Inhibition of the Notch Pathway Promotes Flap Survival by Inducing Functional Neoangiogenesis

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Objective: The Notch pathway seems to function as an antiangiogenic factor, negatively regulating the sprouting effect of vascular endothelial growth factor (VEGF). This function is well defined in embryonic and tumor vasculature. However, little is known about its function in ischemia-induced angiogenesis. In the first part of this study, we investigated the role of Notch in reparative angiogenesis after ischemia. In the second part, we hypothesized that anti-Notch therapy will result in increased angiogenic sprouting. We analyzed the effect of Notch inhibition in the induction of angiogenic sprouting.

Methods: In the first part, we investigated the effect of ischemia on the Notch ligand delta-like ligand 4 (DLL4). Twenty rats were divided equally into 2 groups. In the surgery group, dorsal skin flap was used as model of ischemia. In the control group, no surgical procedure was performed. DLL4 and VEGF gene expressions were assessed. Immunohistochemical staining was used for detection of DLL4 in tissue materials. Plasma levels of VEGF and DLL4 were measured. In the second part, we investigated the effect of Notch inhibition using a gamma-secretase inhibitor (GSI) on inducing neoangiogenesis. Twenty rats were assigned to 2 equal groups. In all animals, dorsal skin flap was raised and sutured back into its bed. Animals in the GSI-treated group received GSI intravenously after surgery for 3 days. Saline was administered in the control group. Necrotic area measurements, microangiography, and histologic evaluations were performed to compare groups.

Results: In the first part, VEGF and DLL expressions increased in ischemic tissues ($P < 0.01$). Immunohistochemical analysis revealed that DLL4 expression was upregulated in capillary endothelial cells after ischemia. Plasma levels for VEGF and DLL4 were higher in the animals that underwent surgery ($P < 0.01$). In the second part, GSI treatment resulted in higher flap survival rates ($P < 0.05$). Microscopic analysis exhibited increase in the number of microvascular structures after GSI treatment ($P < 0.05$). Microangiographic evaluation showed that neovascularization increased in the GSI-applied flaps.

Conclusions: We present an evidence for the importance of the Notch pathway in the regulation of ischemia-induced angiogenesis. Notch inhibition promotes flap survival by creating a neovasculature that has an increase in vascular density.

Key Words: Notch, DLL4, angiogenesis, flap, rat, survival, gamma-secretase inhibitor, DAPT, skin, sprouting, VEGF, vascular endothelial growth factor, delta-like ligand 4

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Ischemia may jeopardize skin flap survival and result in flap necrosis. Surgical delay preserves its value to be the first-choice procedure to increase flap viability. However, its invasive nature, the cost, and the inherent drawbacks of a surgical procedure have urged investigators to seek minimally invasive alternative methods. The role of angiogenesis as a mechanism of surgical delay raised the possibility of inducing the delay effect pharmacologically by promoting preoperative angiogenesis in the flap. Improved flap survival in many models has been observed after manipulation of vascular endothelium growth factor (VEGF), transforming growth factor, basic fibroblast growth factor, and platelet-derived growth factor.[1–7]

Angiogenesis is a dynamic process that correlates with tissue's metabolic, immunologic, and growth demands.[8] During angiogenesis, new vessels are generated from the walls of existing vessels in a process termed *angiogenic sprouting*.[9] The sprouting process is spearheaded by specialized endothelial cells, called "tip cells." Stimulated by vascular endothelial growth factor (VEGF), these tip cells break out their stable position in the vessel wall to guide new forming sprouts.[10] The endothelial cells along the main vessel that follow the tip cells, termed *stalk cells*, proliferate simultaneously to form the new sprouts. Each new sprout eventually connects with adjacent sprouts to form a continuous lumen and thus establishes flow in the new vascular loop.[11,12] What controls the specification of endothelial tip and stalk cells, how is tip cell migration regulated, what controls stalk cells and their proliferation, and how do tip cells communicate during the formation of new connections? To create an organized vessel network with the right density of branches, these individual endothelial cells need to be strictly coordinated. What, then, is the underlying mechanism? During the past years, it has become clear that Notch signaling has an essential role in coordinating endothelial cell behavior during sprouting angiogenesis.

The Notch pathway is an evolutionary conserved signaling system that was first discovered in Drosophila as a mutation causing notched wing. Since then, components of the Notch pathway have been discovered in mammals. Studies have shown that this pathway has a critical role in the regulation of cell fate specification, tissue patterning, and morphogenesis by modulating cell differentiation, proliferation, and apoptosis.[13–15] In mammals, the components of the pathway include 4 single-pass transmembrane receptors (Notch 1-4) and 5 transmembrane ligands (Jagged1, Jagged2, delta-like ligand 1 [DLL1], DLL3, and DLL4; Table 1).

Several Notch receptors and ligands have been identified in endothelial cells during angiogenesis.[16–18] Of the 5 Notch ligands, DLL4 is the primary functional ligand in angiogenesis.[19,20] During sprouting angiogenesis, the tip endothelial cells take the leader role of directing the outgrowth of blood vessel sprouts. When sprouting reaches the appropriate density, the tip endothelial cells produce the Notch ligand DLL4. These transmembrane ligands activate Notch receptors on the adjacent stalk cells through direct interaction.[18,21,22] Receptor activation results in a series of proteolytic cleavages, in which the last step is mediated by the gamma-secretase membrane protein complex.[23] The series of proteolytic cleavages lead to translocation of the Notch intracellular domain into the nucleus and initiation of transcription of target Notch genes.[13,24] Proteins encoded

TABLE 1. Notch Receptors and Ligands

Receptors	Comments
Notch 1	Notch 1 is predominant in the endothelium and the most critical receptor during sprouting angiogenesis. Mice embryos homozygous for a null allele of Notch 1 die early with defects in somitogenesis and severe cardiovascular anomalies.
Notch 2	Notch 2 is critical for the vascular morphogenesis of a more selective group of vascular beds such as vasculature of the eye and glomerular capillary.
Notch 3	Notch 3 is present in smooth muscle cells. This receptor is necessary for the differentiation of arterial identity of vascular smooth muscle cells.
Notch 4	Notch 4 is predominant in the endothelium and the second most important receptor during sprouting angiogenesis after Notch 1. Deletion of Notch 4 does not have the same effect on vascular development; however, double knockouts for Notch 1 and Notch 4 have a more severe vascular phenotype than Notch 1 knockout alone.
Ligands	**Commends**
DLL1	DLL1 is expressed in the endothelium of both arteries and veins during development. Disruption of DLL1 leads to early lethality with generalized hemorrhagic events.
DLL2	Expression of DLL2 has not been reported in vascular cells.
DLL3	Expression of DLL3 has not been reported in vascular cells.
DLL4	DLL4 is primarily found in the endothelium and the most critical ligand during angiogenesis. Mice embryos homozygous for a null allele of DLL4 die early. These mice fail to remodel the primitive vascular plexus.
Jagged	Jagged1 is present in the endothelium. Mice embryos lacking a functional Jagged gene die with vascular anomalies.

