allowed to circulate for 4 min after each injection, providing sufficient time for binding of microbubbles. After 4 min, images were acquired with a resolution of 17 frames per second, followed by a high-power ultrasonic destruction (20 cycles, 10 MHz, mechanical index of 0.59). The system was then reset and images were acquired with the same imaging parameters as above. After 15 min the above procedures were repeated using VEGFR2 antibody conjugated microbubble. The UCA signal difference before and after destruction was used to estimate the relative VEGFR2 expression level [17]. Images were analyzed with the Vevo 770 scanner software (VisualSonics). The UCA used in this study showed no notable toxicity because all test animals recovered without complications after the procedure.

2.4. Myocutaneous skin flap procedure

Male mice of 10–15 weeks of age (4 mice per group) were used. Briefly, mice were shaved on the back and anesthetized with tribromoethanol. 2×2 cm Myocutaneous flaps were raised and detached on all four sides, and then sutured back in place. Flaps were photographed from the day of surgery (day 0) to serve as reference, and every day until the wound healed. On day 7 mice were again anesthetized and photographed under a stereomicroscope so that the scale was the same for all mice. The percentage of live tissue area versus total flap area was analyzed with ImageJ (NIH).

2.5. Immunohistochemistry

On each end-point mice were sacrificed and samples from the living tissue of the ischemic hindlimb or the flaps were collected, fixed in 4% paraformaldehyde (PFA) at 4 °C for 1 h, cryoprotected in 15% sucrose, embedded in 7.5% gelatine, frozen in liquid nitrogen and cryosectioned at 10 μm. Immunostaining of platelet endothelial cell adhesion molecule (PECAM) was performed to show vascular density, while staining of pericyte marker NG2 and smooth muscle cell marker alpha smooth muscle actin (α-SMA) was performed to examine vessel maturity. The rat anti-mouse PECAM antibody was from BD Pharmingen (San Jose, CA), rabbit anti-mouse NG2 antibody was from Chemicon (Temecula, CA), and rabbit anti-mouse α-SMA antibody was from Abcam (Cambridge, UK). Hypoxyprobe-1 and hypoxyprobe antibody (Chemicon, Temecula, CA) were used to stain hypoxic areas. Species-specific secondary antibodies conjugated with Alexa Fluor 488 or 555 were from Invitrogen (Carlsbad, CA). For immunofluorescence staining, the tissue sections were incubated with primary antibody overnight at 4 °C and secondary antibody for 1 h at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Molecular Probes, Eugene, OR). Fluorescent immunostained sections were examined under a Leica DMRA2 fluorescence microscope with Leica HC PL Fluotar 10 and 20×/0.5 NA dry objective, captured using Photometrics CoolSNAP HQ, (Photometrics, Friedland, Denmark), and processed with Metamorph 4.6–5 (Molecular Devices, Sunnyvale, CA). Images were analyzed with NIH ImageJ 1.37v. Vessel density corresponds to the percentage of each section field occupied by a PECAM-positive signal. To measure vascular maturity, mural cell recruitment was assessed by quantifying the percentage of PECAM-positive structures lined by α-SMA-positive coverage.

2.6. Vascular perfusion

To mark vessel perfusion in the ischemic hindlimb model, Rhodamine Ricinus communis agglutinin I (RCA) from Vector Laboratories (Burlingame, CA) (0.5 mg in 100 μl) was injected into the tail vein and allowed to circulate for 7 min before the mice were euthanized. The calf muscle was harvested, frozen on dry ice and stored at −80 °C until analysis.

To mark vessel perfusion in the skin flap model. tribromoethanol anesthetized mice were injected with Biotin-conjugated lectin from *Lycopersicon esculentum* (100 μg in 100 μl of PBS; Sigma, St. Luis, MO) via caudal vein and allowed to circulate for 5 min before the mouse was transcardially perfused with 4% PFA in PBS for 3 min. Flap samples were collected and processed as described above. Biotinylated lectin was visualised with Streptavidin-Alexa 488 (Invitrogen, Carlsbad, CA). The images were obtained and processed as described above. Myocutaneous flap perfusion was quantified by determining the percentage of PECAM-positive structures that were colocalized with lectin positive area.

2.7. Quantitative PCR analysis

Tissue samples were collected by dissecting living skin flap tissue from experimental mice on day 7 of the experiment and snap frozen in liquid nitrogen until RNA extraction (Qiagen RNeasy). First-strand cDNA was synthesized using a SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA). Real-time PCR analysis was performed as previously described [12], using specific primer pairs. Primer sequences are available upon request. Gene expression was normalized to β-actin.

