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ORIGINAL PAPER

Angiogenic response to extracorporeal shock wave treatment in murine skin isografts

Alexander Stojadinovic · Eric A. Elster · Khairul Anam · Douglas Tadaki · Mihret Amare · Stephen Zins · Thomas A. Davis

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Abstract Skin grafts are commonly utilized and proven effective methods of open wound coverage. Revascularization through neoangiogenesis is a pivotal mechanism for skin graft integration and durability. Extracorporeal shockwave treatment (ESWT) has been demonstrated to accelerate wound repair; however, its mechanism-of-action is unclear. We investigated the role of ESWT in early revascularization of full-thickness skin isografts in a murine model. Cohorts of mice were euthanized and skin grafts were harvested 6 h, 2, 4, and 7 days post grafting ± ESWT. Various aspects of graft neovascularization were measured including gross morphology, quantitative microscopy (vessel number, density), immunohistochemistry (CD31), cDNA SuperArrays for 84 angiogenesis-specific genes, and custom-designed 'Wound Repair' TaqMan® Low Density Array (TLDA) cards to assess expression of 188 wound repair genes. We demonstrate that a single administration of ESWT immediately following skin grafting significantly enhances recipient graft revascularization (increased vessel number, size, and density). An augmented early pro-angiogenic and suppressed delayed pro-inflammatory response to ESWT was accompanied by significantly increased expression of both skin graft CD31 and angiogenesis pathway-specific genes, including ELR-CXC chemokines (CXCL1, CXCL2, CXCL5), CC chemokines (CCL2, CCL3, CCL4), cytokines (IL-1β, IL-6, G-CSF, VEGF-A), matrix metalloproteinases (MMP3, MMP9, MMP13), hypoxia-inducible factors (HIF-1α), and vascular remodeling kinase (Mst1), as early as 6 h and up to 7 days post grafting and treatment. These findings suggest that early pro-angiogenic and anti-inflammatory effects of ESWT promote tissue revascularization and wound healing by augmenting angiogenesis and dampening inflammation.

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Keywords Angiogenesis · Extracorporeal shock wave therapy · Skin graft · Wound healing

Abbreviations
ESWT Extracorporeal shock wave treatment

Introduction

Wound healing is a complex, sequential, local cellular, and molecular response encompassing overlapping biological processes generally defined by inflammation, epithelialization, angiogenesis, and matrix deposition [1]. During early wound healing, a vigorous angiogenic response supports the increased metabolic demands of rapidly proliferating and migrating leukocytes, keratinocytes, fibroblasts, and endothelial cell (EC) precursors [2–6]. Ultimately, these early angiogenic and inflammatory responses diminish as wound closure, fibrosis, and remodeling predominate [7, 8]. In chronic wounds, this orderly and intricate biological response to wounding is disrupted. The inability to progress through the inflammatory phase of wound healing to complete epithelialization is characteristic of chronic wounds, where prolonged and aberrant accumulation of matrix metalloproteinases (MMPs) impedes cell migration and downregulation of tissue inhibitors of MMPs, hinders neovascularization essential to restoration of local oxygen delivery (O_2), and interferes with extra-cellular matrix (ECM) remodeling [9–12].

Non-healing wounds can pose difficult challenges to timely recovery, rehabilitation, quality of life and cost-effective medical care. Skin grafts remain a time-tested reliable means of efficient and definitive open wound closure. The ultimate survival of skin grafts is dependent on the ability of the graft to receive essential metabolic nutrients (serum imbibition) and vascular in-growth from the recipient wound bed [13]. The underlying mechanisms of skin graft angiogenesis and vasculogenesis have recently been elucidated in depth and include: recipient bed vascular in-growth, donor graft vascular endothelial regression or involution, and inosculation of donor and recipient vascular endothelium, driven in part by recipient bone marrow-derived endothelial progenitor cells responding to hypoxic signaling pathways [2, 8].

Various therapeutic strategies have been implemented to accelerate epidermal cell proliferation and angiogenesis within the open wound bed; however, attempts to use soluble mediators and growth factors have been unfulfilling [14, 15]. Recent studies have emphasized the importance of the wound stimulatory effects of micromechanical forces ("cellular mechanotransduction") [16, 17]. One application of this principal, micro-deformational wound therapy, utilizes local negative pressure- or vacuum-assisted closure to promote neovascularization, granulation tissue formation, and epithelial cell proliferation and migration within the open wound [18, 19]. Innovative approaches to accelerated wound healing and tissue regeneration have broadened the potential clinical ability of other biomechanical therapeutic modalities [20–22].

One such biomechanical modality, extracorporeal shock wave treatment (ESWT), generates an acoustic pressure wave that penetrates tissue and produces favorable biological responses. The target tissue response occurs through complex and incompletely understood cellular biochemical pathways. We have recently shown in a mouse model of severe full-thickness burn injury that ESWT has significant anti-inflammatory properties associated with a significant decrease in pro-inflammatory chemokine and cytokine synthesis at the wound site coupled with a marked reduction in inflammatory leukocyte recruitment to the treated burn [23]. Furthermore, preliminary findings from earlier studies suggest that local delivery of shock waves may stimulate early expression of angiogenesis-related growth factors including endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), and proliferating cell nuclear antigen (PCNA), associated with vasculogenesis, improved local blood flow, EC proliferation, and accelerated soft tissue repair [24–27].

As skin graft survival relies on neoangiogenesis to revascularize ischemic tissue, and the underlying mechanisms of shock wave-derived molecular effects are poorly understood, the present study evaluates the angiogenic response to ESWT in an established ischemic tissue model of murine full-thickness skin isografting. We demonstrate that a single shock wave treatment immediately after skin grafting significantly augments recipient graft revascularization. This pro-angiogenic response to ESWT is associated with significantly increased CD31-positive EC proliferation and early post treatment VEGF-A expression, upregulation of angiogenesis pathway-specific genes in the skin graft, as well as reduced and delayed inflammatory response to ESWT. The early pro-angiogenic and anti-inflammatory effects of ESWT in ischemic tissue make it an attractive therapeutic modality for promoting wound healing.

