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Comparative analysis of angiogenic gene expression in normal and impaired wound healing in diabetic mice: effects of extracorporeal shock wave therapy

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Abstract Impaired wound healing is a persistent clinical problem which has been treated with mixed results. Studies aimed at elucidating the mechanism of impaired wound healing have focused on small cohorts of genes which leave an incomplete picture of the wound healing process. We aimed to investigate impaired wound healing via a comprehensive panel of angiogenic/inflammation-related genes and wound closure kinetics with and without the application of extracorporeal shock wave therapy (ESWT), which has been demonstrated to improve wound healing. Full-thickness skin from the dorsal surface of "normal" (BALB/c) and "impaired" (db^+/db^+) mice was excised, and wound margin tissue was harvested 2, 7, and 10 days post injury. A separate, but identical wound model was established over 40 days in order to measure wound closure kinetics. Over time, the normal non-ESWT treated wounds exhibited varying patterns of elevated expression of 25–30 genes, whereas wounds with impaired healing displayed prolonged elevated expression of only a few genes (CXCL2, CXCL5, CSF3, MMP9, TGF-α). In response to ESWT, gene expression was augmented in both types of wounds, especially in the expression of PECAM-1; however, ESWT had no effect on wound closure in either model. In addition, multiple doses of ESWT exacerbated the delayed wound healing, and actually caused the wounds to initially increase in size. These data provide a more complete picture of impaired wound healing, and a way to evaluate various promising treatments.

Keywords Acute wound healing · Angiogenesis · Extracorporeal shock wave therapy · Impaired wound healing · Wound closure

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Introduction

Wound healing is a multistep process that starts with the injury itself and involves an orderly and complex interplay amongst cells and soluble mediators [1, 2]. Normal cutaneous healing is characterized by an early spike in

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14. ABSTRACT

Impaired wound healing is a persistent clinical problem which has been treated with mixed results. Studies aimed at elucidating the mechanism of impaired wound healing have focused on small cohorts of genes which leave an incomplete picture of the wound healing process. We aimed to investigate impaired wound healing via a comprehensive panel of angiogenic/inflammation-related genes and wound closure kinetics with and without the application of extracorporeal shock wave therapy (ESWT), which has been demonstrated to improve wound healing. Full-thickness skin from the dorsal surface of "nonnal" (BALB/c) and "impaired" (db+tdb+) mice was excised, and wound margin tissue was harvested 2, 7, and 10 days post injury. A separate but identical wound model was established over 40 days in order to measure wound closure kinetics. Over time, the nonnal non-ESWT treated wounds exhibited varying patterns of elevated expression of 25-30 genes whereas wounds with impaired healing displayed prolonged elevated expression of only a few genes (CXCL2, CXCL5 CSF3, MMP9, TGF-oc). In response to ESWT, gene expression was augmented in both types of wounds, especially in the expression of PECAM-1; however, ESWT had no effect on wound closure in either model. In addition, multiple doses ofESWT exacerbated the delayed wound healing, and actually caused the wounds to initially increase in size. These data provide a more complete picture of impaired wound healing, and a way to evaluate various promising treatments.

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inflammatory cytokine and chemokine expression, recruitment of inflammatory cells and other regulatory cells to the wound site, and followed by inflammation, angiogenesis, provisional matrix synthesis, collagen deposition, fibroblast proliferation, wound contraction and re-epithelialization [1–3]. In chronic wounds, healing is characterized by delayed cellular infiltration and granulation tissue formation, decreased collagen deposition and reduced angiogenesis. This can lead to a hypoxic wound environment and a prolonged inflammation phase [4–8].

Impaired wound healing is a major healthcare issue and a formidable clinical challenge. Wounds exhibiting impaired healing may result from a number of etiologies including diabetes, ischemia, pressure, hypoxia, venous congestion, malnutrition, immunodeficiency, invasive local infection, medications and radiation therapy [8–10]. Open wounds create an ideal environment for opportunistic infections, and, without effective treatment, these wounds become chronic. Adjuncts to surgical debridement and antibiotic therapy, including hyperbaric oxygen, negative pressure wound therapy, topically applied biologically derived molecules, and mesenchymal stem cell therapy have been reported to accelerate chronic wound healing in small animals and humans [8–10]. Alternative noninvasive therapies to treat wounds with impaired healing are warranted.