3. Results

3.1. Microbubble ultrasound analysis indicates improved perfusion and increased VEGFR2 by Dll4-Fc treatment

Microbubble ultrasound technique has been used to determine the vascular perfusion *in vivo*, while a vascular endothelial growth factor receptor 2 (VEGFR2)-targeted ultrasonographic contrast agent (UCA) has been used to determine the extent of VEGFR2 expression in tumor vasculature [17]. A bolus of the UCA was injected and allowed to circulate, followed by acquisition of two sets of images before and after a high-power ultrasonic destruction sequence. The perfusion status in the ischemic hindlimb is reflected by the signal intensity of non-targeted UCA, while the VEGFR2 level in the vasculature is reflected by the extent of the decrease of VEGFR2-targeted UCA signal when all VEGFR2 bound microbubbles are destroyed by the ultrasonic destruction sequence. In this study, the mice with ischemic hindlimb received 2 doses of Dll4-Fc in one week immediately after femoral artery ligation. As shown in Fig. 1C, top panel, the isotype control antibody conjugated (non-targeted) UCA signal was significant higher in 2.5 mg/kg Dll4-Fc treated ischemic hindlimb compared to PBS treated hindlimb (Fig. 1A), close to the lateral control hindlimb (Fig. 1E). This implicates significantly improved perfusion after 2.5 mg/kg Dll4-Fc treatment. However, 0.75 mg/kg Dll4-Fc treatment had no significant benefit on the perfusion (Fig. 1B), and 10 mg/kg Dll4-Fc treatment led to decreased perfusion (Fig. 1C) compared to PBS group. In terms of VEGFR2-targeted UCA signal, it is higher than the non-targeted UCA only in 2.5 mg/kg Dll4-Fc treated hindlimb (Fig. 1C). In addition, destruction of microbubble led to a more significant drop of VEGFR2-targeted UCA signal in 2.5 mg/kg Dll4-Fc treated hindlimb compared to all other groups (Fig. 1A–E). These indicate that the VEGFR2 level was markedly increased in 2.5 mg/kg Dll4-Fc treated hindlimb compared to PBS group, and the higher (10 mg/kg) or lower (0.75 mg/kg) doses of Dll4-Fc did not induce VEGFR2 expression. Based on these results, only 2.5 and 0.75 mg/kg doses were chosen for later experiments.

3.2. Dll4-Fc facilitates the recovery of ischemic hindlimb

After the ligation of right femoral artery, the mice were intraperitoneally injected with PBS or Dll4-Fc (0.75 or 2.5 mg/kg, two times a week) for 4 weeks. In the PBS treated control group, the ligated foot developed signs of vascular insufficiency including pale color, tissue damage, ulceration, and even auto-amputation of toes (Fig. 2A, left). In contrast, the mice treated with 2.5 mg/kg Dll4-Fc did not show overt signs of ischemia in the foot (Fig. 2A, right). 0.75 mg/kg sDll4-Fc also showed protection from signs of vascular insufficiency, but to a less extent (Fig. 2A, middle). The statistics analysis of the ischemic appearance score of the animal feet (Fig. 2B) shows that 2.5 mg/kg Dll4-Fc treatment significantly

