AdVEGF-All6A+ Preconditioning of Murine Ischemic Skin Flaps Is Comparable to Surgical Delay

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Background: Surgical flap delay is commonly used in preconditioning reconstructive flaps to prevent necrosis. However, staged procedures are not ideal. Pharmacologic up-regulation of angiogenic and arteriogenic factors before flap elevation poses a nonsurgical approach to improve flap survival.
Methods: Male Sprague Dawley rats were divided into control ($n = 16$), surgical delay (Delay), AdNull, AdEgr-1, and AdVEGF ($n \geq 9$/group) groups. Delay rats had a 9 cm × 3 cm cranial based pedicle skin flap incised 10 days prior to elevation. Adenoviral groups received 28 intradermal injections (10^9 pu/animal total) throughout the distal two thirds of the flap 1 week prior to elevation. At postoperative day (POD) 0 flaps were elevated and silicone sheeting was placed between flap and wound bed. Perfusion analysis in arbitrary perfusion units of the ischemic middle third of the flap using laser Doppler imaging was conducted preoperatively and on POD 0, 3, and 7. Clinical and histopathologic assessments of the skin flaps were performed on POD 7.
Results: AdVEGF (50.8 ± 10.9 APU) and AdEgr-1 (39.3 ± 10.6 APU) perfusion levels were significantly higher than controls (16.5 ± 4.2 APU) on POD 7. Delay models were equivalent to controls (25.9 ± 6.8 APU). AdVEGF and Delay animals showed significantly more viable surface area on POD 7 (14.4 ± 1.3 cm^2, $P < 0.01$ and 12.4 ± 1.2 cm^2, $P < 0.05$, respectively) compared with Controls (8.7 ± 0.7 cm^2).
Conclusions: AdVEGF preconditioning resulted in flap survival comparable to surgical delay. Adenoviral preconditioning maintained perfusion levels postoperatively while surgical delay did not. *(Plast Reconstr Surg Glob Open 2015;3:e494; doi: 10.1097/GOX.0000000000000453; Published online 27 August 2015.)*

Local, regional, or free flaps may be hampered by partial- and full-thickness necrosis in the critical, distal zone of the reconstruction, leading to additional surgeries and increasing patient morbidity.[1–3] Comorbid conditions such as obesity, diabetes, radiation therapy, or other illness may further complicate flap survival.[4–6] Surgical delay is the most common method used to improve flap perfusion and survival. It remains the gold standard to which other techniques are compared. Surgical delay en-

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tails a second surgery with its inherent risk of surgical complications and increased financial burden. A nonsurgical option improving vascularity, perfusion, and ultimately flap survival would be of great benefit to patient and surgeon.

Vascular endothelial growth factor (VEGF) is a gene family with several isoforms produced in normal, and up-regulated in, wounded tissues.[7–9] $VEGF_{121}$ and $VEGF_{165}$ show more potent initiation of angiogenesis than $VEGF_{189}$ and $VEGF_{206}$.[8,10] VEGF is said to be capable of enhancing tumor growth when transfected into breast cancer cell lines and transplanted into nude mice. In this regard, $VEGF_{121}$ is the most potent isoform, with $VEGF_{189}$ showing no effect on tumor growth.[11]

A combinational VEGF vector, in which the longer isoform $VEGF_{189}$ is preferentially expressed over $VEGF_{121}$, improved safety and efficacy.[9,12] The use of this vector showed improved survival and reduced tumor growth in vivo. This suggests that multi-isoform adenoviral VEGF vector (AdVEGF-All6A+, hereafter AdVEGF) offers a potential therapeutic tool able to initiate angiogenesis with less risk of tumor growth.[12]

Arteriogenesis is the formation of mature vessels surrounded by a smooth muscle cell layer in response to vessel occlusion.[13,14] Early growth response gene-1 (Egr-1), a transcription factor, has been identified as a master switch regulating arteriogenesis. Soon after occlusion occurs in model systems, Egr-1 is dramatically up-regulated and initiates a cascade of key growth factors including Platelet-derived Growth Factor, Transforming Growth Factor, VEGF, and also Matrix Metalloproteinases.[15,16] As arteriogenesis may play a role in skin salvage in our model, an adenoviral Egr-1 vector (AdEgr-1) was also studied.

