

ONLINE FIRST

Evaluating Revascularization and Flap Survival Using Vascular Endothelial Growth Factor in an Irradiated Rat Model

Patrick C. Angelos, MD; Shelley R. Winn, PhD; Darryl S. Kaurin, PhD; John Holland, MD; Mark K. Wax, MD

Objective: To evaluate the role of vascular endothelial growth factor (VEGF) plasmid DNA (pDNA) in improving flap revascularization in a previously developed rat model. Our hypothesis was that the uptake and expression of VEGF pDNA in the wound bed would improve revascularization and flap viability.

Design: Twenty-eight male Sprague-Dawley rats received a total dose of 40 Gy electron beam radiation to the ventral abdominal wall. After a recovery period, they underwent a ventral fasciocutaneous flap procedure with a 2-hour ischemia period. Group 1 (n=14) received topical VEGF pDNA, in vivo cationic polymer, and fibrin sealant. Group 2 (n=14) received topical cationic polymer and fibrin sealant only. Seven of the rats from each group underwent pedicle ligation at 8 or 14 days. The primary outcome measure was per-

centage of flap revascularization 5 days after pedicle ligation.

Results: Rats receiving VEGF pDNA had a significantly higher rate of flap revascularization (90.8% vs 79.8%) after pedicle ligation at 14 days ($P=.045$). At 8 days, rats receiving VEGF pDNA (group 1) had an increased rate of flap revascularization (58.2% vs 42.8%) that approached significance ($P=.11$).

Conclusion: This study demonstrates the potential of VEGF pDNA to improve revascularization and flap viability in previously irradiated tissue.

Arch Facial Plast Surg. 2011;13(3):185-189.

Published online January 17, 2011.

doi:10.1001/archfacial.2010.115

RECONSTRUCTION AFTER SALVAGE surgery for failed radiation or chemoradiation often involves free-tissue transfer and provides a challenge for the surgeon because of the effects of radiation on the host microvasculature. These changes ultimately affect wound healing. Whereas reconstructive flaps may have adequate blood supply to survive, the radiation effects on the host wound bed may delay flap incorporation and revascularization between the flap and host tissue. The effects of radiation therapy on the microvasculature have been described as a decrease in capillary density and diameter as well as excessive fibrosis, endothelial cell damage, and reduced cellular turnover.¹⁻³

Vascular endothelial growth factor (VEGF) is a potent angiogenic cytokine that induces angiogenesis, stimulates endothelial cells to mitogenic responses, and increases microvascular permeability.⁴ In the skin, VEGF is secreted by keratinocytes and fibroblasts and is especially active on dermal vascular structures.⁵ Pre-

viously, 2 of us have shown that the topical application of fibrin-embedded VEGF protein enhanced survival of fasciocutaneous flaps in nonirradiated animals.⁶ Other investigators have also demonstrated improved skin flap survival using VEGF gene therapy in nonirradiated animals.^{7,8} No previous studies, to our knowledge, have examined the effects of VEGF on flap revascularization in previously irradiated animals. The purpose of this study was to evaluate the effects of topical VEGF pDNA on revascularization of a ventral fasciocutaneous flap in an irradiated rat model.

METHODS

After Institutional Animal Care and Use Committee review and approval, 28 male Sprague-Dawley rats (Charles River Laboratories International Inc, Wilmington, Massachusetts) received a total dose of 40 Gy electron beam radiation to the ventral abdominal wall. After a 1-month recovery period, the rats underwent a ventral fasciocutaneous flap procedure with a 2-hour ischemia period. The rats were initially divided into 2 groups, an experi-

Author Affiliations:
Departments of Otolaryngology–Head and Neck Surgery (Drs Angelos, Winn, and Wax) and Radiation Oncology (Drs Kaurin and Holland), Oregon Health & Science University, Portland.