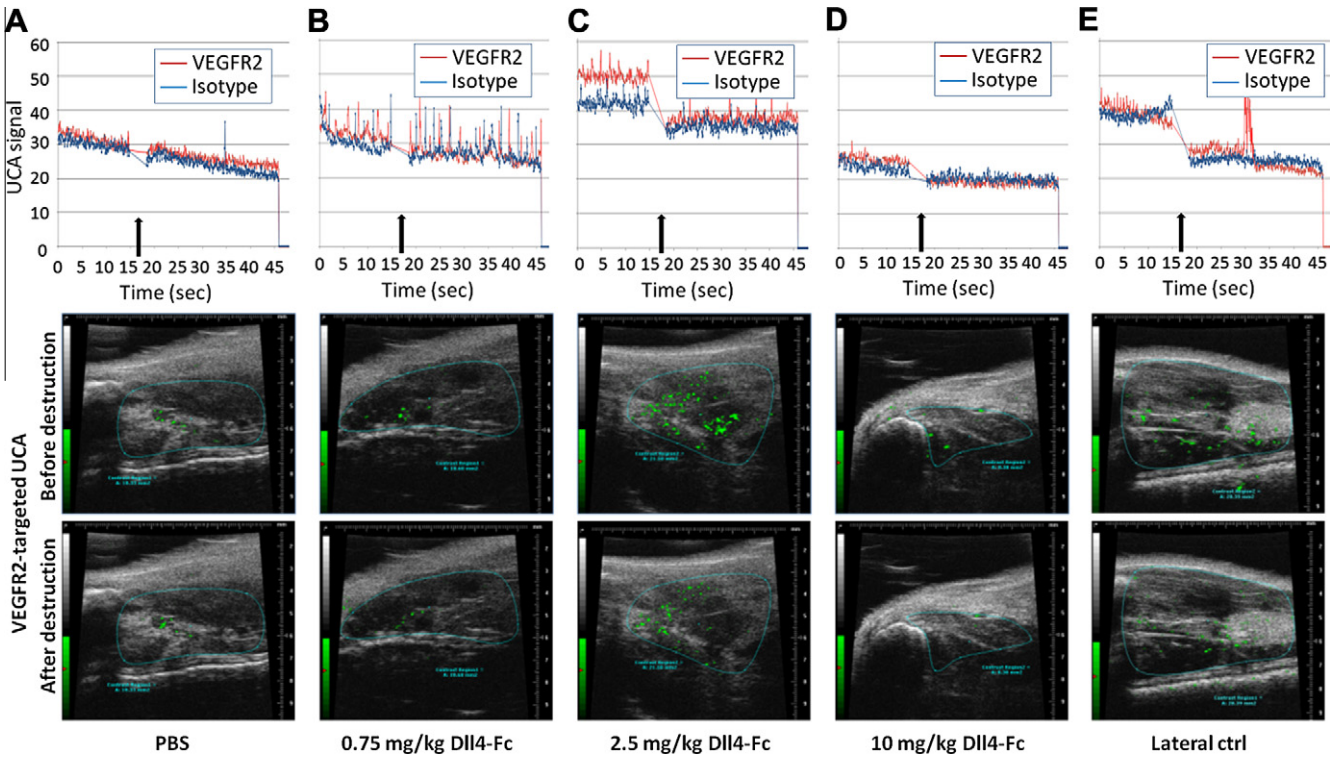

Fig. 1. Dll4-Fc increased perfusion and VEGFR2 expression in ischemia model based on VEGFR2 micro-ultrasound analysis. After femoral artery ligation, the mice with ischemic hindlimb received 2 doses of PBS (A), 0.75 mg/kg Dll4-Fc (B), 2.5 mg/kg Dll4-Fc (C), and 10 mg/kg Dll4-Fc (D) in a week. Isotype control antibody or VEGFR2 antibody conjugated UCA was injected and 2 sets of images of the hindlimb were taken, before and after a high-power ultrasonic destruction sequence (indicated by a black arrow in the top graphs). The measured UCA signal intensity was shown on the top. The representative ultrasound images of hindlimb after VEGFR2 targeted UCA injection were shown on the bottom. Images are superimposed on gray scale B-mode images of the hindlimb. The unligated hindlimb (lateral ctrl) was used as a control showing normal perfusion (E).