We evaluated the effects of AdVEGF and AdEgr-1 preconditioning on perfusion and necrosis in the McFarlane murine ischemic skin flap model because it is well established and has been previously used to test pharmaceutical intervention.[17,18] Two modifications were made to isolate the vascular supply to the flap: (1) a cranial based pedicle was used and (2) a sterile silicone sheet was placed between the flap and wound bed to prevent perfusion from the subcutaneous and lateral sources. Using this model, we found perfusion changes after vector administration or delay can be determined by laser Doppler imaging (LDI).[19]

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METHODS

Experimental Flap Creation

All animal protocols and husbandry were approved by the Institutional Animal Care and Use Committee of Stony Brook University. Eight- to ten-week-old male Sprague-Dawley rats (Charles River, Wilmington, Mass.) were anesthetized using 3–5% isoflurane and secured in a prone position on a sterile field with arms and legs fully extended. The dorsal hair in a 13 cm × 5 cm area, centered 1 cm cranial to the scapulae and extending 1 cm caudal to the iliac crests, was shaved and depilated using Nair (Church & Dwight, Princeton, N.J.). Care was taken to avoid skin trauma or irritation with the clippers and by limiting the time the skin was exposed to Nair and by thorough rinsing.

Five treatment groups were established: Control ($n = 16$), Delay ($n = 9$), AdNull ($n = 10$), AdVEGF ($n = 10$), and AdEgr-1 ($n = 11$). Animals receiving viral vectors (AdNull, AdVEGF, and AdEgr-1) were pretreated with virus 7 days before flap elevation. The 3 cm × 9 cm flap was outlined with surgical marker (Viscot Medical, East Hanover, N.J.), and 28 intradermal injections of 100 μl adenoviral vector each (10^9 pu/animal total) were placed at 1-cm intervals throughout the distal two thirds of the flap (Fig. 1A). This was intended to localize viral effect to areas that were found to become necrotic in prior work.[19] The animals in the Control group underwent no intervention before flap elevation. To simulate surgical delay, we used a modified McFarlane flap described by Holzbach et al.[20] Ten days before flap elevation, a 3 cm × 9 cm cranially based pedicle flap was created by incising the 3 borders without undermining the base and closing with sutures at 1 cm intervals. Following surgery, animals were allowed to recuperate normally with Nonsteroidal Anti-Inflammatory Drug pain relief postoperatively (Fig. 1B).

At flap elevation, animals were anesthetized via 3–5% isoflurane inhalation. A cranially based 3 cm × 9 cm McFarlane flap was created by incising through the paniculus carnosus, leaving a cranial based pedicle 1 cm caudal to the scapulae. This area selection allows for ample blood flow supplied by one or both of the 2 branches of the thoracodorsal artery at the pedicle base.[21] The flap was undermined using blunt dissection and elevated. A 3.5 cm × 9.5 cm × 0.0254 cm sterile silicone sheet (Technical Products of Georgia, Atlanta, Ga.) was placed between the wound bed and flap to prevent vascular ingrowth from the subcutaneous and lateral margins. The flap was then sutured down at 1 cm intervals with the silicone between the wound

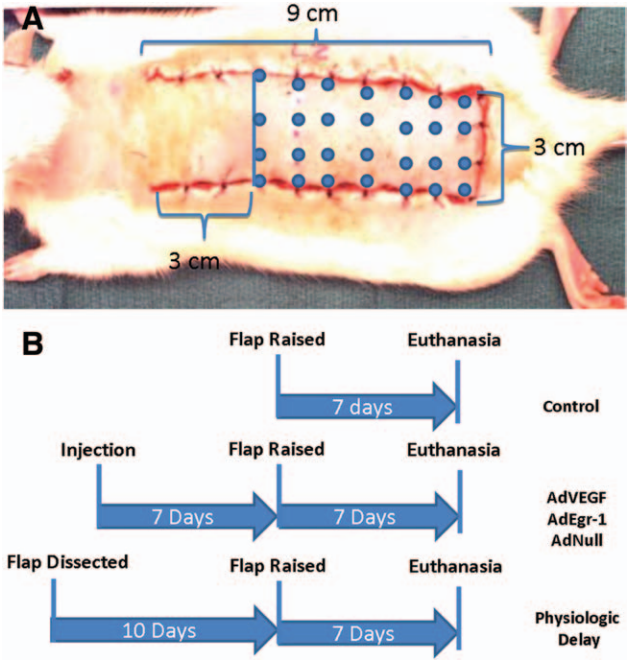

Fig. 1. Methodology and preconditioning for control, adenoviral, and delay experiments. A, Photograph of the skin flap with location of adenoviral vector injection points (blue dots) in the distal two thirds of the flap are outlined. B, Diagrams outlining the time course for each experiment are shown. Control animals undergo flap elevation 7 days before postoperative time course. Adenoviral preconditioned animals undergo 28 intradermal injections 7 days before flap elevation. Delay animals undergo incision of flap margins without undermining 10 days before flap elevation.