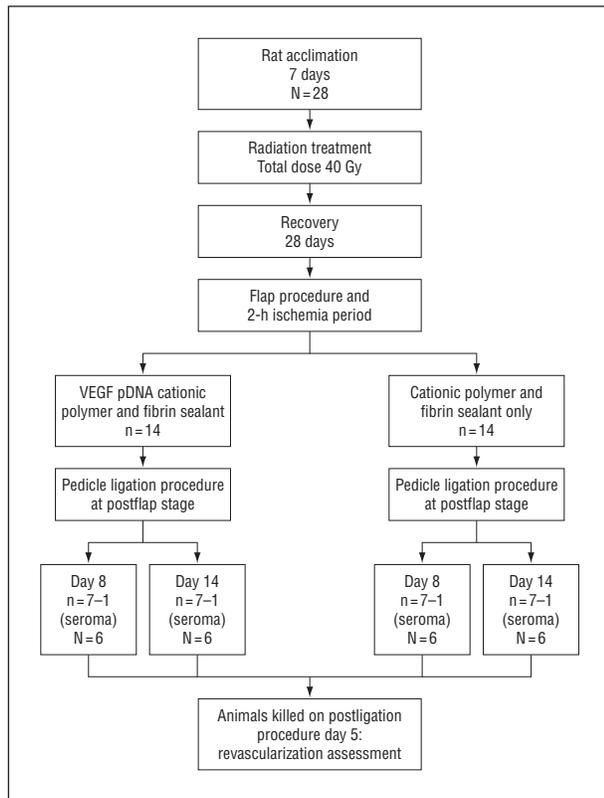


Figure 1. Study overview.

mental arm to receive VEGF pDNA and a control arm to receive only carriers (**Figure 1**). The experimental group (n=14) received topical VEGF pDNA, in vivo cationic polymer (jetPEI; Polyplus-transfection Inc, New York, New York), and fibrin sealant (Tisseel; Baxter International Inc, Deerfield, Illinois). The control group (n=14) received topical cationic polymer and fibrin sealant only. To evaluate the time course of revascularization, 7 rats from each group underwent pedicle ligation at 8 or 14 days. The primary outcome measure was percentage of flap revascularization measured as area of viable skin 5 days after pedicle ligation.

RADIATION PROTOCOL

All rats underwent general anesthesia using isoflurane inhalation during radiation. A lead shield was placed to isolate the templated flap region. A 6-MeV electron beam accelerator (Clinac 2100EX; Varian Medical Systems Inc, Palo Alto, California) was used to irradiate the rats. A bolus material of 2 cm on top of the abdomen was used to improve radiation dose distribution. During a 10-day period, radiation was administered in 5 divided doses of 8 cGy each, totaling 40 Gy.

VENTRAL FASCIOCUTANEOUS FLAP PROCEDURE

After a recovery period of 28 days after radiation, the rats underwent a ventral fasciocutaneous flap procedure. A ventral 3×6-cm fasciocutaneous flap (**Figure 2A**) pedicled on the inferior epigastric artery and vein was raised. Following elevation of the flap, a 20-g temporary occlusion clip was applied to the vascular pedicle for 2 hours to simulate the ischemic period during free-tissue transfer (Figure 2B). Prior to flap inset and closure, experimental or control solution was applied