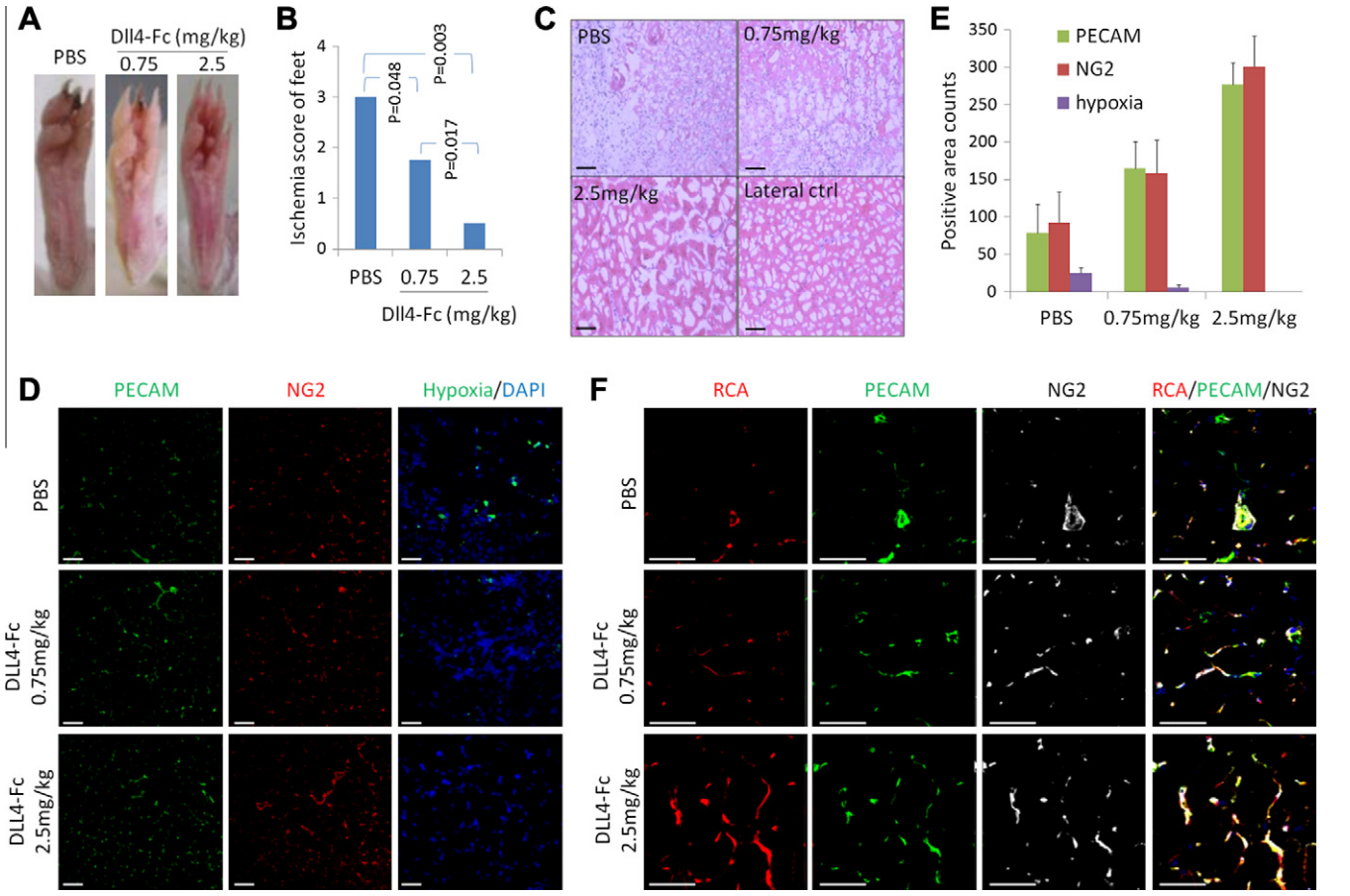

Fig. 2. Dll4-Fc facilitated the recovery from ischemia by promoting angiogenesis and perfusion. (A) Representative pictures showing that the ligated right foot in PBS, 0.75 mg/kg Dll4-Fc, or 2.5 mg/kg Dll4-Fc treated mice. (B) Dll4-Fc treatment improves ischemic foot appearance score. Scale: 0 = normal, 1 = pale foot or gait abnormalities, 2 = necrosis in one toe, 3 = necrosis in more than one toes or toenails falloff, 4 = toe auto-amputation; $n = 4$ per group. Statistics analysis was done with student T-test, 2 tails, and two-sample unequal variance. (C) Haematoxylin Eosin staining of the ischemic mouse calf tissue. The unligated left foot treated with 2.5 mg/kg Dll4-Fc is shown as "lateral ctrl". (D) The mouse muscle tissue was stained with PECAM (endothelial cell marker) antibody, NG2 (pericytes marker) antibody, and hypoxia probe (marking the hypoxia area). Nuclei were counterstained with DAPI. The quantification result of the positive areas was shown in (E). (F) Confocal images showing the perfusion (RCA) as well as blood vessels and pericytes. Bar = 20 μm.

($P = 0.003$) improved the recovery from ischemia. Tissue analysis revealed that the PBS treated ligated hindlimb had sparse muscle component, while 0.75 mg/kg Dll4-Fc treatment led to increased muscle tissue density (Fig. 2C). With 2.5 mg/kg Dll4-Fc treatment, the muscle structure was very similar to the contra-lateral unligated hindlimb control (lateral ctrl) (Fig. 2C).

3.3. Immunofluorescence staining reveals that Dll4-Fc promotes angiogenesis and perfusion in ischemic hindlimb

Four weeks after femoral artery ligation, the mice were sacrificed and the calf muscle tissue was collected for immunofluorescence analysis. PECAM staining indicated that the vessel density increased with the increasing dose of Dll4-Fc (Fig. 2D). Pericyte (NG2 positive cells) numbers also increased in a dose dependent manner to Dll4-Fc (Fig. 2D). Ischemia leads to profound tissue hypoxia. The hypoxia probe staining demarcates the regions of hypoxia and this response was prevented by Dll4-Fc in a dose dependent manner (Fig. 2D). The statistical analysis of the immunostaining signals is shown in Fig. 2E. The confocal imaging of perfusion (RCA), blood vessels (PECAM), and pericytes (NG2) show that in PBS and 0.75 mg/kg Dll4-Fc treated group, many blood vessels were not covered by pericytes and some were not perfused (Fig. 2F). In contrast, in 2.5 mg/kg Dll4-Fc treated group, most of the blood vessels were covered by pericytes and well perfused (Fig. 2F).