Materials and methods

Animals

Seven- to eight-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the Walter Reed Army Institute of Research (WRAIR, Silver Spring, MD) animal facility,

which is certified by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All procedures were conducted using facilities and protocols approved by the Animal Care and Use Committee of WRAIR (protocols #K01-08 and #K06-05). Mice were housed five animals per cage prior to any study intervention, and caged individually post skin transplantation in standard micro-isolator polycarbonate cages. Mice, age 10–12 weeks, weighing 18–22 g were used for skin transplantation. Animal rooms were maintained at a temperature 21 ± 2°C with 50 ± 10% humidity on a 12-h light/dark cycle. Commercial rodent ration (Harlan Teklad Rodent Diet 8604) was available freely, as was acidified (pH = 2.5) water to prevent opportunistic infections.

Skin transplantation

Skin transplantation was conducted as previously described [28]. Briefly, full-thickness donor skin (3 cm × 3 cm) was harvested from the dorsum of euthanized and shaved donor BALB/c mice, and the underside of the donor skin was gently scraped with a scalpel to remove fat and muscle. Donor skin sections were placed in a petri dish containing sterile normal saline and maintained at 4°C until grafted. The dorsal surface of an anesthetized (Ketamine 100 mg/kg and Xylazine 5 mg/kg injected intra-peritoneally; Reckitt Benckiser Pharmaceuticals, Richmond, VA) recipient BALB/c mouse was shaved and skin washed with 70% ethanol. An oval graft bed ~2 cm in maximum diameter was prepared with fine curved scissors by removing an area of epidermis and dermis down to the level of the underlying musculature. Full-thickness skin grafts, 3 cm^2 in surface area, were fitted to the prepared recipient bed without suturing, covered with a liberal amount of Bacitracin ointment (Pharmaderm, Melville, NY), shock wave- or sham-treated, then covered with an adhesive plastic bandage. Once the mice were recovered from anesthesia, they were placed alone in separate cages and maintained under standard conditions in the animal facility. Buprenorphine (Reckitt Benckiser Pharmaceuticals, Richmond, VA), 0.1 mg/kg SC BID, was given on post-operative days 1 and 2 for pain management. No topical wound care was provided aside from the aforementioned Bacitracin.

A total of 90 full-thickness skin grafts, including 46 shock wave-treated and 44 sham-treated skin transplants, were included in this study. Cohorts of mice (4–5 per group at each time point for each experiment) were euthanized 6 h, 2, 4, and 7 days post grafting and treatment. Following euthanasia, the skin grafts and adjacent normal tissue were excised carefully. The underside of the skin was photographed 2, 4, and 7 days post grafting for macroscopic examination of blood vessel formation. Next, the graft and a small amount of peripheral recipient skin tissue were divided into three segments. One segment was processed by formalin fixation and paraffin embedding for histological evaluation, the second section was embedded in Tissue-Tek OCT cryopreservation medium and snap-frozen (in 2-methyl butane using dry ice bath) for immunohistochemistry staining; and, the third segment was dissected into small pieces and stored in RNAlater® (Ambion, Austin, TX) for gene transcript expression studies.

Extracorporeal shock wave treatment

Immediately post grafting, Bacitracin ointment was applied directly to the unfocused lens of the DermaGold™ (Tissue Regeneration Technologies, LLC, Woodstock, Georgia) shock-wave applicator. The ESWT applicator is comprised of parabolic reflector, was gently placed directly on the dorsal skin isograft, which was treated with 200 impulses (energy level = 0.1 mJ/mm^2, frequency = 3 pulses per second). The parabolic reflector permits a large treatment area to be stimulated by the acoustical field. ESWT lasted ~45 s. Sham-treated grafts were treated identically; however, no shock wave impulses were administered. The dose response (ESWT energy level and frequency) is well established for this modality and based on extensive animal and human use testing [22, 23, 29, 30].

Quantitative angiogenesis assessment

At the gross macroscopic level, documentation of new vessel formation on the underside of full-thickness dorsal skin grafts was captured by photographic image analysis using a digital Fuji Finepix Camera (Fuji Imaging, Valhalla, NY) in the presence of a scaled ruler (to ensure standardized assessment at consistent magnification). Images were imported into Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) for reproduction. Images were assessed for visible alterations in graft vascularity under magnification at 4×. Visible vessels with and without ramifications and branches were counted in accordance with the criteria described by Isenberg et al. [31]. For quantification of skin graft microvessels, 5-μm graft cross-sections made from the mid portion of each full-thickness skin graft were made using a Leica CM 1900 cryostat (Leica Microsystems). All graft sections and washes were performed at room temperature. Cryosections were fixed in acetone, endogenous peroxidase inactivated (0.03% H_2O_2, 0.15 mol/l NaN_3), and non-specific binding sites blocked with a 1:10 dilution of normal mouse serum (Sigma Chemical Company, St Louis, MO) in phosphate buffered saline (PBS) for 30 min. Central graft cross-sections were incubated with 1.0 μg/ml rat anti-mouse CD31 antibody (anti-PECAM-1, clone MECA-133; Pharmingen,

San Diego, CA). Graft cross-sections were washed, incubated for 30 min with biotinylated mouse anti-rat IgG (1:250 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA), and visualized using a Streptavidin–Biotin–Peroxidase staining kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The sections were developed using horseradish peroxidase substrate, 3,3′-diaminobenzidine (Sigma Chemical Company, St Louis, MO) for 10 min. Slides were counterstained with Harris Hematoxylin (Sigma Chemical Company, St Louis, MO) to stain nuclei and coverslipped with a permanent mounting medium (Permount, Fisher Scientific, Pittsburg, PA). Digital images of CD31 (PECAM-1)-stained skin graft sections were captured at ×100, ×200, and ×400 magnification using a BX50 Olympus microscope equipped with an Insight Firewire Spot Color Camera and Spot 4.6 photographic and analysis software (Diagnostic Instruments, Inc., Sterling Heights, MI). Two independent observers (blinded to study group assignment) assessed microvessel density in the graft sections by enumerating the number of CD31-positive vessels in consecutive 8–16 high-power fields (at 400× magnification) across each central graft section. Standardized analysis of pixel density using digital analysis software (ImageJ, NIH) was used to enumerate the CD31 (PECAM)-positive area(s) within each skin graft section and the overall percent vascularization.