by Notch genes downregulate the expression of VEGF receptor 2 and restrict the emergence of excessive sprouting via reduction of responsiveness to VEGF[12] (Fig. 1). This helps to create a vascular network with the appropriate density of branches.[18,25]

Although previous studies have established a well-defined role for Notch signaling in embryonic and tumor vasculature, less is known about the functional importance of Notch pathway in ischemia-induced angiogenesis. Thus, we investigated whether the Notch pathway is upregulated during ischemia-induced angiogenesis using a skin model of ischemia in the first part of the study. In the second part, given the fact that the Notch functions as an antiangiogenic factor, we hypothesized that anti-Notch therapy will result in increased formation of endothelial tip cells and, in turn, excessive angiogenic sprouting. We analyzed the potential effect of Notch inhibition in the induction of angiogenic sprouting in a skin flap.

MATERIALS AND METHODS

This study was approved by Baskent University Ethical Committee for Experimental Research on Animals. Forty male Sprague-Dawley rats, weighing between 390 and 430 g, were used in this study. Each animal was anesthetized with an intraperitoneal injection of ketamin hydrochloride (50 mg/kg) and xylazine (10 mg/kg) with periodic supplementation as needed. The study was conducted in 2 parts.

Part 1

In this part of the study, we investigated whether the Notch ligand DLL4 is upregulated during ischemia-induced angiogenesis. Twenty animals were assigned randomly to 2 equal groups. In the surgery group (n = 10), a caudally based dorsal skin flap with the dimensions of 9 × 3 cm was raised and sutured back into its initial position with a silicone sheet placed underneath to avoid vascular ingrowth from the wound bed (Fig. 2). In the control group (n = 10), no surgical procedure was performed.

Gene Expression

To investigate the effect of ischemia on VEGF and DLL4 expressions, VEGF and DLL4 messenger RNA (mRNA) levels were measured using real-time polymerase chain reaction assay. In the surgery group, punch biopsies of full-thickness skin were taken from the center of the outlined flap territory. In the control group, biopsies of cephalic dorsal skin were performed. Whole RNA was extracted from tissue samples using High Pure RNA Tissue Kit (Roche Diagnostics, GmbH, Mannheim, Germany). The extracted RNA was reverse transcripted to complementary DNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, GmbH, Mannheim, Germany). real-time polymerase chain reaction was carried out using LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, GmbH, Mannheim, Germany). Housekeeping gene β-actin served as internal control. Gene expressions were expressed as a fraction of VEGF or

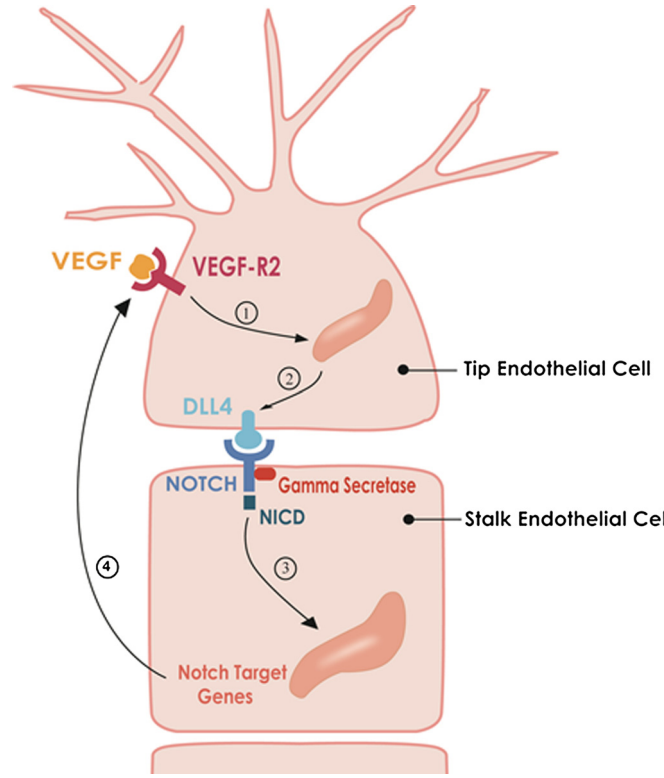

FIGURE 1. 1, Sprouting angiogenesis is spearheaded by the tip endothelial cells under the influence of VEGF. 2, Notch activation occurs through DLL4 signals from tip endothelial cells to Notch receptors on neighboring stalk endothelial cells. 3, Activation of Notch receptors initiates a series of proteolytic cleavages, in which the last step is mediated by the gamma-secretase membrane protein complex. The series of proteolytic cleavages lead to the translocation of the Notch intracellular domain (NICD) to the nucleus and initiation of transcription of target Notch genes. 4, Notch activation restricts the emergence of excessive sprouting through repression of VEGF receptor 2 (VEGF-R2) transcription.

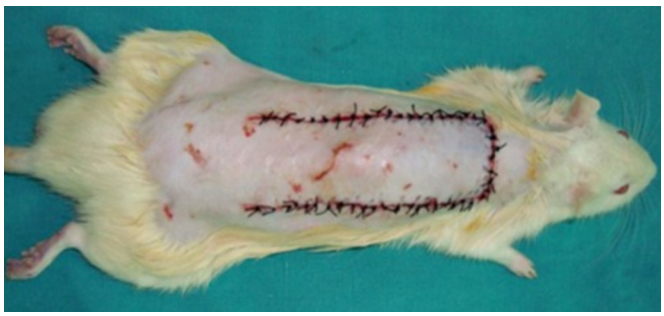

FIGURE 2. A caudally based dorsal skin flap with the dimensions of 9 × 3 cm was raised and sutured back into its initial position with a silicone sheet placed underneath to avoid vascular ingrowth from the wound bed.