Wound healing crucially relies on the formation of new blood vessels in the wound bed [11–13], and has been shown to be significantly impaired in diabetic wounds resulting in decreased time of closure [4–7, 14, 15]. The recent use of low energy Extracorporeal Shock Wave Therapy (ESWT) as a therapeutic modality to stimulate angiogenesis and wound healing in a variety of musculoskeletal disorders has aroused considerable interest [16]. ESWT has been shown to induce neovascularization and modulation of proangiogenic growth factor expression in ischemic skin flap tissue and in a pig model of myocardial infarction resulting in improved blood perfusion [17, 18]. Recently, we showed that ESWT induced robust augmentation of proangiogenic factors following ischemic isograft skin transplantation [19]. Moreover, ESWT has been shown to facilitate the mobilization of endothelial progenitor cells into chronic ischemic tissue [20]. These vascular effects were notably mediated through enhanced expression of nitric oxide synthase (NOS) and vascular endothelial growth factor (VEGF) [17, 21]. We have also demonstrated that proinflammatory gene expression and proinflammatory cell infiltration are attenuated in burns after ESWT treatment postoperatively [22–24]. In the clinical experience, ESWT has been shown to be an effective treatment for certain musculoskeletal disorders [29, 31], and found to modestly enhance the healing and closure of difficult-to-heal wounds; [25] however, the effects of ESWT on acute and impaired soft tissue wound healing in a controlled, standardized, small animal excisional wound model have not been vigorously evaluated. In this study, BALB/c mice were used as a model example of normal wound healing (as opposed to the heterozygous counterpart of our chronic model, Bk.Cg-m Lepr db^{+}/db^{+}) due to the fact that BALB/c mice historically provide a robust example of wound healing at near 100% efficiency, with no underlying genetic disease disposition or pathology. We aimed to determine what augmenting effect, if any, ESWT had on a normal healing system, as well as evaluating any potential negative effects. Although ESWT has been shown to be a promising therapeutic agent for regenerative medicine applications, little is known about the specific mechanisms underlying ESWT-accelerated tissue repair, and/or the proper dosing regimen. It appears physical energy in the form of penetrating acoustic pressure waves induce various intracellular signaling pathways to stimulate neovascularization, enhanced blood supply and accelerated tissue healing/regeneration.

Since angiogenesis plays a key role in normal as well as diabetic wounds, we hypothesized that ESWT might be efficacious in augmenting proangiogenic gene expression resulting in accelerated excisional wound closure in both normal and diabetic mice. We examined the effect of ESWT on wound closure kinetics of full thickness cutaneous wounds and evaluated the transcript expression kinetics of 84 proangiogenic genes during normal and impaired wound healing. Our results demonstrate wounds in non-treated diabetic mice express transcripts for only a few proangiogenic genes and that ESWT does not significantly accelerate healing in either normal or impaired wounds. On the other hand, we show that a single ESWT treatment in the context of an impaired wound was able to turn-on and/or augment for a prolonged period a number of key proangiogenic genes which were previously silent, and that ESWT significantly augments the angiogenic response globally in normal healing wounds. These results point to an interesting phenomenon in which there is a definitive differing molecular signature both with and without ESWT in impaired wounds compared to normal which does not correlate with improved gain of function regarding wound closure.

Materials and methods

Mice

Aged matched (8–12 weeks) female BALB/c and homozygous Bk.Cg-m Lepr db^{+}/db^{+} mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed at the Walter Reed Army Institute of Research/Naval Medical Research Center (WRAIR/NMRC) 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 (protocol #K01–08). Animals were housed 5 to a cage until study initiation, and individually housed thereafter using standard microisolator polycarbonate caging. Animal rooms were kept at 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 control opportunistic infections.

Full-thickness excisional wound

Mice were anesthetized with an intraperitoneal injection of xylaine (10 mg/kg) and ketamine (50 mg/kg). Using fine scissors, a single full thickness, circular 19 mm diameter (280-mm^2) excisional wound area was made at the supra-fascial level, including the panniculus carnosus, on the mid-dorsum of each mouse. Wounds were topically treated with bacitracin immediately after wounding, left uncovered and allowed to desicate. Once mice recovered from anesthesia, they were placed alone in separate cages and maintained under standard conditions in the animal facility (as described above). Buprenorphine (Reckitt Benckiser Pharmaceuticals, Richmond, VA) was given subcutaneously every 12 h (0.3 mg/kg) on postoperative days 1–5 for pain management. No topical wound care was provided aside from the aforementioned bacitracin.

Extracorporeal shock wave treatment (ESWT)

Two hours post injury, mice were anesthetized briefly using isoflurane inhalation. A liberal amount of bacitracin ointment, which serves as a good conductive gel, was applied directly to the wound and wound margin. The unfocused lens of the DermaGold™ (Tissue Regeneration Technologies, LLC, Woodstock, Georgia) shock wave applicator, which comprises a parabolic reflector, was gently placed directly on the ointment-covered wound and treated with 200 impulses (energy level 0.1 mJ/mm^2, frequency 5 pulses per second). The parabolic reflector permits a large treatment area to be stimulated by the acoustical field. ESWT treatment lasted approximately 45 s. Following treatment, the excess bacitracin was removed carefully using sterile gauze. No dressing was applied. Sham-treated wounds were treated identically; however, no shock wave impulses were administered. Treatment for the cohort to be harvested for gene expression analysis was only administered one time due to the fact that multiple doses of ESWT had an adverse affect on wound closure kinetics and gross morphology of the wound bed. Moreover, it has been published that the desired clinical effects of ESWT occur with a single, bi-weekly application [25] which corresponds to a single dose in the normal healing mice (wounds typically fully close at 14 days).