RNA extraction

Total RNA was extracted from the excised skin graft and adjacent marginal tissue and stored in RNAlater® (Ambion, Austin, TX). Briefly, skin tissue was homogenized using Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated using Qiagen RNeasy Lipid Tissue Mini Kit (QIAGEN Inc. Valencia, CA) according to manufacturer's instructions. Isolated RNA was resuspended in 30 µl of 10 mM Tris buffer, pH 7.5. Sample purity, quantity, and quality were assessed by determining the $A_{260/280}$, $A_{260/230}$ ratio on a Nanodrop Spectrophotometer (NanoDrop Technologies Inc. Wilmington, DE), by measuring 28S/18S ribosomal RNA ratio and ascertaining RNA integrity number (RIN) using an Agilent 2100 Bio-Analyzer (Agilent Technologies Inc. Santa Clara, CA). Agilent RNA integrity values for all sampled wound specimens in this study were $\geq$8.5.

Gene expression profiling for angiogenic transcripts

Gene expression profiles were created using the RT^2-Profiler PCR Array: Mouse Angiogenesis Gene Array (SuperArray, Rockville, MD). The total RNA was isolated as described above, and subsequently converted to cDNA using SuperArray's RT^2 First Strand Kit per the manufacturer's instructions (SuperArray, Rockville, MD). The expression of each gene tested at multiple time points (6 h, 2, 4 and 7 days post skin grafting ± ESWT) was expressed as relative gene transcript expression level, normalized to the averaged value of the set of multiple internal controls (housekeeping genes). A gene was considered to be differentially up- or downregulated by shock wave treatment if it was differentially expressed by at least twofold compared with the sham-treated control group.

Quantitative real-time PCR (RT-PCR) gene profiling for wound healing-repair gene transcripts

Quantitative real-time polymerase chain reaction (RT-PCR) was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Custom-designed 'Wound Repair' TaqMan® Low Density Array (TLDA) cards (Applied Biosystems, Foster City, CA) were used to assess skin graft transcript gene expression. The set of TLDA cards comprised 188 individual target assays including respective forward and reverse primers and a dual-labeled probe (5′-6-FAM; 3′-MGB) in quadruplicate on a 384-well card (96 genes per card, including housekeeping genes). Amplification parameters were as follows: one cycle of 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Two tissue samples were processed on each card.

RT-PCR data analysis

RT-PCR data were analyzed using the Sequence Detection System version 2.1 included with the ABI Prism 7900HT SDS (Applied Biosystems, Foster City, CA) or using Microsoft Excel. The threshold cycle (C_t) for each sample was manually set to 0.2 and the baseline was set between 3 and 15 cycles. 18S ribosomal RNA was used as an endogenous housekeeping control for normalization and the comparative C_t method was used to calculate the relative fold expression by$2^{-\Delta\Delta C_t}$. Assays with C_t values greater than 35 cycles were excluded from analysis.

Statistics

Data were analyzed using GraphPad Prism version 4.01 software (GraphPad Software, San Diego, CA, USA). Gene expression is shown as fold change in average C_t value relative to expression levels of naïve skin. A greater than twofold increase in specific gene expression was considered significant. SuperArray (SuperArray, Rockville, MD) statistical analyses were performed using the manufacturer's software, setting $P < 0.05$ for significant differential gene expression between sham- and ESWT-treated tissues. All other results are expressed as mean ± SD. Statistical