DLL4 to β-actin. Relative gene expression data were analyzed using $2^{(-\text{Delta Delta C(T)})}$ method.[26] Primer sequences of the target genes are listed in Table 2.

Whole-Mount Immunohistochemistry

Whole-mount immunohistochemical analysis using specific anti-DLL4 antibodies was used to analyze the expression of endothelial DLL4 in tissue samples of each group. Sections (5 μm) of formalin-fixed paraffin-embedded tissues were processed using standard methods. Sections were incubated with primary antibody (anti-DLL4 antibody; Abcam, Cambridge, England) overnight at 4°C and with appropriate secondary antibody (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester [DAPT]; Abcam, Cambridge, England) for 1 hour at room temperature. All immunostainings were analyzed using a standard light microscope (×40 magnification).

Enzyme-Linked Immunosorbent Assays

After skin biopsies, blood samples were obtained (5 mL for each rat) by cardiac puncture. Measurement of VEGF and DLL4 proteins in the blood was performed by direct enzyme-linked immunosorbent assay (ELISA) using VEGF (VEGF ELISA kit; Ray Biotech, Inc, Norcross Ga) and DLL4 (DLL4 ELISA kit; Life Science, Inc, St Petersburg, Fla) antibodies.

Statistics

Compliance with the normal distribution of continuous variables was checked with the Shapiro-Wilk test. Homogeneity of the groups' variances was checked by Levene test. Parametric test assumptions were available in terms of blood VEGF level variable, so group means were compared by 1-way analysis of variance, and then multiple comparisons between pairs of groups were carried out according to the Tukey honest significant difference test. The groups' variances were not homogeneous in terms of VEGF and DLL4 gene expression variables, so the Kruskal-Wallis test and then the Dunn test were used for comparisons. Two independent groups were compared by the Mann-Whitney *U* test for blood DLL4 level variable. The Pearson correlation coefficient was used to evaluate the correlations between normally distributed variables. Data analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 17.0 (SPSS Inc, Chicago, Ill). A *P* value of less than 0.05 was considered statistically significant. The results of statistical analysis were expressed as number of observations, mean (SD), and median (minimum to maximum) values.

Part 2

In this part of the study, we analyzed the potential effect of Notch inhibition in the induction of angiogenic sprouting in a skin flap model. Because the gamma-secretase protein complex acts as a key enzyme in the activation of the Notch, gamma-secretase inhibitors (GSIs) can be used to potently inhibit Notch signaling. The DAPT has previously been shown to potently inhibit gamma secretase activity.[27] Twenty animals were assigned randomly to 2 equal groups, namely, the control group (n = 10) and the GSI-treated group (n = 10). In both groups, a caudally based dorsal random-pattern skin flap, measuring 3 × 9 cm, was elevated to include the panniculus carnosus. A silicone sheet was placed beneath the flap to prevent revascularization from the wound bed. The flap was sutured back into its original bed. In the control group, 1 mL/kg of saline was administered intravenously per day after surgery for 3 days. The GSI-treated group received 10 mg/kg of DAPT intravenously per day after surgery for 3 days.

Evaluation of the Skin Flap Survival

Flap survival rate was observed on postoperative day 10, when the border of the necrotic area on the flap was defined. Standardized digital photographs of the flaps were collected and transferred to a computer. Digital images were processed using an image analysis software. Dark zones were defined to be necrotic, and the remaining areas were defined to be viable. Survival rate was expressed as percentage of the total flap area.

Histologic Evaluation

After determination of necrotic areas, full-thickness tissue specimen was harvested from distal flap zones (2 rats from each group were excluded for microangiographic evaluation). Specimens were fixed in 10% neutral formalin for 72 hours. The specimens were subsequently embedded into paraffin. Sections of 5-μm thickness were taken from paraffin blocks, and deparaffinized sections were prepared with hematoxylin-eosin for histologic analysis by light microscopy. A semiquantitative assessment of neovascularization was performed to measure the number of capillaries per high-power fields (×40 magnification) using the light microscope.

Microangiographic Evaluation

Microangiography was performed in 2 randomly selected rats from each group after flap elevation. Two 20-gauge catheters were inserted caudally into the aorta and the inferior vena cava. The aorta was perfused with 200 mL of heparinized (10 U/mL) warm saline, which was followed by injection of contrast medium (lead oxide and gelatin), prepared as follows: 200-g commercial grade (96% pure) lead oxide was added to 100 mL of water heated to 50°C, followed by addition of 3-mg gelatin powder, and thoroughly stirred. The mixture was kept warm at a constant temperature in a water bath and stirred from time to time until injection time. The mixture was injected via the abdominal aorta. Thereafter, the flaps were separated from their beds, placed in dampened gauze, and refrigerated at 4°C for 12 hours.

TABLE 2. Primers Used for Real-time Polymerase Chain Reaction Analysis

Gene	Primer
VEGF	
Forward	5′ ACg AAA gCg CAA gAA ATC CC 3′
Reverse	5′ TTA ACT CAA gCT gCC TCg CC 3′
β-Actin	
Forward	5′ Agg gAA ATC gTg CgT gAC AT 3′
Reverse	5′ AAC CgC TCA TTg CCg ATA gT 3′

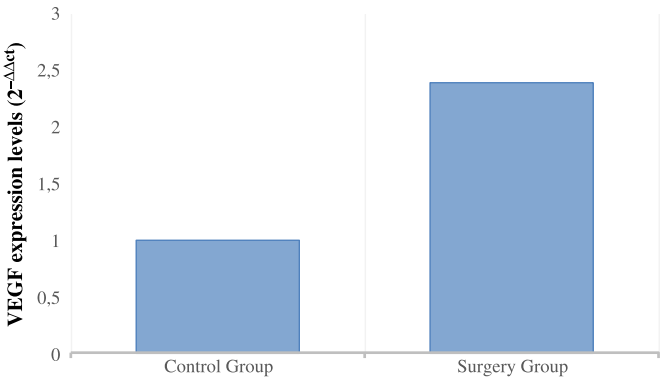

FIGURE 3. VEGF mRNA levels measured in the control group represent our baseline. Levels measured in the surgery group were higher than the baseline ($P < 0.01$).

Images of the flaps were then taken using a mammography device (24 mA s, 8 KA, Mammo Diagnost UC; Philips, Germany).