3.4. Dll4-Fc treated mice displayed improved tissue revascularization in skin flap model

The above results suggest that inhibition of Dll4-Notch signalling with Dll4-Fc can promote angiogenesis while allowing perfusion. However, in hindlimb ischemia model, the delivery of Dll4-Fc to the large ischemic areas in the hindlimb is limited because of the femoral artery ligation. That explains the relative high dose of Dll4-Fc needed to achieve efficient recovery in this model, even though this dose is five to ten times lower than needed to inhibit tumor growth. To test the pro-angiogenic activity of Dll4-Fc, we tested a super-low dose in a dorsal skin flap model in which delivery of the drug is not compromised. In this model a myocutaneous flap is raised and detached on all four sides, and then sutured back in place. Engraftment requires angiogenesis and vascular reperfusion from the lateral unaffected skin into the flap, reconnecting the vascular circuits. A dose of 0.05 mg/kg of Dll4-Fc was tested and found to be effective in promoting engraftment in this model in C57BL/6 mice. Mice were administered with Dll4-Fc every 48 h from day 0 to 7 via i.p. injection. After 7 days skin flaps in Dll4-Fc treated mice had more than twice area of living tissue compared to their respective controls, 75% versus

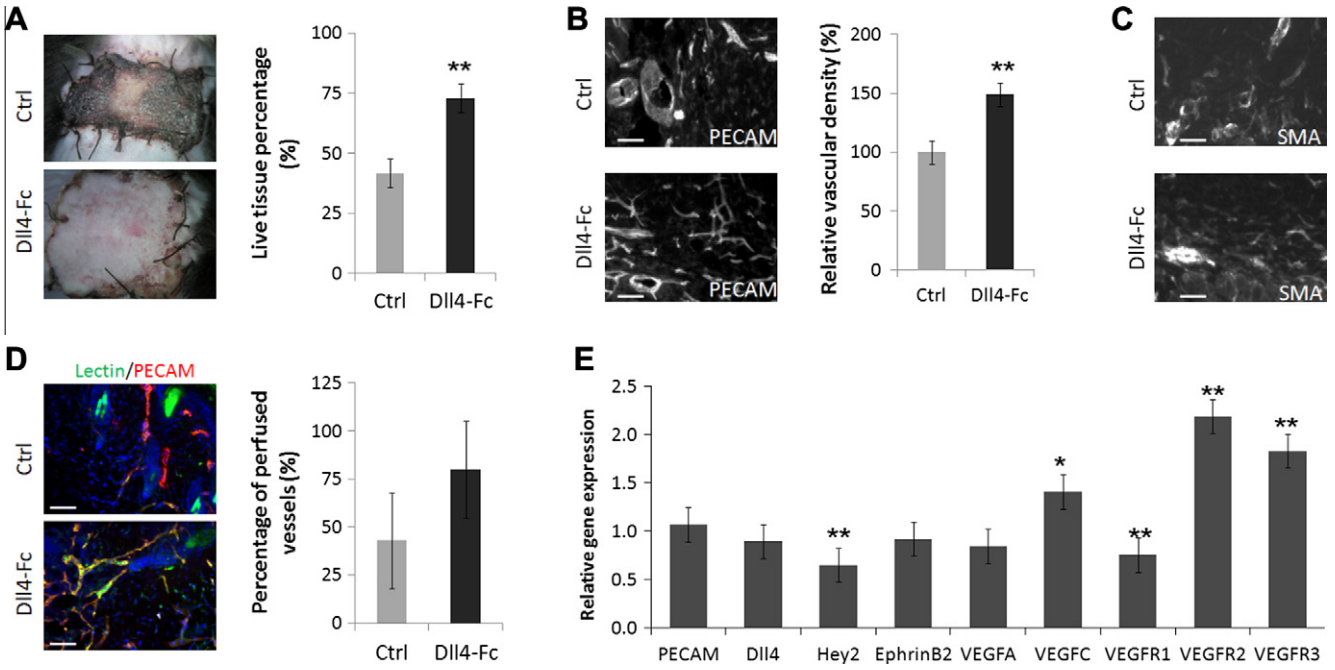

Fig. 3. Low dose Dll4-Fc therapy improves skin flap reperfusion and overall tissue survival. (A) The representative pictures of the wound at day 7. (B) Relative vascular density in the skin flap was analyzed with PECAM staining. The quantification of vessel density is shown on the right. (C) Recruitment of smooth muscle cells was analyzed with SMA staining. (D) Lectin perfusion assays (Lectin is in green and PECAM stained vessels are in red) revealed that the vessel perfusion in Dll4-Fc-treated flaps was improved compared to control. The quantification of the percentage of perfused vessels is shown on the right. (E) Living skin flap tissue samples were collected and subjected to quantitative RT-PCR analysis. Expression levels were corrected for PECAM-1 mRNA levels to compensate for variations in vascular density between samples. Statistical analyses were performed using Mann–Whitney-Wilcoxon test. All results are presented as mean ± SEM. *P*-values < 0.05 and <0.01 were considered significant (indicated in the figures with *) and highly significant (indicated with **), respectively. Bar = 30 μm.