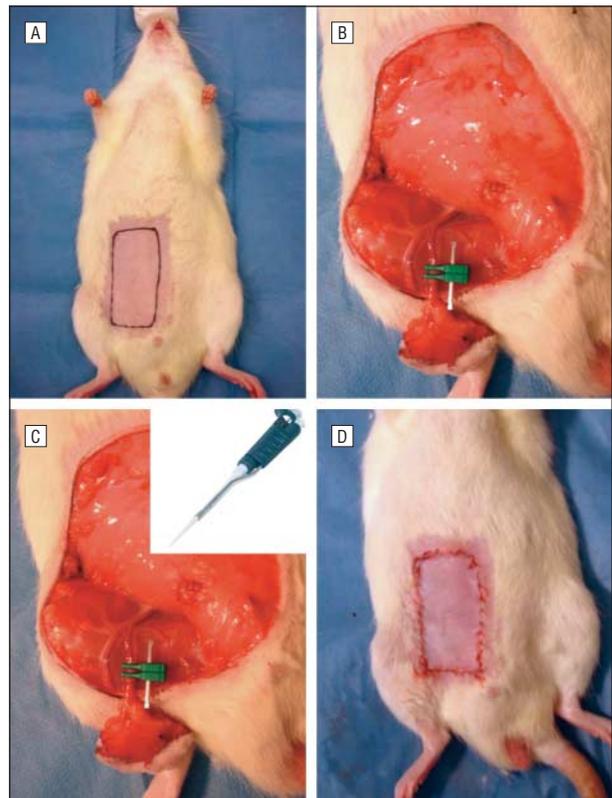


Figure 2. Flap procedure overview. A, 3×6 cm—Ventral fasciocutaneous flap (outlined in black). B, Temporary occlusion clip on the vascular pedicle at the inferior aspect of the raised flap. C, Application of topical solution via micropipette. D, Closed flap.

topically (Figure 2C). During this period, the flap was inset (Figure 2D) and the rats were awakened from anesthesia. At the end of the 2-hour period of simulated ischemia, the rats were briefly reanesthetized, the corner of the flap was elevated, the occlusion clip was removed, and the wound was reapproximated. The rats were monitored daily for signs of pain and discomfort and treated with analgesics as needed. They then underwent ligation of the inferior epigastric vein and artery at the previously outlined intervals.

VEGF pDNA AND CARRIERS

Preparation of VEGF pDNA was performed using recombinant polymerase chain reaction technology similar to that previously described.⁹ The messenger RNA (mRNA) of VEGF with alternate splicing amino acid number 165 (VEGF₁₆₅) was isolated from U937 histiocytic lymphoma cells (ATCC CRL-1593). Complementary DNA (cDNA) was synthesized from 1-μg polyadenylated RNA by a cDNA synthesis kit (Pharmacia Corporation, Kalamazoo, Michigan). cDNA application via polymerase chain reaction occurred using the VEGF₁₆₅-specific primers: *Forward*: 5'GAAACCATGAACTTTCT-GCTG and *Reverse*: 5'TCACCGCCTCGGCTTGTCACA.

This encodes the entire region and includes the signal sequence. The polymerase chain reaction product was ligated to pCEF1α-DNT-IgSP with the Eukaryotic TA Cloning Kit—Unidirectional (Invitrogen, Carlsbad, CA). The ligation mixture was transformed, colonies were selected, and the mixture was sequenced from both directions to ensure appropriate cDNA sequencing (**Figure 3**). For the present studies, the pDNA expressing the VEGF₁₆₅ was purified via Qiagen Plasmid Maxi Kits (Qiagen Inc, Valencia, California), and aliquots were nicked