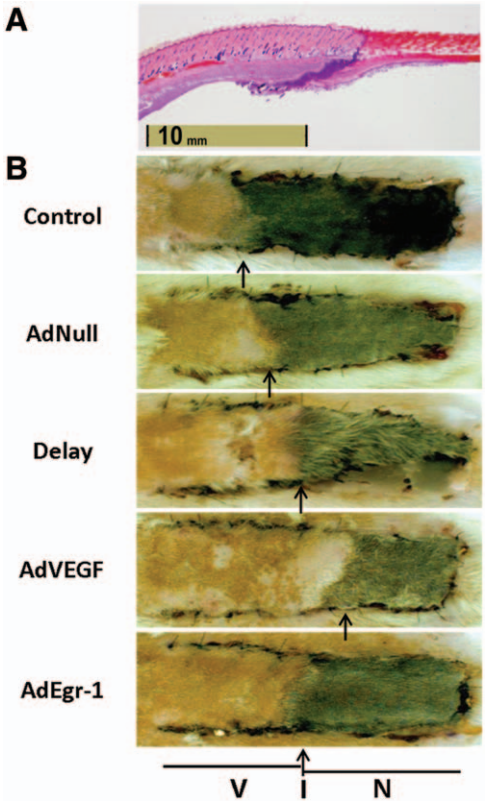

Fig. 2. Longitudinal hematoxylin and eosin (H&E) section of skin flap harvested on POD 7. A, Histopathologic representation of cross section of representative flap stained with H&E. B, Representative photographs of flap necrosis on POD 7 for each group. Black arrows denote mean interface (I) between viable (V) and necrotic (N) tissue.

margins. Euthanasia was performed 7 days post flap elevation via CO_2 asphyxiation followed by cervical dislocation.

Clinical and Histopathologic Assessment

Clinical assessments were performed on postoperative day (POD) 7 (Fig. 2). For each animal, before euthanasia, total viable surface area was determined by blinded clinical assessment (by R.P.G.) and reported in $cm^2 \pm$ standard error (Fig. 3). For our purposes, necrotic tissue was defined as dark and dusky skin lacking capillary refill and turgor compared with normal skin. These observations were supported by histopathologic assessment of proximal/viable, middle/ischemic, and distal/necrotic zones of the flap (Figs. 2, 4).

At the time of euthanasia, the flap was excised and biopsy samples were taken, fixed in 10% buffered formalin for 24 hours, processed in graded alcohols, xylene, and paraffin embedded, and then sectioned at 5 µm for H&E staining. Sections were examined and photographed by a board-certified dermatopathologist (S.A.M.) blinded to study conditions. Samples were examined by dissecting microscope and conventional light microscope (Olympus SZX7 and BX 61 with DP71 camera, Center Valley, Pa.), and photomicrographs were taken throughout the length of the flap.

LDI Analysis

Preoperatively and on POD 0, 3, and 7, LDI was performed in triplicate using the Perimed PIM3 (Perimed, Kings Park, N.Y.), with the scanning monitor head centered at mid-flap. Imaging at 255×555 pixels was performed over an area of 5 cm × 11 cm in triplicate, with aggregate scan times of 6 minutes per flap.