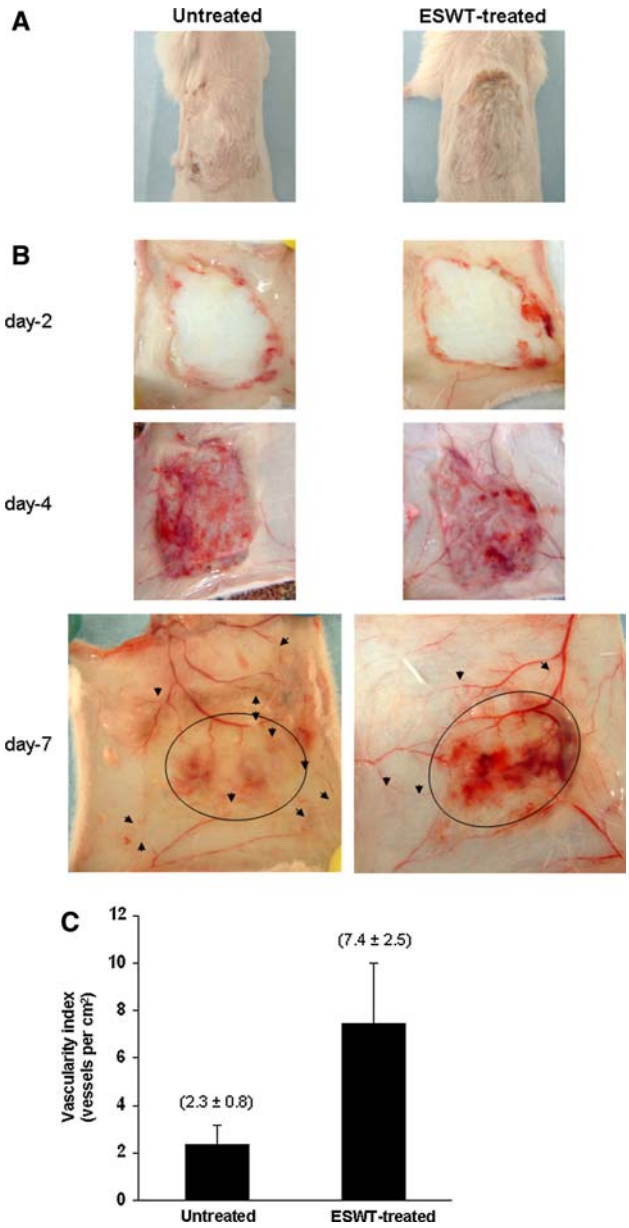

Fig. 1 ESWT stimulates angiogenesis and tissue revascularization in a murine skin grafting tissue ischemia model. Full-thickness skin from syngeneic donor mice was grafted onto the dorsum of adult recipient BALB/c mice treated with either a single ESWT application (200 impulses of ESWT, energy level = 0.1 mJ/mm^2, frequency = 3 pulses per second) or sham-treated at the time of graft placement over the wound bed. **A** Representative photographs of sham-treated and ESWT-treated isografts on BALB/c recipient mice 7 days post grafting ($n = 16$ mice per group) demonstrate no gross macroscopic differences in either isograft appearance, graft acceptance, or the overall healing process. **B** Macroscopic analysis of the vessels in the underside of ESWT-treated and sham-treated isografts reveals more pronounced angiogenesis in ESWT-treated isografts. Representative images ($n = 12$ mice per group) of the underside of isografts surgically excised from sham-treated and ESWT-treated mice on days 2, 4, and 7 post skin transplantation. The margin of the graft edge is delineated with the *elliptical outline*. The *black arrow heads* point to those vessels that are obviously undergoing vascular regression as determined by microscopic assessment of the images at ×4 magnification. **C** Vascularity of the donor skin graft and the adjacent recipient tissue at the peripheral graft edge was measured on day 7 post skin transplantation. Results represent the mean ± SD number of visible vessels (×4 magnification) per cm^2 ($P < 0.001$, $n = 12$ mice per group)

analysis of variance was used to compare mean values of specific covariates between study groups, and Mann–Whitney U test was used to determine the level of significance of differences between sample means. Values of $P < 0.05$ were considered statistically significant.

Results

ESWT enhances ischemic tissue angiogenesis and revascularization

We evaluated the effects of ESWT on ischemic tissue angiogenesis and revascularization using a well-established murine model of skin transplantation. Full-thickness skin isografts were transplanted from syngeneic BALB/c donor mice onto the dorsum of adult recipient BALB/c mice. At the time of placement of the skin graft over the open recipient bed (devoid of overlying dermis), isografts were treated with 200 impulses of ESWT (energy level = 0.1 mJ/mm^2, frequency = 3 pulses per second) or identical sham treatment absent the delivery of shock waves. No gross macroscopic differences in isograft appearance, graft acceptance, or overall healing were noted in the short-term (6 h to 1 week post transplant; Fig. 1A; $n = 16$) or over a 30-day observational period. Cohorts of treated mice were euthanized at 6 h and on days 2, 4, and 7 post transplantation, at which time isografts were excised surgically and graft angiogenesis–revascularization quantified. Figure 1B ($n = 12$) shows the vascular anatomy of the central skin isograft undersurface demonstrating a marked macroscopic neovascular response in both ESWT- and sham-treated mice. The undersurface of all grafts demonstrated gross morphology consistent with viability and well-perfused appearance. Histological examination of multiple hematoxylin and eosin (H&E)- or Masson's trichrome-stained sections from the central cross-section portion of each graft on day 7 post transplantation failed to reveal any significant degree of hemorrhage or necrosis (data not shown). Gross inspection revealed marked granulation tissue development on day 4 post skin grafting followed by significant granulation tissue regression, which was evident on day 7 post skin grafting. Macroscopic analysis of the vessels in the underside of ESWT-treated and sham-treated isografts on day 4 and 7 post grafting revealed more pronounced graft edge vascularity (neovascularization, which we interpret as new blood vessel formation and vessel

in growth) in ESWT-treated isografts (Fig. 1B). In addition, day 7 ESWT-treated grafts demonstrated a greater number and more prominent blood vessels visible at the periphery (less vessel regression observed, black arrows), which progressed centrally in the treated skin isografts (Fig. 1C; 7.4 ± 2.5 vessels/cm^2 vs. 2.3 ± 0.8 vessels/cm^2; $n = 12$; $P < 0.001$). It is important to note that full-thickness skin grafts revascularize primarily from the graft edges, unlike split-thickness grafts, which have been shown to revascularize not only from the graft edges but also from the underlying recipient wound bed [8, 32]. Also, the hypodermal/adipose dermis/fascial fat layer (adipose dermis) and panniculus carnosus were all removed from the donor skin graft prior to transplantation.

Next, quantitative immunohistochemical analysis of ESWT effects on microvessel revascularization of ischemic skin tissue was conducted. Two independent observers (blinded to treatment assignment) assessed microvessel density on CD31-stained frozen tissue sections by counting the number of CD31-positive vessels per high-power field (×400 magnification). Eight to sixteen consecutive high-power fields across each graft section were enumerated (Fig. 2A, B). In addition, digital analysis software (ImageJ, NIH) was used to quantitate the total CD31-positive area(s) within each skin section, and the overall percent vascularization (Fig. 2C). Representative images of anti-CD31 immunohistochemical staining in Fig. 2A revealed significantly more newly