35% (Fig. 3A). In the control group, many of the PECAM positive vessels were not perfused based on lectin staining (Fig. 3D). These vessels could be vessels left in the skin flap after surgery, which lacked the connection with the vasculature in the underlying tissue and hence failed to be perfused (Fig. 3D). Dll4-Fc treated skin flaps had 50% more blood vessels compared to their respective controls (Fig. 3B). All these blood vessels were covered with smooth muscle cells (Fig. 3C) and were perfused well (Fig. 3D). This indicates that new vessels sprouting from underlying tissue migrated into skin flap and established network with the vascular remnants.

In order to study biomarkers and surrogate markers of response for the observed phenotype we performed quantitative RT-PCR analysis of selected angiogenesis related genes (Fig. 3E). RNA was extracted from living skin flap tissue. Expression levels were corrected for PECAM-1 mRNA levels to compensate for variations in vascular density between samples. Dll4 expression was slightly reduced in Dll4-Fc treated skin flaps, probably reflecting the similar reduction observed in VEGF-A expression levels, which is one of its main inducers, although differences were not statistically significant. Reduced Hey2 expression levels confirmed the inhibitory effect of Dll4-Fc on Notch signaling. Ephrin-B2 expression was slightly reduced. VEGF-C expression was increased. Among the VEGF receptors, expression of VEGFR1 was decreased and expression of VEGFR2 and VEGFR3 was increased, indicating a trend to increase the angiogenic potential.

3.5. Skin flap tissue of *Dll4*$^{+/-}$ mice displayed improved survival

To further address the Dll4 dose dependent function in the regulation of myocutaneous flap reperfusion in genetic models, we used both *Dll4*$^{+/-}$ mice and endothelial-specific *Dll4* gain-of-function mutant mice. *Dll4*$^{+/-}$ mice were found to have a higher percentage of living myocutaneous flap tissue than their control mice, 75% versus 25% (Fig. 4A). Vascular density in *Dll4*$^{+/-}$ mice was almost 60% higher than in controls (Fig. 4B) although smooth cell recruitment was impaired (Fig. 4C). Mice overexpressing Dll4 in endothelial cells (*Dll4OE*) underwent the same surgical procedure but displayed impaired revascularization and therefore poorer outcomes in myocutaneous flap recovery. After 7 days *Dll4OE* mice had less than 10% of the skin flap still alive, whereas controls displayed approximately 30% (Fig. 4D). Skin flap vasculature in *Dll4OE* mice was less dense than in their respective controls (Fig. 4E) but smooth muscle cell recruitment was enhanced (Fig. 4F).

4. Discussion

Dll4 inhibits angiogenesis by down-regulating VEGF receptor expression. Inhibition of Dll4 paradoxically inhibited tumor growth while increasing angiogenesis. New vessels however had poor maturation, and were mostly non-perfused, thus reducing tumor vascular function and leading to increased hypoxia, tumor apoptosis and necrosis. We postulated since Dll4/Notch inhibition has a negative correlation with vessel maturation and positive correlation with vessel proliferation, there may exist a maturation threshold and a proliferation threshold. When high dose Dll4/Notch inhibitor is used, the vessel maturation is below its threshold, leading to non-functional vessels and poor perfusion, even though the vessels are highly proliferating (Fig. 4G, blue area). When very low dose Dll4/Notch inhibitor is used, the vessel proliferation is below its threshold, leading to insufficient neovascularisation and poor perfusion (Fig. 4G, green area). However, when low/intermediate dose of Dll4/Notch inhibitor is used, the vessel maturation is above its threshold, allowing perfusion as well as increase in vascular proliferation (Fig. 4G, red area). Therefore, the low/intermediate dose Dll4-Fc may be used to promote reperfusion and facilitate the recovery from ischemia.