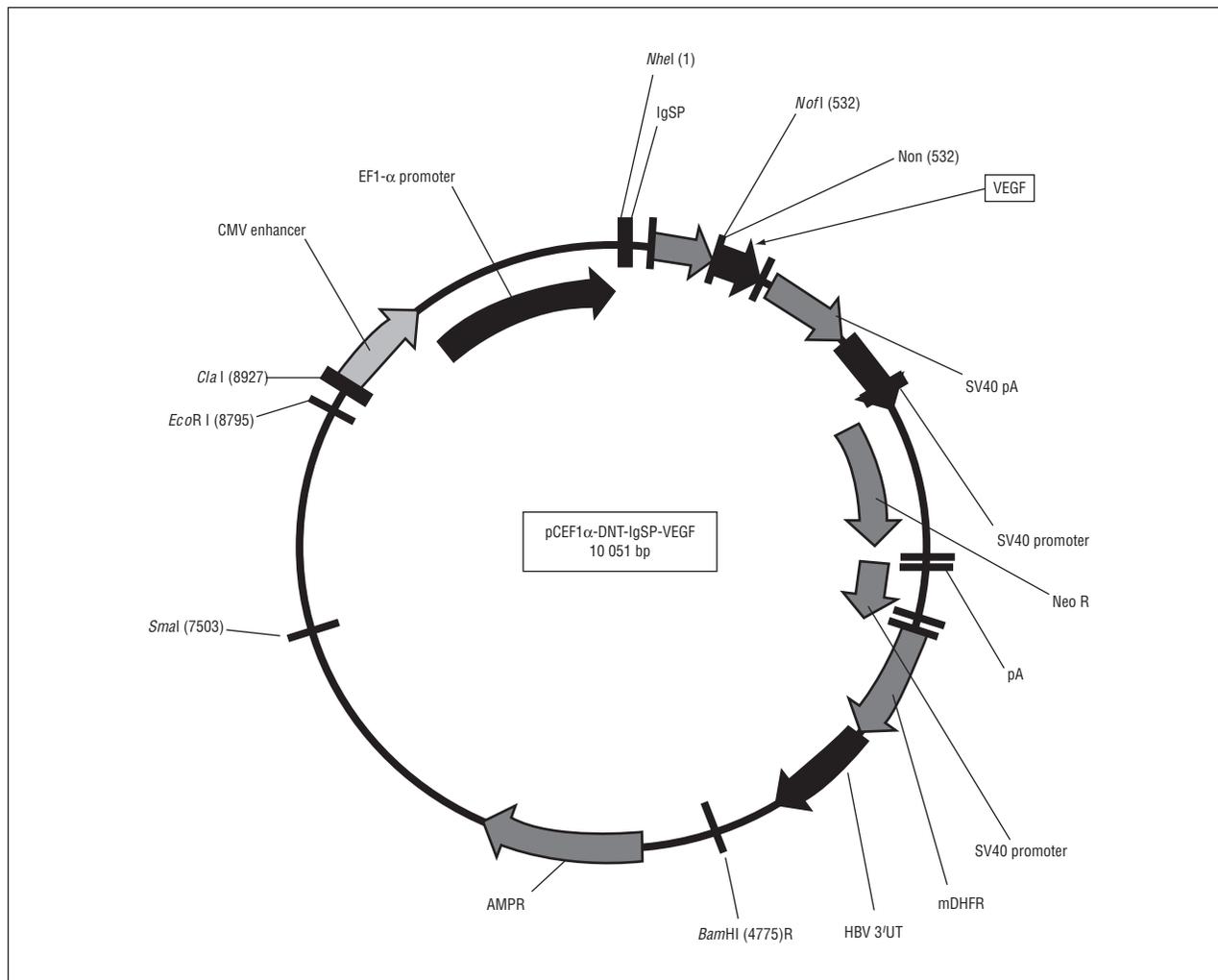


Figure 3. Plasmid DNA expressing vascular endothelial growth factor (VEGF). The pCEF1 α -DNT-IgSP-VEGF plasmid expression vector has a cytomegalovirus (CMV)-enhancer element that upregulates the elongation factor 1- α (EF1- α) promoter. The circular plasmid DNA construct contains ampicillin resistance for bacterial expansion and a neomycin analogue that confers resistance for selection in eukaryotic systems. Numbers in parentheses correspond to the specific base pair site(s) in the sequence of the location of the restriction endonuclease sites. AMPR indicates ampicillin resistant, HBV 3'UT, hepatitis virus B 3' untranslated region; IgSP, immunoglobulin signal peptide; mDHFR, mouse dihydrofolate reductase; Neo R, neomycin resistance; *NheI*, restriction endonuclease site; and pA, polyadenylation.

at appropriate restriction sites and run on 1% agarose gels for quality assurance.

After the vascular clip was applied, the rats were given 1 of 2 treatments. The control group was treated with a buffered solution in a polycation complex (in vivo cationic polymer) and further suspended in fibrin sealant. The experimental group received 100 μ g of pDNA-expressing, VEGF-condensed cationic polymer and further suspended in fibrin sealant. One milliliter of the viscous solutions was evenly distributed over the exterior surface of the 3 \times 6-cm elevated flap.