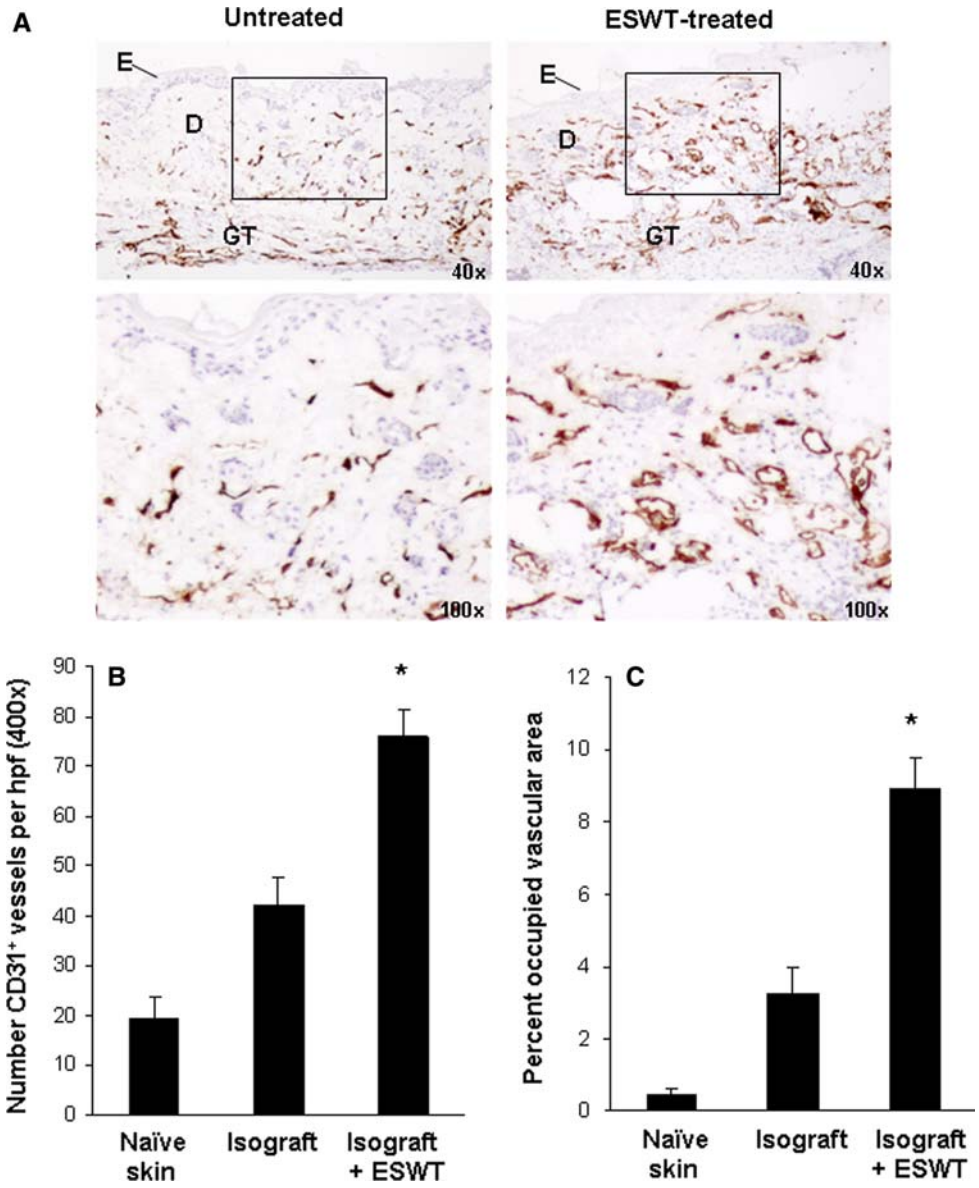

Fig. 2 **A** Microscopic immunohistochemical analysis (×40 and ×100 magnification) of CD31-stained tissue sections on 7 days post skin grafting shows a significant 1.8-fold increase in the capillary-vessel density (**B**) and a 2.7-fold increase in vascular area (**C**) in the graft-wound bed in ESWT-treated isografts compared with sham-treated isografts. Representative images from multiple sections from three independent animals in each group are shown. *E* epithelium; *D* dermis; *GT* granulation tissue. Vessel density measurements were determined by two independent observers that counted the number of CD31-positive vessels in consecutive 8–16 high-power fields (×400 magnification) across each graft section. The percent vascular areas were determined as CD31-positive areas within the graft bed using digital analysis software (ImageJ NIH). All values represent the mean ± SD ($n = 6$ animals). * $P < 0.01$, ESWT-treated compared to sham-treated isografts