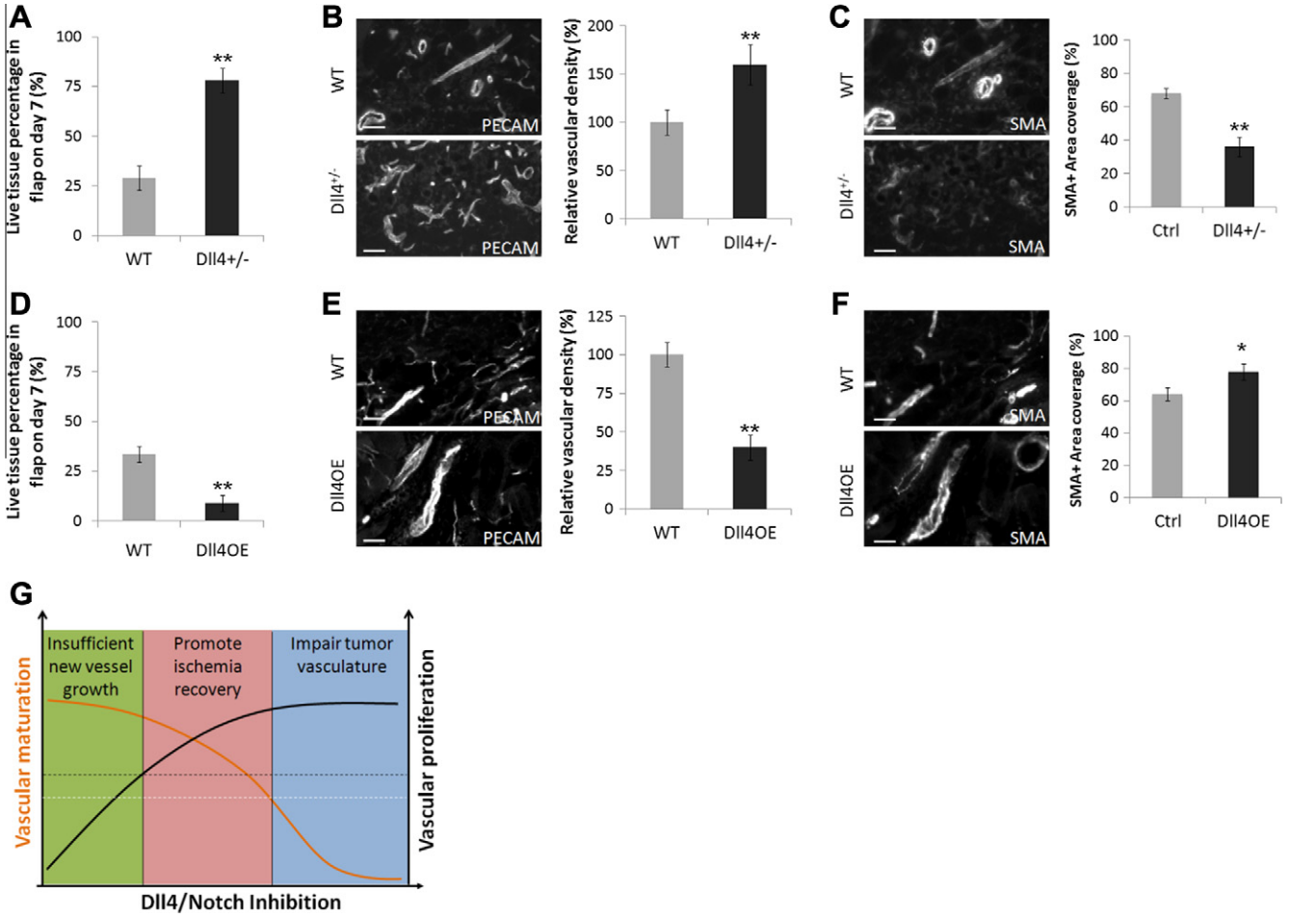

Fig. 4. Opposite effect of Dll4 heterozygous deletion and overexpression on skin flap recovery. Live skin area percentage seven days after skin flap surgical procedure was compared between wild type and *Dll4*$^{+/-}$ mice (A), and wild type and *Dll4OE* mice (D). Vascular density (B and E) and smooth muscle cell recruitment (C and F) were analyzed with immunostaining. The quantification analysis result of each Immunofluorescence study is shown on the right of each picture. Statistical analyses were performed as described in Fig. 3. (G) A scheme showing the effect of Dll4/Notch inhibition on vascular maturation and proliferation. Along with Dll4/Notch inhibition, endothelial cell proliferation increases (black line) and vessel maturation decreases (brown line). There is a threshold level of vascular proliferation indicated with the black dashed line and a threshold level of maturation indicated with the white dashed line. Bar = 30 μm.

We thus sought out to test the activity of various doses of Dll4-Fc in hindlimb ischemia model and dorsal skin flap model. We found that low dose Dll4-Fc can promote the growth of new vessels and yet these vessels are mature and functional, leading to better perfusion and less hypoxia. Thus both ischemic hindlimb and skin flap recovered much faster when treated with low dose Dll4-Fc. Quantitative RT-PCR analysis revealed that Dll4/Notch signalling blockage leads to increased expression of pro-angiogenic factors, such as VEGFC. VEGFR2/VEGFR1 ratio in endothelial cells is also increased, consistent with the general outcome of Dll4 blockade and also predicting elevated pro-angiogenic response. This increase in VEGFR2 is also confirmed in a VEGFR2-targeted microbubble study. In addition, VEGFR3 expression is also increased, again contributing to the increased vascular responsiveness and sprouting. However, elevated angiogenesis is not accompanied by much impaired vessel maturation, which is normally seen in high dose inhibition of Dll4 signalling. In fact, pericytes/smooth muscle coverage on newly formed blood vessels is almost intact and the expression of Ephrin-B2, a marker for arterial endothelial and mural cell is not affected.