Statistics

Pearson correlation coefficient was used to see whether there is a linear relationship between samples. Normality assumption was checked by the Shapiro-Wilk test. Normality conjunction with no correlation implies independence, so assumption of independent samples was also satisfied. Thus, all assumptions of parametric test were satisfied. Because sample sizes are small ($n < 30$), t test was used, and significance level was taken as 0.05. Equality of variances (homoscedasticity) of samples was checked by the Levene test ($\alpha = 0.05$), and calculation of t test was arranged on the basis of these results. Data analyses were performed using the SPSS, version 18.0.

RESULTS

Part 1

Gene Expression

Vascular endothelial growth factor and DLL4 mRNA levels measured in the control group represent our baseline. For both parameters, expression levels measured in ischemic tissues were higher than the baseline ($P < 0.01$). Vascular endothelial growth factor and DLL4 expression levels are shown in Figures 3 and 4.

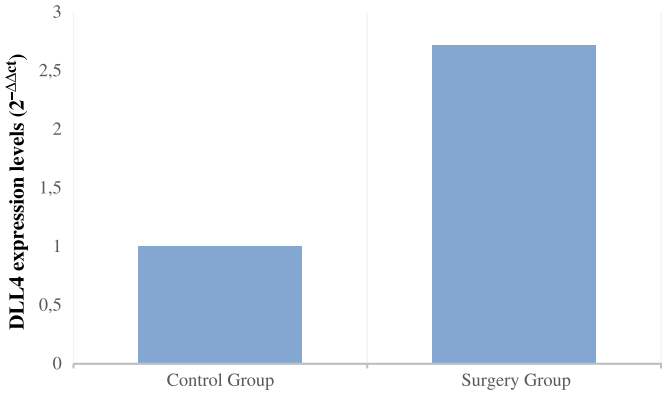

FIGURE 4. DLL4 mRNA levels measured in the control group represent our baseline. Levels measured in the surgery group were higher than the baseline ($P < 0.01$).

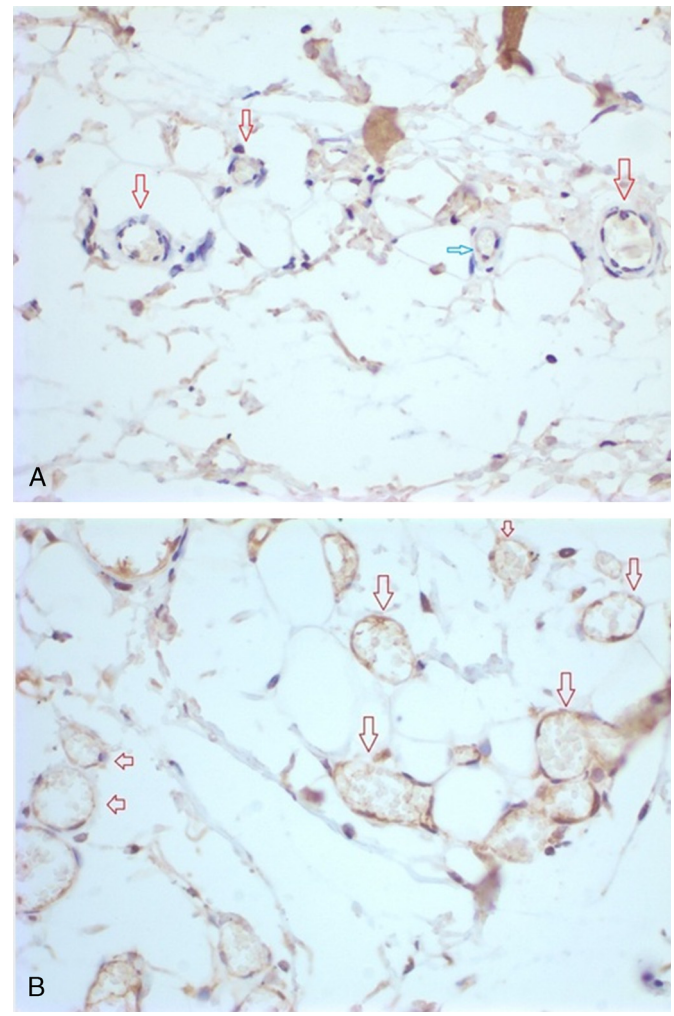

FIGURE 5. A, Weak and patchy expression of DLL4 in microvascular endothelial cells of normoperfused tissues (blue arrow). B, DLL4 expression is upregulated in capillary endothelial cells after ischemia (red arrows).

Whole-Mount Immunohistochemistry

Immunohistochemical analysis using specific anti-DLL4 antibodies revealed weak and patchy expression of DLL4 in microvascular

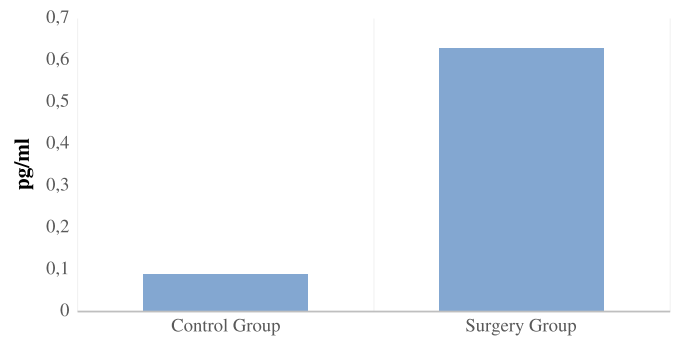

FIGURE 6. The mean of plasma levels for VEGF were significantly higher in the animals that underwent the surgical procedure when compared with the control group ($P < 0.01$).

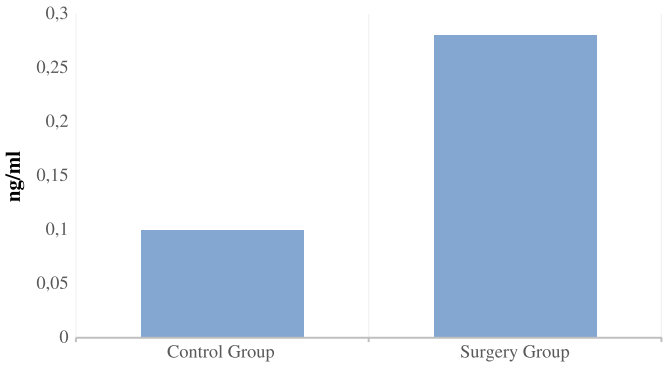

FIGURE 7. The mean of plasma levels for DLL4 were significantly higher in the animals that underwent the surgical procedure when compared with the control group ($P < 0.01$).