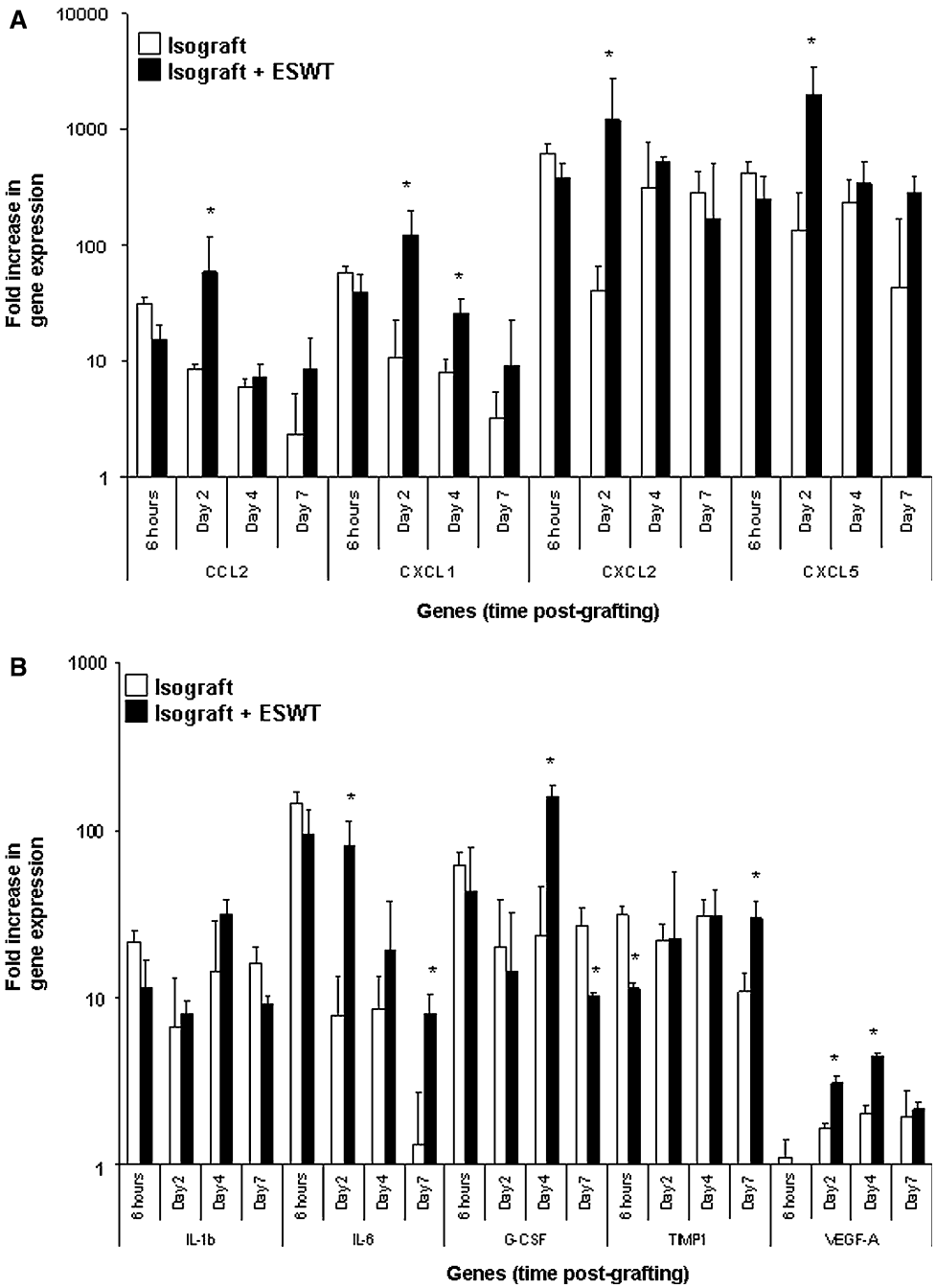

Fig. 3 ESWT enhances local pro-angiogenic gene expression in full-thickness murine skin isografts. Analysis of pro-angiogenic ELR$^+$-CXC and CC chemokines and pro-angiogenic cytokine gene expression at skin graft margins of untreated and ESWT-treated BALB/c mice. Quantitative RT-PCR on angiogenesis pathway-specific SuperArrays was performed as described in the "Materials and methods" and representative results from two independents experiments are shown at study points: 6 h, 2, 4, and 7 days post grafting ± ESWT ($n = 4$ mice per treatment group at each time point). Gene expression profiles were generated. **A**, **B** Fold change in the expression of genes within transplanted skin, including a small rim of marginal native recipient tissue, when normalized to gene expression levels in control-untreated naïve skin [untreated (□) and ESWT-treated (■) isografts]. Note differences of scales on *y* axis. Data illustrated are from one of two independent experiments with similar results and are expressed as the mean ± SD of two technical repeats per tissue sample. * $P < 0.05$, ESWT-treated compared to sham-treated isografts

formed CD31-positive blood vessels within the wound bed of ESWT-treated isografts in comparison with sham-treated isografts (Fig. 2B; 76.1 ± 5.2 vs. 42.4 ± 5.2 vessels per hpf; $n = 6$ per group; $P < 0.01$). In addition, change in vessel size during revascularization in ESWT-treated isografts was more pronounced than that of sham-treated skin grafts. In accordance with this finding, quantitative analysis 1 week post grafting revealed that the relative graft wound bed area occupied by blood vessels (as measured by the percentage of CD31-positive area in the wound bed per hpf) was 2.7-fold greater (Fig. 2C; 8.9 ± 0.8 vs. 3.3 ± 0.7; $n = 6$ per group; $P < 0.001$) in ESWT-treated grafts than in sham-treated control isografts.

ESWT enhances early expression of pro-angiogenic genes in ischemic skin grafts

In order to identify pro-angiogenic and wound repair genes that may mediate the effects of ESWT on angiogenesis, we analyzed the expression profiles of isolated mRNA transcripts in tissue digests by quantitative RT-PCR analysis. Native recipient skin at the periphery of the graft was tested at 6 h. Transplanted skin including a small rim of marginal

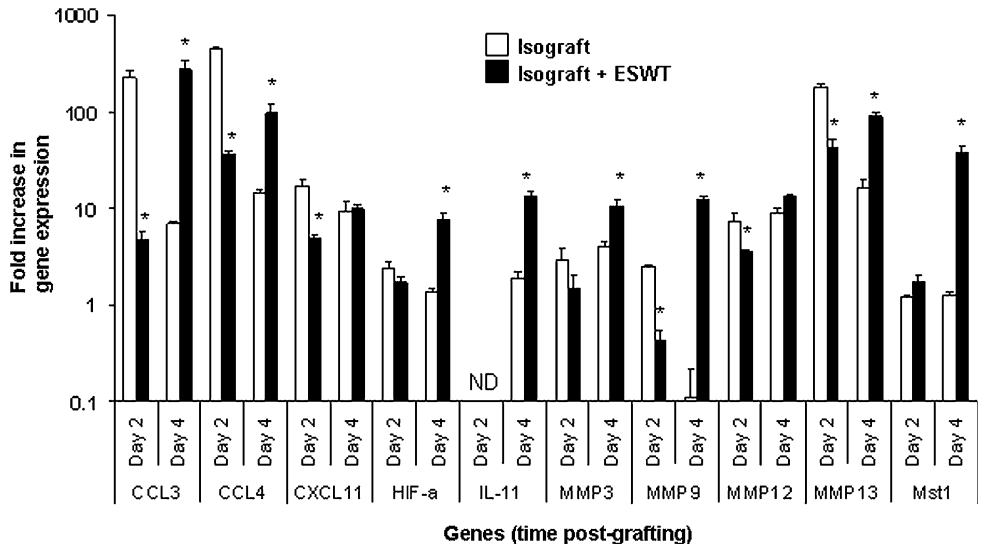

Fig. 4 The analysis of additional candidate wound repair genes utilizing custom-designed 188-gene TaqMan® Low Density Array (*TLDA*) cards by quantitative RT-PCR was performed 2 and 4 days post full-thickness skin grafting ± ESWT. ESWT-treated isografts demonstrated significantly increased expression of CC chemokines, pro-inflammatory cytokines, matrix metalloproteinases, hypoxia-inducible factor-1α, and Mst1. Gene expression profiles were generated. Fold change in the expression of genes within transplanted skin, including a small rim of marginal native recipient tissue, when normalized to gene expression levels in control-untreated naïve skin [untreated (□) and ESWT-treated (■) isografts]. Data illustrated are from one of two independent experiments with similar results and are expressed as the mean ± SD of two technical repeats per tissue sample. * $P < 0.05$, ESWT-treated compared to sham-treated isografts

native recipient tissue was tested 2, 4, and 7 days post ESWT or sham treatment.