Assessment of wound healing using planimetric analysis

Photographs of wounds in the presence of a scaled ruler were taken with a Panasonic HDC-SD1 AVCHD 3CCD Flash Memory High Definition Camcorder (Panasonic Corp, Osaka, Japan) every other day. For quantification of open wound area, raw digital files were imported into NIH Image J software (v1.37) for processing and planimetry was used to calculate wound surface area.

Tissue collection

Mice were euthanized by CO_2 inhalation followed by cervical dislocation at days 2, 7, and 10 post wounding for tissue acquisition ($n = 5$ animals per strain at each time point in both the control and the ESWT treatment groups). From the circumference of the entire wound, ~2–3 mm of tissue from the wound edge/margin and down to the underlying musculature was harvested using fine sterile scissors. Wound margin tissue was used since angiogenesis initiates from pre-existing blood vessels that were damaged as a result of the experimental injury model used. Wound margin tissue provides the most accurate portrayal of actual angiogenesis activity for the gene panel assessment. Collected tissue was finely minced, placed in RNALater (Ambion, TX), and stored at 4°C for future analysis.

RNA isolation and cDNA conversion

Total RNA was isolated from wound margin tissue as previously described [22]. Briefly, ~100 mg of tissue was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and RNA was isolated using the commercially available Qiagen RNeasy Lipid Tissue Mini Kit (QIAGEN, INC, Valencia, CA) according to manufacturer's instructions. Sample quantity and quality were ascertained via the NanoDrop Spectrophotometer (NanoDrop, Wilmington, DE) and Agilent 2100 Bioanalyzer (Quantum Analytics, Inc., Foster City, CA). RNA Integrity Numbers (RIN) of greater than 8.5 were used in this study. First strand cDNA synthesis was generated from 1 μg total RNA using the ReactionReady First Strand cDNA Synthesis kit according to manufacturer's instructions (SABiosciences, Fredrick, MD). Samples were stored at −20°C until array analysis.

RT-PCR via superarray angiogensis RT profiler platform

Samples were analyzed for the expression of 84 known transcripts directly related to angiogenesis and extracellular matrix composition through the use of the Mouse Angiogenesis RT^2 Profiler. Arrays and RT^2 Real-Timer SyBR Green/

ROX PCR Mix were purchased from SABiosciences and used according to manufacturer's instructions. One microgram (μg) cDNA was used per sample per array. PCR was performed on ABI Prism 7900HT Sequence Detector (Applied Biosystems, Foster City, CA). Per experiment, a set of 5 housekeeping genes (HKG) was included. For data analysis the ΔΔCt method was used. The relative gene expression of the genes was calculated as ΔC_t sample = (C_t sample GENE) − (C_t sample HKG). Then, the relative gene expression (RGE) = 2 power − (ΔC_t sample1 − ΔC_t naïve skin samples). Comparisons were then made between treatment groups and a gene was said to be differentially regulated if there was a fivefold difference in expression. Assays with C_t values greater than 35 cycles were considered not expressed.

Immunohistochemistry for PECAM-1/CD31 expression

Wound sites and adjacent normal skin were excised, fixed with 4% formaldehyde in a buffered zinc solution (Z-fix), embedded in paraffin, and sectioned (5 μm). Slides were deparaffinized using xylene followed by graded baths of ethanol. A DAKO Autostainer Plus Universal Staining System (DAKO, Carpenteria, CA) was used for the automated immunohistochemical staining. A few sections from each tissue were mounted onto slides, and stained with hematoxylin-eosin. Antigen retrieval was performed using Citra (BioGenex, San Ramon, CA) for 30 min in a steamer. Briefly, deparaffinized- tissue sections were incubated at room temperature for 60 min with goat antimouse PECAM-1/CD31 polyclonal antibody (1506R: Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 10% rabbit serum (1:250). Then sections were incubated with biotinylated rabbit antigoat secondary antibody (K1015: Santa Cruz Biotechnology) for 60 min followed by streptavidin-conjugated horseradish peroxidase (DAKO). Antigen–antibody complexes were visualized by incubation with DAB substrate and counterstained with Hematoxylin (DAKO). The sections were counterstained with Hematoxylin (DAKO, Carpenteria, CA) and then coverslipped. Negative reagent controls were processed identically, however, the primary antibody was substituted with normal rabbit serum at a 1:250 dilution. Microvessels stained with CD31 (brown) were quantified in 4–8 random microscopic (×400) fields tissue section by an independent observer.

Western Blot analysis for PECAM-1

The protein lysates were prepared from frozen mouse skin tissue by powdering the tissue in liquid nitrogen in a mortar. Proteins were extracted in lysis buffer: 10 mM $Na_4P_2O_7$, 50 mM NaF, 50 mM NaCl, 1 m M EDTA, 50 mM HEPES, 1% Triton X-100, adjusted to pH = 7.5, with 2 mM Na_3VO_4, 1 mM PMSF, and 1 tablet/10 ml buffer of complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA). Cell lysates were standardized for total protein content using BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (20 μg per sample) were separated by 4–12% BisTris gels (Invitrogen Carlsbad, CA, USA) and transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen). Membranes were blocked using blocking buffer (5% milk, 0.1% Tween-20 in PBS) for 1 h at room temperature and incubated overnight 4°C with 1:2,000 anti-PECAM-1 (SC-28188; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer. Anti-rabbit IgG secondary antibody 1:5,000 (SC-2004, Santa Cruz) was applied for 1 h at room temperature and blots were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) and FujiFilm imager (FujiFilm, Edison, NJ). Blots were stripped and probed for β-actin (AC-15; Sigma, St. Louis, MO, USA). Relative quantities and data were normalized from scanned immunoblots using the β-actin values and intensity levels were quantified using Adobe Photoshop CS3 software. Shown are the representative data from wound margin/granulation tissue obtained from separate mice.