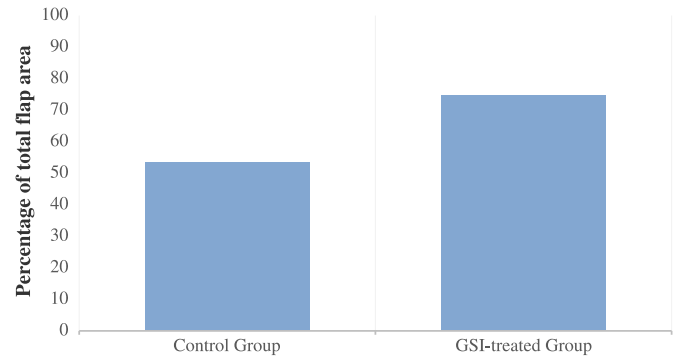

FIGURE 9. GSI treatment (64.815% [2.95%]) resulted in a significantly improved flap survival rate compared with the control group (53.559% [2.59%]) ($P < 0.05$).

endothelial cells of normoperfused tissues (Fig. 5A). Conversely, DLL4 expression was upregulated in capillary endothelial cells after ischemia (Fig. 5B).

Enzyme-Linked Immunosorbent Assays

The means of plasma levels for VEGF and DLL4 were significantly higher in the animals that underwent the surgical procedure when compared with the control group ($P < 0.01$). The median values of plasma levels for VEGF and DLL4 are shown in Figures 6 and 7.

Part 2

Flap Survival

Ten days postoperatively, necrotic and viable regions were clearly demarcated (Fig. 8). Gamma-secretase inhibitor treatment (74.815% [2.95%]) resulted in a significantly improved flap survival rate compared with the control group (53.559% [2.59%]; $P < 0.05$) (Fig. 9).

Microangiographic Evaluation

Angiograms revealed no significant difference in number and calibration of large arteries and arterioles between the groups. After evaluation of small-diameter vessels, neovascularization and vessel diameters were found to be increased in the GSI-applied flaps (Fig. 10).

Histologic Evaluation

The semiquantitative assessment of the number of capillaries (mean [SD]) exhibited a significant difference between the GSI treatment group (5 [1] capillaries per field) and the control group (1 [1] capillary per field) ($P < 0.05$) (Fig. 11).

DISCUSSION

Angiogenesis, or the formation of new blood vessels from preexisting ones, is essential to establish a functional vascular circuit during embryonic development and in adult life. During angiogenic sprouting, endothelial cells migrate extensively and proliferate in

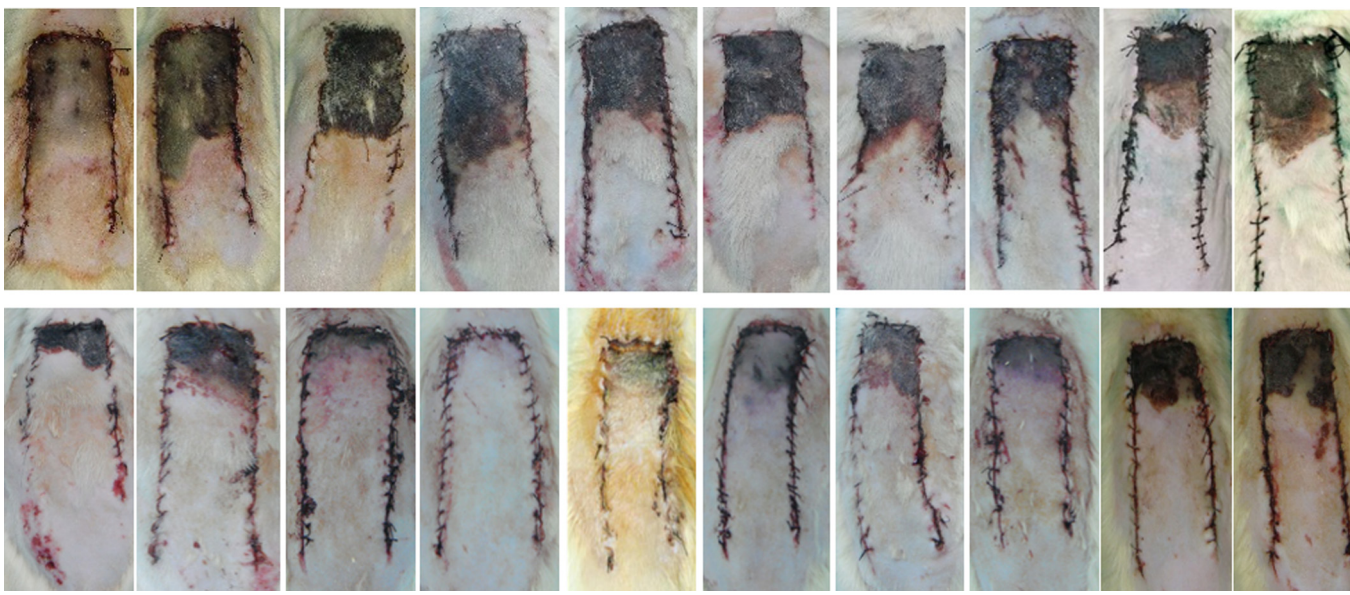

FIGURE 8. Representative view of survival of skin flaps on the 10th postoperative day in rats from the control group (top) and the GSI-treated group (bottom).