Ischemic stress produced marked increases, relative to naïve skin, in pro-angiogenic ELR$^+$-CXC chemokines (CXCL1, CXCL2, CXCL5) and cytokines (IL-1β, IL-6, and G-CSF) within 6 h post skin grafting (Fig. 3A, B; $n = 6$ per group per time point; $P > 0.05$). Although pro-angiogenic chemokine and cytokine expression peaked early after treatment in either study treatment group, transcript levels for these angiogenic factors in ESWT-treated isografts persisted or increased significantly. Consistent with these findings, VEGF-A signals were significantly increased, albeit at relatively lower levels than other pro-angiogenic signals, on 2 and 4 days post skin grafting in ESWT-treated versus sham-treated grafts.

In addition, we observed significant changes in the differential expression of CC chemokines (CCL2, CCL3, CCL4), pro-inflammatory cytokines (IL-11), MMPs (MMP-3, MMP-9, MMP-13), hypoxia-inducible factor-1α (HIF-1α), and the mammalian sterile 20-like kinase 1 (Mst1) vascular remodeling-related gene in skin samples obtained from ESWT-treated isografts on 2 and 4 days post skin grafting (Fig. 4; $n = 4$ per group per time point). Compared with sham-treated skin grafts on day 2, ESWT-treated isografts had a 7.4-fold increase in expression of monocyte chemoattractant protein-1 (MCP-1, CCL2), a known pro-angiogenic chemokine produced by endothelial cells (ECs), smooth muscle cells, epithelial cells, and monocytes. Furthermore, ESWT-treated skin grafts on day 2 had significantly decreased local inflammatory responses as assessed by marked lower expression of macrophage-derived inflammatory protein-1α (MIP-1α, CCL3) and macrophage inflammatory protein-1β (MIP-1β, CCL4) (48.6-fold and 12.3-fold reduction, respectively) in comparison with sham-treated grafts.

Note, in comparison with sham-treated isografts, the early enhanced angiogenic response in ESWT-treated skin grafts on day 2 was accompanied by a delayed but marked increased gene expression in wound-graft macrophage-derived factors including MIP-1α (CCL3), MIP-1β (CCL4), and MMP-13 on day 4. These macrophage-derived factors along with the upregulated expression of Mst-1 are known to be pivotal in wound healing and vascular-tissue remodeling.

Discussion

The current study harnesses low-energy unfocused shock waves to illustrate positive angiogenic effects of physical energy (in the form of an acoustic pressure wave) in a murine model of tissue ischemia. The understanding of the stimulatory effects of shock waves on ischemic full-thickness skin revascularization has been extended through characterization of specific changes and early differential expression of specific pro-inflammatory genes. Our findings

suggest that a single treatment of 200 shock wave impulses at an energy flux density 0.1 mJ/mm^2 applied immediately following isogenic full-thickness skin transplantation in a murine model results in significantly enhanced graft revascularization whilst suppressing inflammation and enhancing extracellular matrix remodeling.

Various targeted therapeutic approaches have been explored to address the problem of threatened soft tissue viability, particularly in chronic wounds and remote aspects of skin flaps compromised by ischemic necrosis. Topical growth factor and cytokine therapy involving single pro-angiogenic proteins as well as adenovirus-mediated VEGF, TGF-β1, and angiopoietin-1 gene therapy have had partial therapeutic neoangiogenic effects in ischemic flaps and grafts [15, 16, 33–37]. In an effort to improve the therapeutic index, biomechanical approaches using non-invasive modalities have been utilized to stimulate angiogenesis in ischemic tissue. These include shock wave therapy, electrical stimulation, and ultrasound [26, 29, 30, 38–46]. However, the clinical decision algorithm specifying type, timing, sequence, combination, and dose intensity of these adjunctive modalities for a given wound remains to be defined.

Neovascularization of ischemic tissue is central to wound healing and is dependent both on local resident EC activation, invasion, migration, and proliferation (angiogenesis), and on formation of new vasculature stemming in part from differentiation of bone marrow-derived endothelial precursor cells (vasculogenesis) [3, 6, 47]. The regulation of angiogenesis is complex and multi-dimensional, reflecting the balance between pro-angiogenic and angiostatic factors expressed by different cell types in response to hypoxic signals [7, 33].

Angiogenesis and inflammation have been shown to be distinct, though inter-related processes; however, the temporal dominance of one biological process over the other may profoundly influence tissue repair and overall healing outcome [48]. Whereas most factors that stimulate angiogenesis have a concomitant pro-inflammatory effect [1, 48, 49], a single ESWT administration at the time of skin grafting in this study was associated with a predominant early pro-angiogenic response and a delayed inflammatory response. Relative to sham-treated grafts, shock wave-treated grafts showed early and significantly enhanced increases in pro-angiogenic gene expression of ELR$^+$-CXC chemokines (CXCL1, CXCL2, CXCL5), CC chemokines (MCP-1/CCL2), and cytokines (IL-1β, IL-6, IL-11, G-CSF). In contrast, sham-treated skin grafts showed enhanced early local inflammatory responses exceeding observed pro-angiogenic effects. The dominant pro-inflammatory response in sham-treated grafts is evident by marked (>100-fold) tissue gene expression of MIP-1α (CCL3) and MIP-1β (CCL4) when compared with ESWT-treated grafts. During the same study time points, sham-treated animals showed a 10- to 100-fold lower level of pro-angiogenic gene expression (CXCL1, CXCL2, CXCL5). Conversely, ESWT-treated skin grafts demonstrated markedly delayed as well as reduced local inflammatory responses evidenced by significantly lower expression of macrophage-derived factors, MIP-1α and MIP-1β, relative to sham-treated skin. ESWT was associated with marked differential expression of interleukin-1β (IL-1β) and interleukin-6 (IL-6), cytokines shown previously to indirectly stimulate angiogenesis through VEGF-A induction [50, 51]. Collectively, our observations suggest that an important shift in the balance of pro-angiogenic chemokines–cytokines and pro-inflammatory mediators, supporting an enhanced and prevailing early angiogenic response to ESWT. These results are consistent with those of others reporting angiogenesis, wound healing progression, formation of new vascular granulation tissue, and tissue regeneration. These findings have particular relevance when one considers the fact that scarless healing is impeded by on-going inflammation [1, 49].