Results from our models of Dll4 gain- and loss-of-function confirmed the importance of Dll4 dosage in the regulation of angiogenesis and vessel maturation. *Dll4*$^{+/-}$ mice were revealed to have improved skin flap revascularization, although impaired vascular maturation, leading to a 2.7-fold increase in live tissue after days, relative to control mice. *Dll4OE* mice had reduced skin flap revascularization with improved vascular maturation, leading to a 4-fold reduction in skin flap survival. Taken together, these results suggest the low dose of Dll4/Notch inhibitor may have the therapeutic potential for promoting revascularization and reperfusion of ischemic tissues.

Acknowledgement

This work was supported in part by the National Cancer Institute (RO1 CA 079218-07 to P.S.G.) and the Portuguese Science and Technology Foundation (PTDC/CVT/71084/2006 to A.D.).

References

[1] G.C. Gurtner, S. Werner, Y. Barrandon, M.T. Longaker, Wound repair and regeneration, Nature 453 (2008) 314–321.
[2] S. Werner, R. Grose, Regulation of wound healing by growth factors and cytokines, Physiol. Rev. 83 (2003) 835–870.
[3] P.C. Hsieh, C. MacGillivray, J. Gannon, F.U. Cruz, R.T. Lee, Local controlled intramyocardial delivery of platelet-derived growth factor improves postinfarction ventricular function without pulmonary toxicity, Circulation 114 (2006) 637–644.

[4] J.A. Balfour, S. Noble, Becaplermin, Bio Drugs 11 (1999) 359–364.
[5] M.G. Franz, D.L. Steed, M.C. Robson, Optimizing healing of the acute wound by minimizing complications, Curr. Probl. Surg. 44 (2007) 691–763.
[6] J.R. Shutter, S. Scully, W. Fan, W.G. Richards, J. Kitajewski, G.A. Deblandre, C.R. Kintner, K.L. Stark, Dll4, a novel notch ligand expressed in arterial endothelium, Genes Dev. 14 (2000) 1313–1318.
[7] A. Duarte, M. Hirashima, R. Benedito, A. Trindade, P. Diniz, E. Bekman, L. Costa, D. Henrique, J. Rossant, Dosage-sensitive requirement for mouse Dll4 in artery development, Genes Dev. 18 (2004) 2474–2478.
[8] R. Benedito, A. Duarte, Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles, Gene Expr. Patterns 5 (2005) 750–755.
[9] J.S. Scehnet, W. Jiang, S.R. Kumar, V. Krasnoperov, A. Trindade, R. Benedito, D. Djokovic, C. Borges, E.J. Ley, A. Duarte, P.S. Gill, Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion, Blood 109 (2007) 4753–4760.
[10] I. Noguera-Troise, C. Daly, N.J. Papadopoulos, S. Coetzee, P. Boland, N.W. Gale, H.C. Lin, G.D. Yancopoulos, G. Thurston, Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis, Nature 444 (2006) 1032–1037.
[11] J. Ridgway, G. Zhang, Y. Wu, S. Stawicki, W.C. Liang, Y. Chanthery, J. Kowalski, R.J. Watts, C. Callahan, I. Kasman, M. Singh, M. Chien, C. Tan, J.A. Hongo, F. de Sauvage, G. Plowman, M. Yan, Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis, Nature 444 (2006) 1083–1087.
[12] A. Trindade, S.R. Kumar, J.S. Scehnet, L. Lopes-da-Costa, J. Becker, W. Jiang, R. Liu, P.S. Gill, A. Duarte, Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos, Blood 112 (2008) 1720–1729.
[13] C.K. Williams, J.L. Li, M. Murga, A.L. Harris, G. Tosato, Up-regulation of the notch ligand delta-like 4 inhibits VEGF-induced endothelial cell function, Blood 107 (2006) 931–939.
[14] G. Thurston, I. Noguera-Troise, G.D. Yancopoulos, The delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth, Nat. Rev. Cancer 7 (2007) 327–331.
[15] J.A. Clayton, D. Chalothorn, J.E. Faber, Vascular endothelial growth factor-A specifies formation of native collaterals and regulates collateral growth in ischemia, Circ. Res. 103 (2008) 1027–1036.
[16] D. Djokovic, A. Trindade, J. Gigante, M. Badenes, L. Silva, R. Liu, X. Li, M. Gong, V. Krasnoperov, P.S. Gill, A. Duarte, Combination of Dll4/Notch and ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis, BMC Cancer 10 (2010) 641.
[17] D.J. Lee, A. Lyshchik, J. Huamani, D.E. Hallahan, A.C. Fleischer, Relationship between retention of a vascular endothelial growth factor receptor 2 (VEGFR2)-targeted ultrasonographic contrast agent and the level of VEGFR2 expression in an in vivo breast cancer model, J. Ultrasound Med. 27 (2008) 855–866.