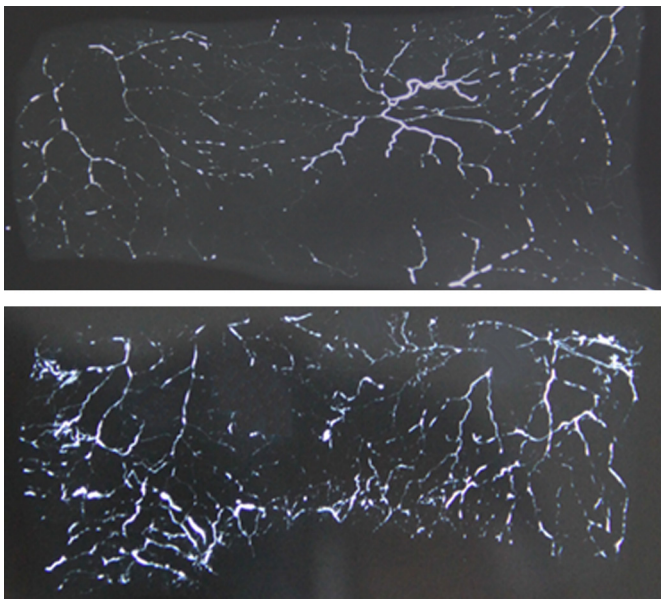

FIGURE 10. Microangiographic views of the skin flap areas in the control rat (top) and the GSI-treated rat (bottom) at 10 days. Angiograms revealed no significant difference in number and calibration of large arteries and arterioles between the groups. After evaluation of small-diameter vessels, neovascularization and vessel diameters were found to be increased in the GSI-applied flaps.

response to proangiogenic factors.[10,28] Given the need to regulate angiogenic sprouting at several different steps, it is likely that Notch signaling is used throughout this process. Notch activation restricts the emergence of excessive sprouting through repression of VEGF receptor transcription and consequent reduction of responsiveness to VEGF.[12]

The importance of the Notch was first described by its function in the regulation of embryonic vascular development. Growing evidence supports the involvement of the Notch ligand DLL4 in the regulation of sprouting angiogenesis in the murine retina and developing zebrafish.[18,25] Besides its regulator function in angiogenic sprouting, the Notch pathway has a critical role during the whole process of developmental angiogenesis.[29–32] In the early embryogenesis, the Notch modulates the migration of angioblasts from the lateral mesoderm toward the dorsal aorta and induces their specification to endothelial cells.[33] In later stages, Notch signaling controls endothelial cell specification into arterial or venous identity. Activation of Notch in the endothelium results in establishing the arterial identity. Conversely, when the Notch signaling pathway is suppressed by chicken ovalbumin upstream promoter-transcription factor II, vessels acquire vein identity.[34,35]

The contribution of Notch in postnatal angiogenesis is the focus of intense investigation. Most of these studies are in the field of tumor vascularization. In various models, strong DLL4 expression was observed in the majority of tumor vessels, contrasting with significantly lower vascular expression in adjacent normal tissues.[20,36] In humans, DLL4 expression was analyzed in tumors from the kidney, the bladder, the colon, the brain, and the breast.[37–39] Besides tumor vasculature, recent data suggest that the Notch is involved in the regulation of angiogenesis in response to ischemic injury to the retina, the myocardium, and the brain.[40–43] It has been shown that the Notch has a protective function against ischemia in these tissues. In the first part of this study, we have shown, for the first time, that the Notch is activated in a skin model of ischemia. We found that DLL4 is weakly expressed in normoperfused tissues but remarkably induced after ischemia. In addition, immunohistochemical analysis using specific anti-DLL4 antibodies revealed weak and patchy expression of DLL4 in microvascular endothelial cells of normoperfused tissues. Conversely, DLL4 expression was upregulated in capillary endothelial cells after ischemia. These findings are compatible with the possibility that the Notch, through its ligand DLL4, plays a role in the regulation of sprouting during ischemia-induced angiogenesis.

To investigate the functional importance of the Notch, we used GSIs to downregulate the function of the Notch in ischemic skin flaps. Increased number of small-diameter vessels was observed histologically and microangiographically in the GSI-treated group. These findings provide evidence supporting the use of anti-Notch therapy to increase vascularization, improving the rate of flap survival. The mechanism of GSIs in promoting flap survival is strikingly different to that of classic proangiogenic therapies, which work by promoting angiogenesis directly in the flap. Notch inhibition works by increasing vascular density through improving VEGF's sprouting effect without any change in blood VEGF levels. We found no significant difference between the control and GSI-treated groups in VEGF mRNA and plasma VEGF levels.

The concept of therapeutic angiogenesis relies on supporting endogenous vascular growth to improve tissue perfusion. Administration of angiogenic factors to induce therapeutic angiogenesis resulted in remarkable success in preclinical studies.[2,7,44–47] However, only partial success was achieved in clinical trials.[48,49] One of the reasons accounting for this discrepancy is that delivery of single growth factors may represent a simplification of the cooperative molecular interaction necessary for the establishment of a functional vascular network. Inhibition of the Notch uses the interaction between Notch and VEGF pathways to promote angiogenesis. Notch-based therapies could then be used to treat conditions in which the formation of new blood vessels is essential, such as wound healing. In this case, angiogenesis starts immediately after the injury and is important for the formation of granulation tissue. In a recent study, Notch inhibition accelerated wound regeneration by creating a neovasculature that has a slight increase in vascular density.[50] There was an effective increase in local blood supply to the wound site.

Insights into the mechanisms of the delay phenomenon helped us to develop pharmacological manipulations to augment skin flap survival. The use of growth factors to induce a delay effect has been reported.[2,7,44–47] Flap survival rates with preoperative VEGF administration were found to be equivalent to surgical delay.[51] However, exogenous VEGF application has not found utility in the clinical setting because of the high cost and potential side effects in

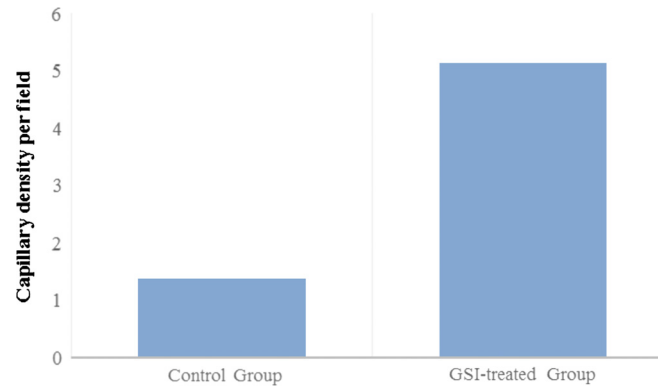

FIGURE 11. Histologic assessment of the number of capillaries exhibited a significant difference between the GSI treatment group (5 [1] capillaries per field) and the control group (1 [1] capillary per field) ($P < 0.05$).

efficient human doses. In the second part of this study, Notch inhibition provides an alternative method in inducing the delay effect pharmacologically. Instead of using exogenous factors, the angiogenic effects of available local VEGF molecules are augmented. The resultant increase in flap survival after Notch inhibition adds up to identify the Notch pathway as a potential target of inducing the delay effect pharmacologically. Moreover, Notch inhibition can be used in conjunction with surgical delay. Inhibition of the Notch in surgically delayed flaps can speed up angiogenic sprouting. This may help in reducing the long period required for surgical delay.