LDI analysis, using the Perimed analysis software (Perimed), was used to calculate average perfusion values over 3 regions of interest—the proximal/viable zone, the middle/ischemic zone, and the distal/necrotic zone, each 3 cm × 3 cm. Within the middle/ischemic zone, the 3 cm × 3 cm beginning 4 cm distal to the base of the flap average perfusion values were determined for each flap. No normalization was performed, as there was no need to account for

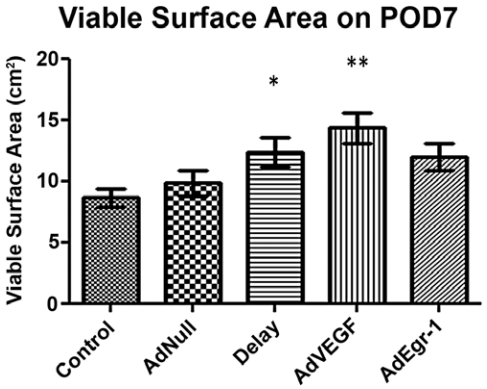

Fig. 3. Quantifying viable surface area. Viable surface area, defined as nonnecrotic flap tissue, was determined by blinded observer for Control (n = 16), AdNull (n = 10), Delay (n=9), AdVEGF (n=10), and AdEgr-1 (n=11) on POD 7. *P<0.05, **P < 0.01 compared to Control.

variable dose with this analysis technique. Perfusion was reported in arbitrary perfusion units (APU) ± standard error, which corresponds to signal return from erythrocytes in viable skin.[19]

Statistical Analysis

Statistical analysis was performed using Prism 5.0 (GraphPad, LaJolla, Calif.), and data sets were reviewed for normal distribution with the Grubbs Test for Outliers (α < 0.05). Statistical significance for clinical and perfusion measurements was determined using the Student's t test versus Control.

RESULTS

Viable Surface Area

Three distinct zones were observed in each flap on POD 7: a proximal/viable zone with normal histology, a middle/ischemic zone showing edema as well as epidermal and follicular atrophy, and a distal/necrotic zone demonstrating karyolysis, pyknosis, and karyorrhexis as shown in the representative images (Figs. 2, 4). Clinical assessment of the viable surface area also showed that Delay (12.4±1.2 cm²; P < 0.05) and AdVEGF (14.4±1.3 cm²; P < 0.01) had significantly more viable surface area when compared with Control (8.7±0.7 cm²) and AdNull (9.9±1.0 cm²). AdEgr-1 (12.0±1.1 cm²) showed increased viable surface area compared with Control, yet this difference failed to reach significance (P = 0.058; Fig. 3).

Perfusion Analysis

Perfusion analysis focused on the middle/ischemic third of the flaps, which often contained the interface between viable and necrotic tissue because the proximal third of the flaps consistently remained viable and the distal third consistently became necrotic. Preoperative average perfusion for each treatment group (Delay: 207.4±17.0 APU, P < 0.05; AdVEGF: 176.1±10.3 APU, P < 0.01; AdEgr-1: 179.7±23.4 APU, P < 0.01; and AdNull: 174±18.6 APU, P < 0.01) had significantly lower perfusion levels when compared with Control (266±17.9 APU).

On POD 0, immediately post flap elevation, Delay showed significantly higher perfusion levels (63.3±7.4 APU; P < 0.05) than Control (44.3±2.2 APU; Fig. 4) or AdNull, AdVEGF, and AdEgr-1 (39.2±4.0 APU, 40.2±2.6 APU, and 38.0±2.0 APU, respectively).

On POD 3, both Delay (44.5±7.4 APU; P < 0.05) and AdVEGF (47.9±8.2 APU; P < 0.05) showed significantly higher perfusion levels compared with Control (26.50±4.94 APU), whereas AdNull (31.8±5.1 APU) and AdEgr-1 (38.4±5.3 APU) showed no significant difference in perfusion levels.

On POD 7, both AdVEGF (50.8±10.9 APU; P < 0.01) and AdEgr-1 (39.3±10.6 APU; P < 0.05) showed significantly higher perfusion in the middle third of the flap compared with Control (16.5±4.2 APU), whereas AdNull and Delay (24.3±6.1 APU and 25.9±6.8 APU, respectively) showed no significant difference in perfusion levels (Table 1).

DISCUSSION

AdVEGF preconditioning showed improved flap survival compared with the Control group and was comparable to the Delay group at completion of the time course on POD 7. Preoperatively, all treatment groups showed significantly reduced perfusion levels within the middle third of the flap compared with control; this was likely caused by the pre-elevation surgery in the Delay group and adenoviral administration in the other groups. Immediately after flap elevation, the Delay group showed higher perfusion levels in the middle/ischemic zone compared with all other groups. However, these levels declined on POD 3 and were not significantly different from Control by POD 7. Preconditioning with either AdEgr-1 or AdVEGF maintained perfusion in the middle third of the flap, resulting in significantly higher perfusion levels on POD 7 compared with either Delay or Control, peaking with 2.4- and 3.0-fold higher perfusion levels than controls, respectively. These data indicate that adenoviral preconditioning may offer comparable outcomes to surgical delay, reducing flap necrosis.