Statistics

Statistical differences were assessed using the paired t test (GraphPad PRISM 4.0). Significance was assumed at $p < 0.05$. Data are shown as mean ± SD, unless otherwise mentioned.

Results

Wound closure is significantly delayed in the db^+/db^+ diabetic mice

A single full thickness (280-mm^2) excisional wound was made at the suprafascial level, including the panniculus carnosus, on the mid-dorsum of control wild type BALB/c mice and db^+/db^+ diabetic mice. Open wound area (Fig. 1A) was calculated as a percentage of original wound areas at days 2–35 post injury. At days 4–22 post injury, nondiabetic wild type BALB/c mice showed significantly ($p < 0.05$) faster rates of excisional wound closure than diabetic db^+/db^+ mice (Fig. 1B). By day 14, wound closure on wild type mice was 88.9 ± 4.4% compared to 24.2 ± 8.3% on diabetic mice with impaired wound healing. Acute wounds in the wild type mice were completely reepithelialized and closed by day 22 with the greatest rate of epithelialization and wound contraction observed between days 4 and 14 post wounding. Conversely, wounds on db^+/db^+ mice during the same time interval post injury appeared moist and largely unhealed with little

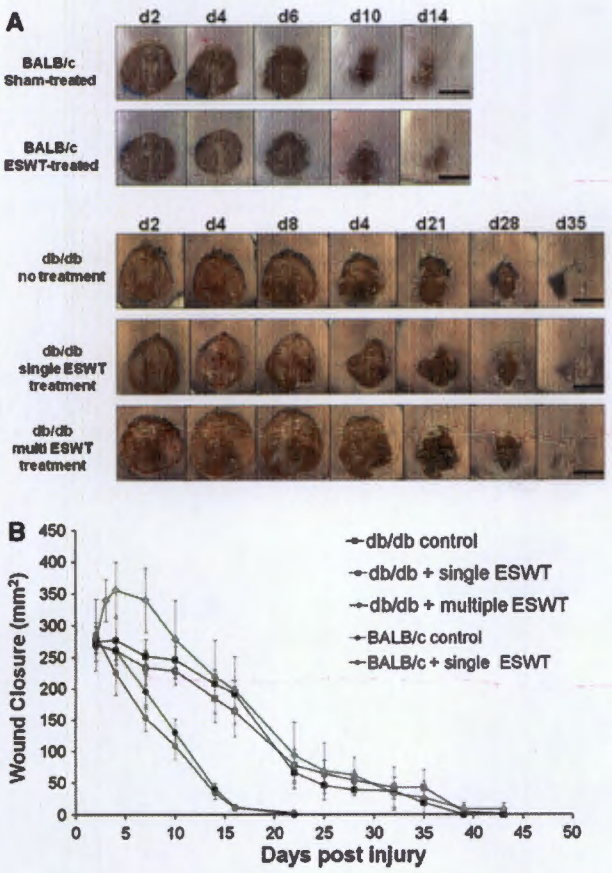

Fig. 1 **A** Representative photographs of open excisional wounds at various time points and treatments. **B** The kinetics of acute normal excisional wound closure in wild type BALB/c mice ± ESWT treatment [*gray filled square*, *black filled square*], the kinetics of impaired excisional wound closure in diabetic db^+/db^+ mice ± ESWT [*gray circle*, *black circle*], and the kinetics of impaired excisional wound closure in db^+/db^+ mice + 5 doses of ESWT at 2 h and days 1, 3, 5 and 7 post injury [*gray filled diamond*]. Multiple doses of ESWT caused a significant initial increase ($p < 0.05$) in wound size (wound retraction) over the first 7 days. Each point represents the mean of the percentage of area of the initial wound size ± standard deviation of the mean ($n = 7$–10 mice per treatment group per time point)

evidence of new tissue ingrowth. At day 40 post injury, the majority of wounds on db^+/db^+ mice were fully closed and reepithelialized.