Recently, several groups have investigated whether inhibition of the Notch pathway might affect tumor angiogenesis and growth. Inhibition of Notch signaling caused increased vascular density and vascular sprouting in tumors. Surprisingly, this vascular overgrowth phenotype resulted in tumor growth inhibition.[36,52,53] Perfusion studies demonstrated that the hypersprouting tumor vasculature was nonfunctional, and consequently, anti-Notch treated tumors exhibited increased levels of hypoxia with resultant growth inhibitory effects on tumors.[36,52–54] It seems that excessive branching results in a highly chaotic vascular network that lacks the hierarchy essential for efficient directional blood flow.[36,52,53] In 1 set of studies, reduced pericyte coverage with increased vascular leakage was observed in tumors treated with Notch inhibitors.[55,56] Such an increase in vascular leakiness associated with impaired vascular integrity may explain a rapid decrease in tumor perfusion. Another possibility is that the abnormalized network as a whole loses the organization necessary to support adequate perfusion.[52,54] Alternatively, it is plausible that initiation of hypersprouting structures that are devoid of lumens would result in a loss of perfusion.[57,58]

In contrast to tumoral tissue, Notch inhibition in ischemic flap tissues promoted flap survival by inducing functional angiogenesis. We think that the nonfunctional nature of the tumoral neovasculature observed in anti-Notch therapy was a consequence of the high dosage of Notch inhibitor used. By modulating therapy dosage, functional proangiogenic effect may be achieved. DLL4 inhibition accelerated wound regeneration by creating a neovasculature that has a slight increase in vascular density.[50] Another possible explanation of the nonfunctional nature of tumoral angiogenesis is the fact that blood vessel in a given solid tumor may, in fact, be mosaic vessels, composed of endothelial cells and tumor cells.[59] In addition, tumor vessels differ from normal vessels in multiple ways. Their structural irregularity, heterogeneity, and leakiness can be regarded as bizarre hallmarks of a propensity to break all the rules of normal blood vessel construction. Most tumor vessels have an irregular diameter and abnormal branching pattern, and they do not fit well into the usual classification of arterioles, capillaries, or venules.[60–62] Thus, inducing sprouting on these abnormal vessels will likely lead to abnormal vascular network.

CONCLUSIONS

Together, our results present an evidence for the functional importance of the Notch pathway in the regulation of ischemia-induced angiogenesis. Proangiogenic factors from the hypoxic tissue induce both the sprouting response and the production of the Notch ligand required to coordinate the response into functional tubular morphogenesis. This fact makes Notch signaling a potential target for therapeutic angiogenesis through its ligand DLL4. Notch inhibition promotes flap survival by creating a neovasculature that has an increase in vascular density. Further characterization of Notch interaction with other signaling pathways might help identify novel targets for therapeutic angiogenesis.

REFERENCES

1. Nall AV, Brownlee RE, Colvin CP, et al. Transforming growth factor beta 1 improves wound healing and random flap survival in normal and irradiated rats. *Arch Otolaryngol Head Neck Surg*. 1996;122:171–177.
2. Carroll CM, Carroll SM, Schuschke DA, et al. Augmentation of skeletal muscle flap survival using platelet derived growth factor. *Plast Reconstr Surg*. 1998;102:407–415.
3. Carroll SM, Carroll CM, Stremel RW, et al. Vascular delay and administration of basic fibroblast growth factor augment latissimus dorsi muscle flap perfusion and function. *Plast Reconstr Surg*. 2000;105:964–971.
4. Ishiguro N, Yabe Y, Shimizu T, et al. Basic fibroblast growth factor has a beneficial effect on the viability of random skin flaps in rats. *Ann Plast Surg*. 1994;32:356–360.
5. Kryger Z, Zhang F, Dogan T, et al. The effects of VEGF on survival of a random flap in the rat: examination of various routes of administration. *Br J Plast Surg*. 2000;53:234–239.
6. Li QF, Reis ED, Zhang WX, et al. Accelerated flap prefabrication with vascular endothelial growth factor. *J Reconstr Microsurg*. 2000;16:45–49.
7. Zhang F, Fischer K, Komorowska-Timek E, et al. Improvement of skin paddle survival by application of vascular endothelial growth factor in a rat TRAM flap model. *Ann Plast Surg*. 2001;46:314–319.
8. Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386:671–674.
9. Yancopoulos GD, Davis S, Gale NW, et al. Vascular-specific growth factors and blood vessel formation. *Nature*. 2000;407:242–248.
10. Gerhardt H, Golding M, Fruttiger M, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol*. 2003;161:1163–1177.
11. Blum Y, Belting HG, Ellertsdottir E, et al. Complex cell rearrangements during intersegmental vessel sprouting and vessel fusion in the zebrafish embryo. *Dev Biol*. 2008;316:312–322.
12. Leslie JD, Ariza-McNaughton L, Bermange AL, et al. Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. *Development*. 2007; 134:839–844.
13. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science*. 1999;284:770–776.
14. Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol*. 2006;7:678–689.
15. Fiuza UM, Arias AM. Cell and molecular biology of Notch. *J Endocrinol*. 2007;194:459–474.
16. Iso T, Hamamori Y, Kedes L. Notch signaling in vascular development. *Arterioscler Thromb Vasc Biol*. 2003;23:543–553.
17. Hofmann JJ, Luisa Iruela-Arispe M. Notch expression patterns in the retina: an eye on receptor-ligand distribution during angiogenesis. *Gene Expr Patterns*. 2007;7:461–470.
18. Hellstrom M, Phng LK, Hofmann JJ, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*. 2007;445:776–780.
19. Benedito R, Duarte A. Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles. *Gene Expr Patterns*. 2005;5:750–755.
20. Mailhos C, Modlich U, Lewis J, et al. Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation*. 2001;69:135–144.
21. Lobov IB, Renard RA, Papadopoulos N, et al. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci U S A*. 2007;104:3219–3224.
22. Claxton S, Fruttiger M. Periodic Delta-like 4 expression in developing retinal arteries. *Gene Expr Patterns*. 2004;5:123–127.
23. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*. 2009;137:216–233.
24. Schroeter EH, Kisslinger JA, Kopan R. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature*. 1998;393:382–386.
25. Siekmann AF, Lawson ND. Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature*. 2007;445:781–784.
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402–408.
27. Zou Y, Cao Y, Yue Z, et al. Gamma-secretase inhibitor DAPT suppresses glioblastoma growth via uncoupling of tumor vessel density from vessel function. *Clin Exp Med*. 2013;13:271–278.
28. Gerhardt H. VEGF and endothelial guidance in angiogenic sprouting. *Organogenesis*. 2008;4:241–246.
29. Phng LK, Gerhardt H. Angiogenesis: a team effort coordinated by notch. *Dev Cell*. 2009;16:196–208.
30. Krebs LT, Xue Y, Norton CR, et al. Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev*. 2000;14:1343–1352.