VEGF is a potent growth factor; the utility of which has been shown in several angiogenic models, with time courses spanning 2 weeks to 1 year.[7–9] In this model, adenoviral vectors were injected intradermally sequestering the virus to the affected

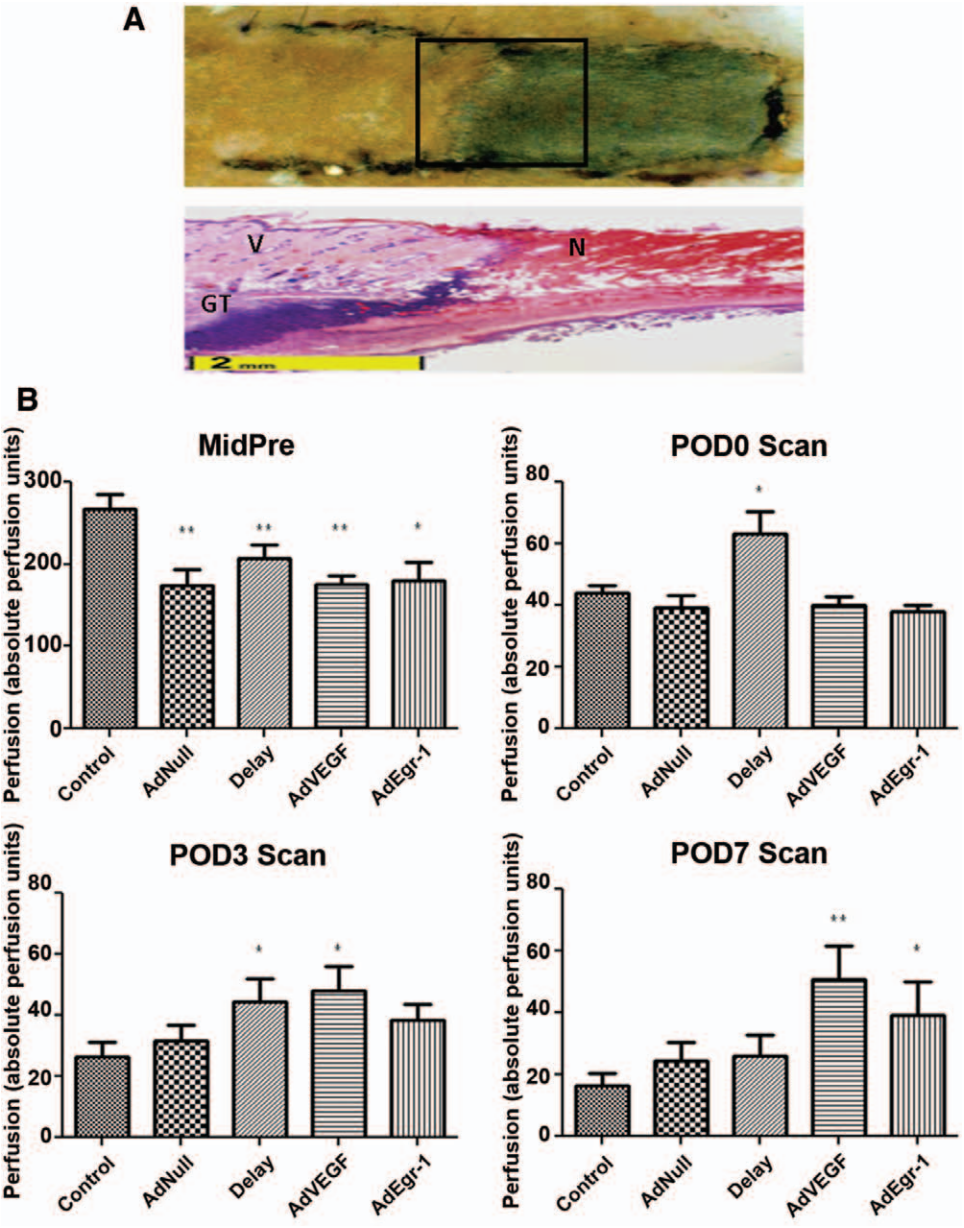

Fig. 4. Perfusion levels in the middle/ischemic region of the skin flap throughout the time course. A, Histopathologic cross section of a representative skin flap. Black arrows denote mean interface (I) between viable (V) and necrotic (N) tissue is noted. GT designates granulated tissue. B, Perfusion levels were measured within a 3 cm × 3 cm middle/ischemic zone in the center of the skin flap (black box) via LDI for Control (n = 16), AdNull (n = 9), Delay (n = 9), AdVEGF (n = 10), and AdEgr-1 (n = 12) preoperatively and on POD 0, 3, and 7. *P < 0.05, **P < 0.01 compared to Control.