In experimental models of repair, overt inflammation has been shown to delay healing and contribute to increased wound scarring. The importance of pro-inflammatory mediators and leukocyte infiltration in the overall healing and angiogenic response is evident in skin wounds of mice deficient for the TNF receptor TNF-Rp55 (decreased PMN and macrophage infiltration resulting in increased angiogenesis and accelerated healing-wound closure) or antagonist for IL-1 (increased leukocyte infiltration resulting in decreased angiogenesis and delayed healing-wound closure) [52, 53]. Consistent with our findings, recently published studies demonstrate the ability of ESWT to stimulate angiogenesis and suppress pro-inflammatory responses in ischemic skin flap segments [29, 30] and acute burn wounds [23]. Single application of low-energy ESWT immediately after extended dorsal rodent epigastric island skin flap elevation significantly enhances flap perfusion and healing of distal ischemic skin necrosis [29, 30]. This effect of ESWT appears to occur through upregulation of endothelial and fibroblast VEGF-A, upregulation of basal epidermal and fibroblast PCNA, downregulation of ischemic tissue TNF-α, and reduction in leukocyte infiltration into the distal skin flap zone of ischemia [29, 30]. A recent study of rodent hind limb ischemia demonstrated a preconditioning effect of ESWT through upregulation of chemoattractant SDF-1 mRNA and quantitative increase in VEGF$^+$ myocytes in non-ischemic limbs [38]. Applying the same ESWT dose regimen used in this study, we demonstrated previously that ESWT of burn wounds 1 h post wounding significantly blunts PMN and macrophage infiltration into the wound [23]. ESWT treatment potently attenuates both CC- and CXC-chemokine

expression, acute pro-inflammatory cytokine expression, and extracellular matrix proteolytic activity at the wound margin [23]. Major sources of these mediators at the wound edge include infiltrating PMNs and macrophages. Of note, a large full-thickness burn wound without eschar excision is extremely inflammatory in nature in comparison with smaller or equal in size incisional or excisional wounds. Additionally, several groups have reported a beneficial effect of ESWT in the reversal of ischemic heart disease in large animal models thought to be secondary to a pro-angiogenic effect [25, 54]. However, the biological mechanism of therapeutic shock waves in wound healing remains incompletely understood, and an area of active translational research.

Sequential and spatial regulation of MMPs is critical for rapid neovascularization and normal wound healing. ECs from chronic wounds have been shown to be deficient in enzyme and growth factor production; these cells demonstrate impaired migration, proliferation, and formation of new capillaries [9–11]. Similarly, keratinocytes in non-healing wounds have functional impairment apparent in reduced ability to migrate, proliferate, and synthesize cytokines, provisional matrix, and basement membrane. During ischemic injury, EC interactions with neighboring ECs are disrupted and ECM elements are digested; this is facilitated by soluble collagen-cleaving proteolytic enzymes such as MMPs [4]. Activated ECs along with monocytes/macrophages, fibroblasts, and smooth muscle cells release VEGF-A, transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF). These paracrine pro-angiogenic cytokines enable EC mobilization, ECM infiltration, and migration along with cytokine gradients, proliferation, and neovessel formation [3, 50].

Correspondingly, we show here differential expression of VEGF-A and MMPs in ESWT-treated isogenic skin grafts early post grafting consistent with these defined mechanisms. Collectively, the findings from the present study contribute to a clearer understanding of pro-angiogenic chemokine, cytokine, and MMP responses of ischemic tissue to shock wave treatment at a molecular level. We speculate that the early pro-angiogenic and anti-inflammatory effects of ESWT may promote wound healing not only by augmenting angiogenesis and dampening early inflammation but also by inducing EC and keratinocyte cell proliferation and migration. Conversely, the early differential expression of transcripts for angiogenic cytokines and chemokines in ESWT-treated grafts may act to sustain a prolonged physiological inflammatory response that favors persistent neovascularization and delays hemodynamic remodeling, which is governed in part by other mediators (“angiogenesis-associated inflammation”). Follow-on molecular and cellular studies to elucidate definitively these mechanisms are warranted.

Although not the principal aim of our current study, sufficient work preceding ours exists to address the role of host versus donor vasculature in ischemic skin graft revascularization [8, 29]. Early revascularization is attributable to newly formed vascular anastomoses between graft and recipient wound bed vessels; this occurs mainly in the central portion of the recipient bed and graft [32]. It would also be important to distinguish the participation of preexisting versus newly formed vessels in this model. This is beyond the scope of the current study and is the focus of future investigations using GFP recipient/donor mice. It is important to re-emphasize that in our studies we used full-thickness skin grafts which, unlike split-thickness skin grafts known to revascularize from the periphery (graft edges) and underlying recipient wound bed, revascularize primarily from the peripheral graft edges [8]. This is a very important distinction between graft types.

The rationale for this experiment is based on a number of hypothesis-generating though provisional findings in the literature using shock waves to treat ischemic tissue in preliminary undeveloped studies. The aim of our study was to conclusively determine whether ESWT has a pro-angiogenic effect on ischemic tissue wound healing and whether ESWT could augment recipient skin graft revascularization. A skin graft is an ideal model for such an inquiry, as it is devascularized prior to grafting and its subsequent survival is dependent upon revascularization. The finding that ESWT augments angiogenic processes formed the basis of gene expression networks that are differentially regulated in ESWT-treated grafts when compared with untreated grafts, which may be contributory to enhanced vascular in-growth. Follow-on localization studies to determine precisely the source of the gene products and molecular signals will help to define mechanistically relevant target cells.

Therapeutic shock wave application has unique characteristics making it a suitable wound healing adjunct including non-invasiveness, ease of application, minimal risk and drug interactions, cost effectiveness, and comparable efficacy to existing treatments. We have found low-energy ESWT at energy flux densities of 0.5–0.1 mJ/mm^2 to be safe clinically, as this level of stimulation is not associated with apoptosis or tissue necrosis [22]. Although the mechanisms of therapeutic shock waves are being elucidated and are currently incompletely understood, data accumulated thus far indicate that ESWT is a feasible and clinically relevant approach to a variety of acute and chronic ischemic conditions. Therapeutic shock waves are currently undergoing clinical evaluation as a means to facilitate cutaneous tissue repair (chronic and difficult to heal acute wounds), and regeneration in thermal injury, and as a means of accelerating healing in autologous skin graft donor sites.

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