31. Roca C, Adams RH. Regulation of vascular morphogenesis by Notch signaling. *Genes Dev*. 2007;21:2511–2524.
32. Duarte A, Hirashima M, Benedito R, et al. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev*. 2004;18:2474–2478.
33. Sato Y, Watanabe T, Saito D, et al. Notch mediates the segmental specification of angioblasts in somites and their directed migration toward the dorsal aorta in avian embryos. *Dev Cell*. 2008;14:890–901.
34. You LR, Lin FJ, Lee CT, et al. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature*. 2005;435:98–104.
35. Grego-Bessa J, Luna-Zurita L, del Monte G et al. Notch signaling is essential for ventricular chamber development. *Dev Cell*. 2007;12:415–429.
36. Noguera-Troise I, Daly C, Papadopoulos NJ, et al. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Novartis Found Symp*. 2007;283:106–120; discussion 121–125, 238–241.
37. Patel NS, Dobbie MS, Rochester M, et al. Up-regulation of endothelial delta-like 4 expression correlates with vessel maturation in bladder cancer. *Clin Cancer Res*. 2006;12:4836–4844.
38. Jubb AM, Turley H, Moeller HC, et al. Expression of delta-like ligand 4 (Dll4) and markers of hypoxia in colon cancer. *Br J Cancer*. 2009;101:1749–1757.
39. Jubb AM, Soilleux EJ, Turley H, et al. Expression of vascular notch ligand delta-like 4 and inflammatory markers in breast cancer. *Am J Pathol*. 2010; 176:2019–2028.
40. Qin X, Zhang Z, Xu H, et al. Notch signaling protects retina from nuclear factor-kappaB- and poly-ADP-ribose-polymerase-mediated apoptosis under high-glucose stimulation. *Acta Biochim Biophys Sin (Shanghai)*. 2011; 43:703–711.
41. Yang Y, Duan W, Jin Z et al. New role of Notch-mediated signaling pathway in myocardial ischemic preconditioning. *Med Hypotheses*. 2011;76:427–428.
42. Zhao Y, Chen X, Ma L, et al. Electroacupuncture pretreatment induces tolerance against focal cerebral ischemia through activation of canonical Notch pathway. *BMC Neurosci*. 2012;13:111.
43. Zhou XL, Wan L, Xu QR, et al. Notch signaling activation contributes to cardioprotection provided by ischemic preconditioning and postconditioning. *J Transl Med*. 2013;11:251.
44. Gurunluoglu R, Ozer K, Skugor B, et al. Effect of transfection time on the survival of epigastric skin flaps pretreated with adenovirus encoding the VEGF gene. *Ann Plast Surg*. 2002;49:161–169.
45. Huemer GM, Shafighi M, Meirer R, et al. Adenovirus-mediated transforming growth factor-beta ameliorates ischemic necrosis of epigastric skin flaps in a rat model. *J Surg Res*. 2004;121:101–107.
46. Lineaweaver WC, Lei MP, Mustain W, et al. Vascular endothelium growth factor, surgical delay, and skin flap survival. *Ann Surg*. 2004;239:866–873; discussion 873–875.
47. Khouri RK, Brown DM, Leal-Khouri SM, et al. The effect of basic fibroblast growth factor on the neovascularisation process: skin flap survival and staged flap transfers. *Br J Plast Surg*. 1991;44:585–588.
48. Simons M, Ware JA. Therapeutic angiogenesis in cardiovascular disease. *Nat Rev Drug Discov*. 2003;2:863–871.
49. Henry TD, Annex BH, McKendall GR, et al. The VIVA trial: vascular endothelial growth factor in ischemia for vascular angiogenesis. *Circulation*. 2003; 107:1359–1365.
50. Trindade A, Djokovic D, Gigante J, et al. Low-dosage inhibition of Dll4 signaling promotes wound healing by inducing functional neo-angiogenesis. *PloS One*. 2012;7:e29863.
51. Vourtsis SA, Spyriounis PK, Agrogiannis G, et al. Does VEGF have an effect on the survival of a long random skin flap by its application at the recipient area? *Chirurgia*. 2012;107:494–500.
52. Ridgway J, Zhang G, Wu Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature*. 2006;444:1083–1087.
53. Scehnet JS, Jiang W, Kumar SR, et al. Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood*. 2007;109:4753–4760.
54. Thurston G, Noguera-Troise I, Yancopoulos GD. The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat Rev Cancer*. 2007;7:327–331.
55. Djokovic D, Trindade A, Gigante J, et al. Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis. *BMC Cancer*. 2010;10:641.
56. Kalen M, Heikura T, Karvinen H, et al. Gamma-secretase inhibitor treatment promotes VEGF-A–driven blood vessel growth and vascular leakage but disrupts neovascular perfusion.*PloS One*. 2011;6:e18709.
57. Strilic B, Kucera T, Eglinger J, et al. The molecular basis of vascular lumen formation in the developing mouse aorta. *Dev Cell*. 2009;17:505–515.
58. Iruela-Arispe ML, Davis GE. Cellular and molecular mechanisms of vascular lumen formation. *Dev Cell*. 2009;16:222–231.
59. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res*. 2004;10:6897–6904.
60. Less JR, Posner MC, Skalak TC, et al. Geometric resistance and microvascular network architecture of human colorectal carcinoma. *Microcirculation*. 1997;4:25–33.
61. Less JR, Skalak TC, Sevick EM, et al. Microvascular network architecture in a mammary carcinoma. *EXS*. 1992;61:74–80.
62. Konerding MA, Miodonski AJ, Lametschwandtner A. Microvascular corrosion casting in the study of tumor vascularity: a review. *Scanning Microsc*. 1995; 9:1233–1243; discussion 1243–1244.