ESWT does not accelerate cutaneous wound closure in either wildtype normal mice or db^+/db^+ diabetic mice

To assess the effects of ESWT on acute and impaired wound healing, we treated wild type BALB/c mice and db^+/db^+ diabetic mice with a single treatment of ESWT (200 impulses at an energy level 0.1 mJ/mm^2, frequency 5 pulses per second), 2 h post injury, and with multiple treatments (5:2 h and days, 1, 3, 5, 7 post injury). The single dose used has been previously shown to be sufficient to induce optimal angiogenesis in a previous reported ischemic cutaneous wound healing study [19]. At various time points post injury, wounds were assessed for percent and time of closure. In comparison to untreated control wounds, ESWT had a modest effect over the first 7 days in BALB/c mice (p values < 0.02), however there was no effect with a single dose in db^+/db^+ diabetic mice (Fig. 1B). Multiple doses of ESWT caused a significant initial increase ($p < 0.05$) in wound size (wound retraction) over the first 7 days, and thereafter followed the wound closure profile of a single dose.

db^+/db^+ mice exhibit attenuated angiogenic gene expression after acute injury

We evaluated the angiogenic response in wounds that exhibited normal and impaired healing. Wound edge/margin tissue was harvested from the circumference of wounds at 2, 7, and 10 days post injury. Transcripts of 84 Angiogenesis-related genes were analyzed using quantitative real-time PCR low density microarray analysis.

To provide a graphical representation of all the results, we converted our quantitative RT-PCR data into a "heat" map (Fig. 2). The color gradient indicates normalized transcription signal values (fold changes above or below expression values detected in naïve, strain-specific, non-injured skin). Genes that had mean expression signals that were within ±5-fold of baseline controls in naïve skin were considered to be not significant. The gene expression patterns were relatively uniform amongst animals in each treatment group ($n = 5$ per strain, per treatment group). We identified 24 angiogenic gene transcripts that were not expressed or were downregulated during diabetic impaired wound healing when compared to normal healing. Of the 84 gene transcripts measured, only transcripts for CXCL2 (MIP-2α) and CXCL5 (ENA-78) were common and highly expressed early in both wound types; however, the kinetics of the expression of these 2 genes were significantly different between wound types (Fig. 3A). In comparison to acute wound healing, we observed an elevated and prolonged upregulation of CXCL2, CXCL5, CSF3, TGFα, and MMP9) in diabetic wounds with impaired healing. ESWT markedly increased and prolonged CXCL2, CXCL5, and CSF3 mRNA expression in both normal and diabetic wounds without having any gross observable changes in wound appearance or significant effect on time of wound closure. This finding is unexpected, because cells in these tissues are cells that are already involved in very active protein synthesis of these potent neutrophil attracting chemokines. Surprisingly, CXCL1, IL-6, MMP19, and MMP9 were not expressed or were underexpressed in acute wound healing; however, expression levels were markedly augmented in ESWT-treated acute wounds (Fig. 2). Globally, the effect of ESWT treatment was much more pronounced in impaired wounds. An additional 21 genes were elevated over 5-fold at