EVALUATION OF FLAP REVASCULARIZATION

Percentage of flap viability was evaluated on postligation procedure day 5 as a marker for flap revascularization. Viable flap area was characterized by warm, pink, hair-bearing skin. Non-viable flap area was characterized by dry, hard, hairless eschar. The rats were given general anesthesia, and standardized digital photographs of the ventral flap were taken. Three qualified masked observers were used to delineate viable and non-viable areas by tracing template etchings. Then, cutouts of vi-

able tissue from the template were weighed to express a percentage of the total template weight, which effectively gave the area percentage of viable flap tissue. Rats were excluded for evaluation if they developed a clinically significant hematoma, seroma, or infection.

STATISTICAL ANALYSIS

To determine the number of rats needed, a power analysis was performed using our preliminary data; it indicated that a minimum of 6 rats for each group would be required to complete a meaningful statistical analysis of flap revascularization to obtain $P < .05$ of significance. A Mann-Whitney test was used to evaluate whether a difference in percentage of flap viability existed between the experimental and control groups at 8 and 14 days to pedicle ligation.

RESULTS

We observed varying degrees of revascularization between the 4 groups, with a trend toward increased re-

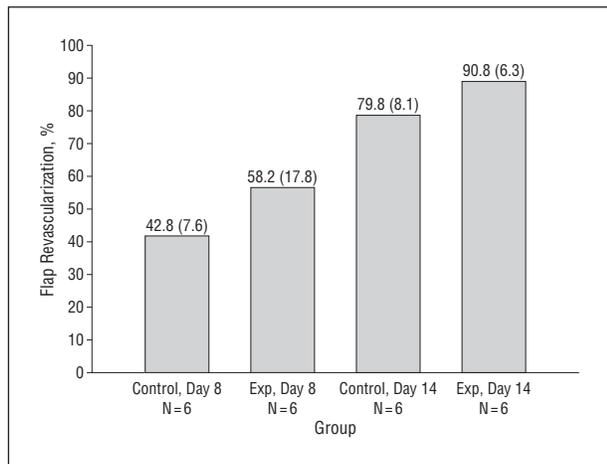


Figure 4. Average percentage flap revascularization results by group (mean percentage). The day number corresponds to day of pedicle ligation. Exp indicates the experimental group.

vascularization in the 2 groups with longer duration to pedicle ligation (**Figure 4**). Rats receiving VEGF pDNA had a significantly higher rate of flap revascularization (90.8% vs 79.8%) after pedicle ligation at 14 days ($P = .045$). At 8 days, rats receiving VEGF pDNA (group 1) had an increased rate of flap revascularization (58.2% vs 42.8%) that approached significance ($P = .11$). One rat from each of the 4 groups developed a seroma, and those rats were excluded from analysis.

COMMENT

Microvascular reconstruction after salvage surgery for failed radiation or chemoradiation is a difficult challenge. The long-term viability and healing of a reconstructive flap is not only dependent on the vascular pedicle but also on revascularization from the surrounding host tissue. It has been shown that previous radiation therapy leads to microvascular compromise in the host tissue that manifests as excessive fibrosis, endothelial cell damage, and reduced cellular turnover.^{1,3} Because of these effects on the host tissue, revascularization between an irradiated wound bed and a reconstructive flap may be diminished.

Vascular endothelial growth factor is an attractive agent for potential therapeutic application because it may improve the microvasculature in previously irradiated tissue and, in turn, the revascularization of remote flap tissue. Several previous studies have evaluated the effects of exogenous VEGF application on skin graft survival.^{4,7,8,10-13} These studies include evaluation of local and systemic application of VEGF protein or VEGF pDNA in various vectors. In a previous study, 2 of us⁶ evaluated the ability of topical application of fibrin-embedded VEGF pDNA and VEGF protein to enhance revascularization and survival of fasciocutaneous flaps in nonirradiated animals. We found that the use of a gene-activated matrix, consisting of pDNA entrapped in a polymer matrix carrier, was an effective method for gene transfer. Studies have shown that matrix carriers serve as a scaffold to hold DNA in situ until endogenous fibro-

blasts arrive.^{14,15} Other studies have also shown that fibrin-embedded administration of VEGF pDNA does not influence its angiogenic properties but enhances skin flap survival.^{8,16}