tissue similar to the methods using topical VEGF for treatment in diabetic wounds healing.[22] The use of intradermal injections eliminates the possibility of treatment loss if the cream rubs off, while further reducing the off target effects of VEGF. This may explain why AdVEGF preconditioning translates to increased flap survival when AdEgr-1 administration does not. VEGF isoforms are able to initiate vasculogenesis in addition to acting as growth factors and activating other key angiogenic regulators. AdVEGF infection results in increases of secreted VEGF isoforms within 24 hours.[12] Although it is accepted that surgical delay increases perfusion via the release of choke vessels within the skin,[23] the mechanism by which VEGF maintains perfusion in this study is unknown; however, the shortened time course of this experiment suggests that neovascularization is not the sole mechanism. The vasodilation effect of this

Table 1. Perfusion Levels Throughout Postelevation Time Course

APU Levels	Control	AdNull	Delay	Delay AdVEGF	AdEgr-1
Preoperative	**266.0±17.9**	174.0±18.6	207.4±17.0	176.1±10.3	179.9±23.4
POD 0	44.2±2.2	39.2±4.0	**63.3±7.4**	40.2±2.6	38.0±2.0
POD 3	26.5±4.9	31.8±5.1	44.5±7.4	**47.9±8.2**	38.4±5.3
POD 7	16.4±4.2	24.3±6.1	25.9±6.8	**50.8±10.9**	39.3±10.6

Arbitrary perfusion units (APU) are listed for each group throughout the 7-day postelevation time course. Bold numbers indicate the highest treatment group perfusion level on each day.

protein may also play a role.[1,24] Mechanistic studies are currently underway to address this.

By contrast, AdEgr-1 intervention did not significantly improve flap survival. However, a trend toward increased viable surface area and significantly improved perfusion suggest this vector's therapeutic potential because peak perfusion levels may not occur for several weeks.[15,16] Egr-1 is a transcription factor and its up-regulation must activate downstream growth factors, matrix metalloproteinases, adhesion molecules, and chemoattractants before reperfusion.[23] Given the nature of this gene, it is possible that the 14-day time course in this study may be long enough for Egr-1 to initiate signal transduction required for reperfusion but too short for this reperfusion to impact flap survival. Extending the preoperative injection time may show significantly improved skin flap survival.

Although VEGF is not inherently tumorigenic, that is, capable of creating de novo tumors, potential risks of inducing angiogenesis may include enhanced tumor growth when VEGF is injected into already established neoplasm.[11] This would be a contraindication for its use in cancer-related reconstruction. We used a viral vector specifically designed to improve efficiency while enhancing safety.[9,12] This vector preferentially expresses the longer $VEGF_{189}$ isoform while limiting the expression of $VEGF_{121}$, which has been linked to tumor growth in vivo[11] resulting from increased angiogenesis in neoplasm.[12] Furthermore, use of adenoviral vectors mitigates the risks of tumor growth as genes are only expressed for a short duration,[25] with an expression effect lasting 14 days as demonstrated in other skin models.[26] This transient pulse-like nature of gene expression further reduces the inherent tumor growth risks of VEGF. Finally, in previous studies on tumorigenicity, AdVEGF vectors were injected intravenously and therefore had systemic effect.[12] In this model, adenoviral vectors were injected intradermally sequestering the virus to the affected tissue and further reducing the off target effects of VEGF.

AdVEGF preconditioning offers the potential of improved flap survival comparable to surgical delay without the inherent complications of a surgical procedure. AdEgr-1 preconditioning showed a positive trend in improved flap survival when compared with control. This preliminary study suggests that future work on optimizing preconditioning by injecting AdEgr-1 at earlier time course with and without AdVEGF warrants investigation.

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