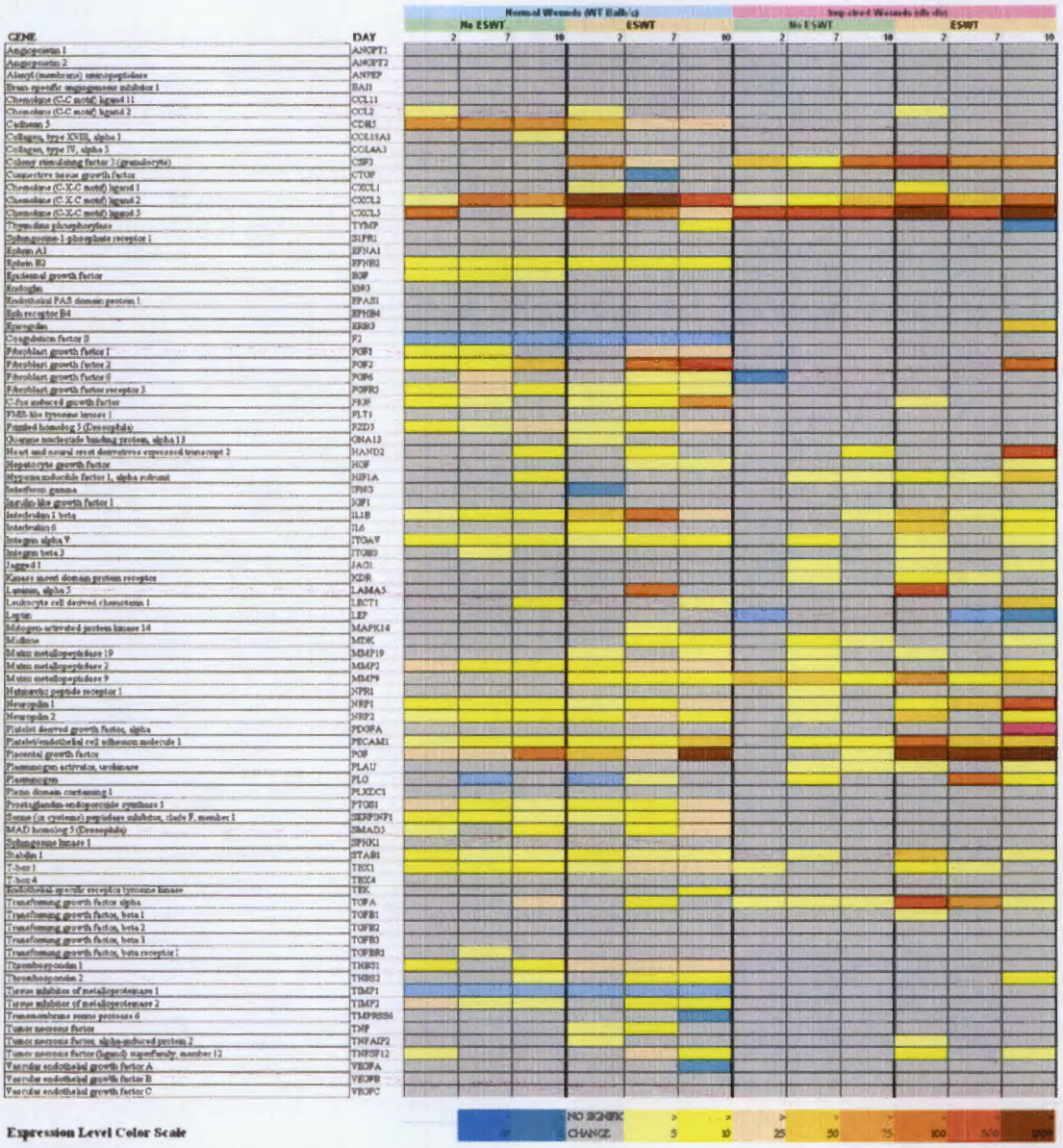

Fig. 2 The expression level of 84 mouse angiogenic related gene targets whose expression was statistically significantly different (gray = within ± 5-fold expression) from uninjured, strain-specific, control skin has been converted to a "heat" map with *light yellow-maroon* representing upregulation of transcripts, and *light blue*-to *royal blue* representing downregulation of transcripts. Gene transcripts that had C_t values ≥ 35 at day 0 include TIMP, PLG, and F2 (diabetic mice); BAI1, TYMP, FGF6, and PGF (WT BALB/c); and TBX4, HAND2, CSF3 (common to both strains)

day 2 post injury, including CCL2, CXCL2, HIF-1a, IL-1β, IL-6, MMP10, NRP1, NRP2, PIGF, and TEK. ESWT-induced expression of the genes resulted in an early spike more associated with normal healing wounds, and either remained slightly elevated, or waned towards baseline values by day 10 post injury.

In BALB/c mice PECAM-1 (CD31) mRNA is highly expressed at days 2, 7 and 10 post injury (Fig. 3B). ESWT-treatment significantly enhanced CD31 mRNA synthesis at days 2 and 10 post injury. In contrast, wounds from diabetic db^+/db^+ mice at days 2 and 7 post injury have very modest levels of PECAM-1 mRNA production. Interestingly, a single ESWT-treatment markedly ameliorated this defective production to a level equal to or greater than those detected in wounds from BALB/c ± ESWT.

ESWT promotes angiogenesis in wounds

To quantify granulation tissue vascularization, anti-PECAM-1 immunohistochemical analysis was performed on