In the present study, we have shown that topical application of VEGF pDNA significantly improves revascularization of a ventral fasciocutaneous flap in an irradiated rat model after pedicle ligation at 14 days postoperatively. At 8 days postoperatively, we found that VEGF pDNA application did not lead to significantly increased revascularization and flap viability.

Very few previous studies, to our knowledge, have evaluated the effects of VEGF in an irradiated field. In a study of skin graft survival in irradiated rats, Richter et al¹⁷ evaluated the effect of local application of VEGF protein. They found that exogenously administered VEGF significantly decreased the graft failure rate but microvascular density was not different between groups. The lack of effect on microvascular density may be due to an increase in newly formed vessels in the recipient bed and not in the transplant bed. Previous studies have also shown similar findings of improved graft survival without a change in vessel density in the transplanted tissue.^{12,13,18,19}

The potential clinical application of this study involves the exogenous use of naturally occurring growth factors and cytokines to augment the protracted wound healing and revascularization of reconstructive flaps in previously irradiated patients. Promising results in soft tissue wound healing may translate into healing in microvascular reconstruction of bone (such as that of the mandible), and further studies are needed for these types of applications. The limitations of this study include a small number of rats in each group; the time between radiation and surgery, which may vary clinically; and the fact that the flap and tissue bed have been irradiated. Typically, the reconstructive flap is taken from a nonirradiated field and brings a fresh blood supply to the wound bed. In this model, previous radiation may affect the revascularization potential of the flap. It will be interesting to see whether future studies demonstrate a change in microvascular density or VEGF mRNA expression in this previously irradiated flap. Long-term studies are needed regarding the potential neoplastic effects or neoplasia potentiating effects of using VEGF gene therapy in a previously cancer-laden wound bed.

Accepted for Publication: November 1, 2010.

Published Online: January 17, 2011. doi:10.1001/archfacial.2010.115

Correspondence: Patrick C. Angelos, MD, Department of Otolaryngology–Head and Neck Surgery, Oregon Health & Science University, Mail Code PV01, 3181 SW Sam Jackson Park Rd, Portland, OR 97239 (angelosp@ohsu.edu).

Author Contributions: Drs Angelos and Wax had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Angelos, Winn, Kaurin, Holland, and Wax. *Acquisition of data:* Angelos, Winn, Kaurin, Holland, and Wax. *Analysis and interpretation of data:* Angelos, Winn, Kaurin, and Wax. *Drafting of the*

manuscript: Angelos and Wax. Critical revision of the manuscript for important intellectual content: Angelos, Winn, Kaurin, Holland, and Wax. Statistical analysis: Angelos and Kaurin. Obtained funding: Angelos and Wax. Administrative, technical, and material support: Winn, Holland, and Wax. Study supervision: Holland and Wax. Financial Disclosure: None reported.