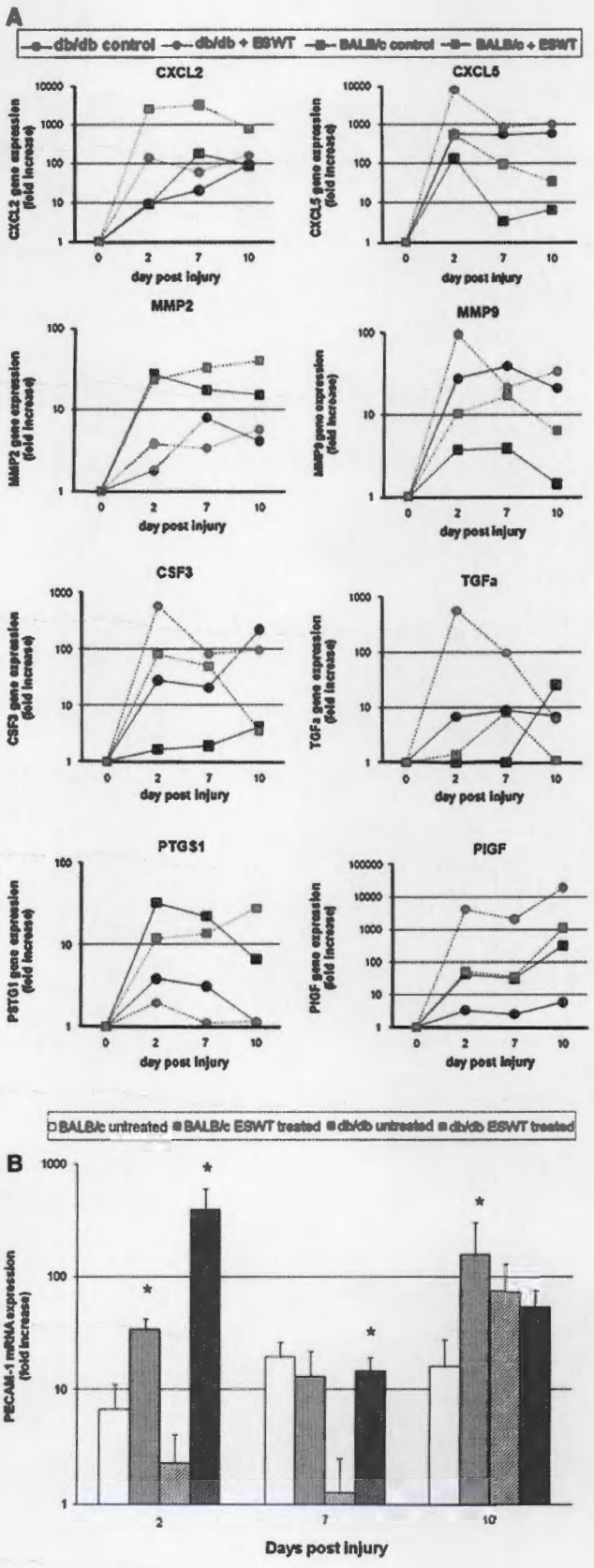

Fig. 3 Real time PCR analysis of early angiogenic gene expression in normal wounds ± ESWT treatment [*gray square*, *black square*], and delayed healing wounds ± ESWT [*gray filled circle*, *black filled circle*]. **A** Wound margin/edge expression for CXL2, CXCL5, MMP2, MMP9, CSF3, TGFα, PSTGA, PIGF and **B** PECAM-1/CD31 was analyzed at different time points ($n = 5$ wounds per time point) after injury by real-time PCR. The relative gene expression of the genes was calculated as ΔC_t sample = (C_t sample GENE) − (C_t sample HKG). Then, the relative gene expression (RGE) = 2 power − (ΔC_t sample1 − ΔC_t naïve skin samples). Values represent the mean ($n = 5$ mice per group). * $p < 0.05$ versus sham-treated

central wound sections at the time of approximately 40–50% wound closure, at day 6 and 11 after injury in BALB/c and db^+/db^+ mice, respectively. A modest 44% and significant 202% increase in blood vessel density was observed in ESWT-treated BALB/c versus ESWT-treated db^+/db^+ mice, respectively, when compared to paired sham-treated control mice (Fig. 4A). Western blot analysis for tissue protein expression confirmed the enhanced production of PECAM-1 (Fig. 4B).

Discussion

In this study, we evaluated the mRNA expression profiles of wound healing-related mediators over the first 10 days of cutaneous wound healing for acute and impaired wounds and the effects of Extracorporeal Shock Wave Therapy. Our findings show that wound healing in the db^+/db^+ diabetic mouse is impaired and characterized by delayed wound closure, a prolonged inflammatory phase and the absence or reduced expression of key proangiogenic mediators. A marked difference in patterns of angiogenesis-related genes was seen over a period of 10 days between wounds that heal normally versus wounds in diabetic mice that demonstrate impaired acute wound healing. Our study identified 27 transcripts whose expression varied significantly. In response to ESWT treatment, angiogenic gene transcript expression patterns were either unchanged or augmented respective to sham treatment, including a number of key genes in db^+/db^+ wounds that were previously dormant or had low expression without ESWT. In contrast, many key angiogenic factors expressed in normal wounds remained dormant in either the absence or presence of ESWT treatment. Analysis of treated wounds showed that ESWT improved granulation tissue vascularization. While ESWT markedly augmented some pro-angiogenic gene expression responses during both normal and impaired wound healing processes, we observed no improvement in the kinetics of excisional wound healing or time-to-wound closure in mice exposed to ESWT. It is conceivable impaired wound healing in the db^+/db^+ mouse may be due to a prolonged aberrant expression of several mediators (CXCL5, CSF3, MMP9) or the lack of expression of others (IL-1b, FGFs, EGF, Cdh5, Efnb2, Fzd5, IL-1b, MMP2, Ptgs1). These data suggest that the expression of many key genes related to angiogenesis and wound healing are suppressed or not activated in diabetic wounds.

The establishment and testing of new, noninvasive, adjunctive therapeutic options in combination with conventional therapy to treat and optimize the wound microenvironment of complex and difficult-to-heal wounds is needed [26]. Various modalities which deliver physical energy to produce a therapeutic biological effect, a process