REFERENCES

1. Denham JW, Hauer-Jensen M. The radiotherapeutic injury—a complex “wound.” *Radiother Oncol.* 2002;63(2):129-145.
2. Doll C, Durand R, Grulkey W, Sayer S, Olivetto I. Functional assessment of cutaneous microvasculature after radiation. *Radiother Oncol.* 1999;51(1):67-70.
3. Dormand EL, Banwell PE, Goodacre TE. Radiotherapy and wound healing. *Int Wound J.* 2005;2(2):112-127.
4. Scalise A, Tucci MG, Lucarini G, et al. Local rh-VEGF administration enhances skin flap survival more than other types of rh-VEGF administration: a clinical, morphological and immunohistochemical study. *Exp Dermatol.* 2004;13(11):682-690.
5. Detmar M, Yeo KT, Nagy JA, et al. Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. *J Invest Dermatol.* 1995;105(1):44-50.
6. McKnight CD, Winn SR, Gong X, Hansen JE, Wax MK. Revascularization of rat fasciocutaneous flap using CROSSEAL® with VEGF protein or plasmid DNA expressing VEGF. *Otolaryngol Head Neck Surg.* 2008;139(2):245-249.
7. Lubiatowski P, Goldman CK, Gurunluoglu R, Carnevale K, Siemionow M. Enhancement of epigastric skin flap survival by adenovirus-mediated VEGF gene therapy. *Plast Reconstr Surg.* 2002;109(6):1986-1993.
8. Michlits W, Mittermayr R, Schäfer R, Redl H, Aharinejad S. Fibrin-embedded administration of VEGF plasmid enhances skin flap survival. *Wound Repair Regen.* 2007;15(3):360-367.
9. Winn SR, Chen JC, Gong X, Bartholomew SV, Shreenivas S, Ozaki W. Non-viral-mediated gene therapy approaches for bone repair. *Orthod Craniofac Res.* 2005;8(3):183-190.
10. Khan A, Ashrafpour H, Huang N, et al. Acute local subcutaneous VEGF₁₆₅ injection for augmentation of skin flap viability: efficacy and mechanism. *Am J Physiol Regul Integr Comp Physiol.* 2004;287(5):R1219-R1229.
11. Kryger Z, Zhang F, Dogan T, Cheng C, Lineaweaver WC, Buncke HJ. 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(3):234-239.
12. O'Toole G, MacKenzie D, Lindeman R, et al. Vascular endothelial growth factor gene therapy in ischaemic rat skin flaps. *Br J Plast Surg.* 2002;55(1):55-58.
13. Taub PJ, Marmur JD, Zhang WX, et al. Locally administered vascular endothelial growth factor cDNA increases survival of ischemic experimental skin flaps. *Plast Reconstr Surg.* 1998;102(6):2033-2039.
14. Endo M, Kuroda S, Kondo H, Maruoka Y, Ohya K, Kasugai S. Bone regeneration by modified gene-activated matrix: effectiveness in segmental tibial defects in rats. *Tissue Eng.* 2006;12(3):489-497.
15. Peng L, Cheng X, Zhuo R, et al. Novel gene-activated matrix with embedded chitosan/plasmid DNA nanoparticles encoding PDGF for periodontal tissue engineering. *J Biomed Mater Res A.* 2009;90(2):564-576.
16. Jozkowicz A, Fügl A, Nanobashvili J, et al. Delivery of high dose VEGF plasmid using fibrin carrier does not influence its angiogenic potency. *Int J Artif Organs.* 2003;26(2):161-169.
17. Richter GT, Bowen T III, Boerma M, Fan CY, Hauer-Jensen M, Vural E. Impact of vascular endothelial growth factor on skin graft survival in irradiated rats. *Arch Facial Plast Surg.* 2009;11(2):110-113.
18. Banbury J, Siemionow M, Porvasnik S, Petras S, Browne E. Improved perfusion after subcritical ischemia in muscle flaps treated with vascular endothelial growth factor. *Plast Reconstr Surg.* 2000;106(7):1541-1546.
19. Zhang F, Oswald TM, Lin L, Wang S, Lin S, Lineaweaver WC. Improvement of full-thickness skin graft survival by application of vascular endothelial growth factor in rats. *Ann Plast Surg.* 2008;60(5):589-593.

Announcement

Visit www.archfacial.com. As an individual subscriber you may view articles by topic. Topic Collections group articles by topic area within a journal and across *JAMA* and the *Archives Journals*. The Topic Collections displayed on each journal site show the topic areas most relevant to that journal's readership. You may use the Topic Collections list in 3 ways:

1. From the Collections page, select the topic of interest to view all articles in that topic in 1 journal or all journals.
2. From an article page, click on the topic collections associated with that article to view other articles on that topic.
3. Sign up to receive an alert when new articles in *JAMA* and the *Archives Journals* are published on the topics of your choice.