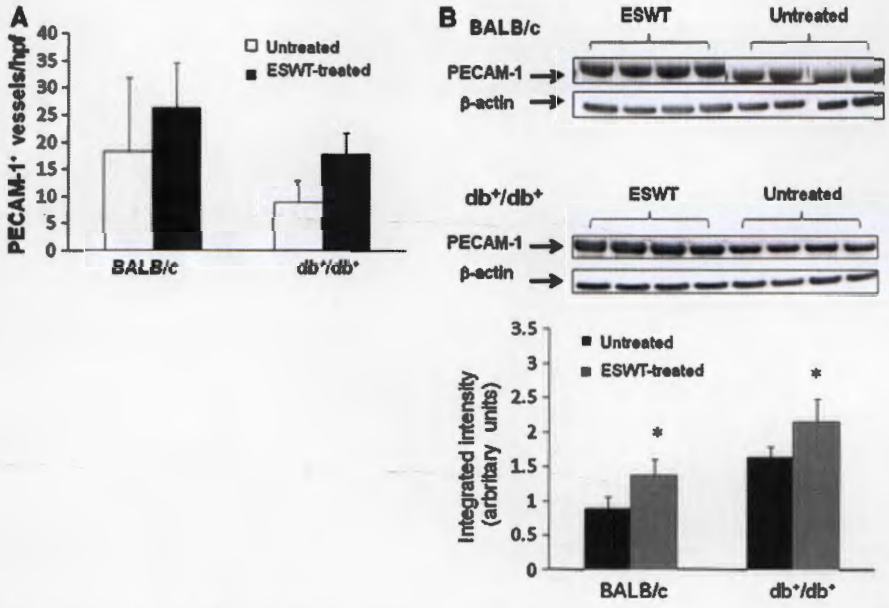

Fig. 4 Quantitation of Angiogenesis: **A** The number of CD31-positive vessels in the wound margin/granulation tissue as an index of angiogenesis, counted in five different fields, of sham and ESWT treated excisional wounds in BALB/c and db^+/db^+ mice at the time of 40–50% wound closure (day-6 for BALB/c wounds and day 11 for db^+/db^+ wounds). All values represent the mean ± SD from 5 mice in each group. **B** Representative Western immunoblot analysis and quantitative assessment of PECAM-1/CD31 protein expression in skin wound specimens obtained from BALB/c and db^+/db^+ mice given sham or ESWT treatment (single dose). Relative quantities and data were normalized from scanned immunoblots using the β-actin values and intensity levels were quantified using Adobe Photoshop CS3 software. Each points represents mean (SD of 4–5 mice at the time of 40–50% wound closure. * $p < 0.05$ versus sham-treated

referred to as biomechanical transduction, demonstrate favorable effects on a number of facets of wound healing [14, 25, 27–29]. Low energy ESWT, ultrasound, and pulsed electromagnetic fields have been used as adjunctive therapies to accelerate wound healing in a variety of tissues via molecular/cellular biochemical pathways that are incompletely understood [14, 17–20, 22, 24, 25, 29]. Clearly one modality will not be optimal for all wounds and data to guide selection of type, duration and sequence of adjunctive modalities based on unique biochemical profiles of an individual wound is lacking. Importantly, these devices are noninvasive, easy to use and have been shown to be effective in reducing pain and accelerating wound healing in difficult-to-heal/chronic pressure, diabetic and venous ulcers [25, 28, 30]. In a clinical feasibility study we demonstrated that ESWT is safe, feasible and well tolerated by patients with acute and chronic soft tissue wounds [25]. In addition to treating difficult-to-heal wounds, ESWT has been used to treat severe angina pectoris, ischemic heart disease and various musculoskeletal afflictions including calcific tendinopathy of the rotator cuff, lateral epicondilitis, and chronic plantar fasciopathy [16, 31–34]. More recently, our group has shown that ESWT is able to elicit strong strong proangiogenic activity and dampen trauma-induced proinflammatory responses in vivo. A single treatment of ESWT shortly after skin isograft transplantation induces a robust increase in dermal vascularization, giving rise to more numerous and strikingly larger vessels [19, 22]. In keeping with these results, we show in this study that the granulation tissue of the ESWT-treated excisional wounds shows increased vascularization with respect to control wounds.

Chemokines assigned to the CXC family and CSF3, which primarily regulate neutrophil chemotaxis, and MMPs play a key role in the control of both angiogenesis and inflammation early in the wound healing process [4, 7, 12, 35]. A major observation of our study was the remarkable prolonged upregulation of CXCL2, CXCL5, CSF3, TGFa, PECAM-1 and MMP9 in diabetic wounds. In comparison to normal healing, our analysis revealed a delayed appearance of CXCL2 during impaired wound healing with a sustained increase in CXCL5 (>500-fold) and CSF3 (>100-fold) throughout the 10 day observation period. Surprisingly, ESWT markedly increased and prolonged CXCL2, CXCL5, and CSF3 expression in diabetic wounds without having any measurable effect on time of wound closure.

TGFα is predominantly expressed in keratinocytes and has a profound autocrine mitogenic effect on keratinocytes, augments angiogenesis in wound healing [36, 37], and is upregulated in keratinocytes after skin injury [38]. However, TGFα deficient mice exhibit a normal healing phenotype [39, 40] suggesting that this growth factor is dispensable for fibroblast proliferation and/or angiogenesis and that its loss can be compensated by other EGFR ligands.

During wound repair, PECAM-1 on leukocytes and on endothelial cells is critically involved in the transendothelial migration processes at inflammatory sites and endothelial cell migration and the formation of new blood vessels [41]. Interestingly, it has been shown that PECAM-1 activation can be strongly induced by mechanical stress forces, indicating that it is a mechotransduction molecule [42]. Our findings further support the hypothesis that the application of physical energy or a mechanical stimulus can influence cellular events to produce a favorable biological response through mechanotransduction. Importantly this mechanotransduction appears to have a significant influence on cell-to-cell interactions, particularly regarding directed endothelial cell migration, vascular remodeling and regeneration [43]. Recent studies with mechanical wound effects through microdeformational therapy administered by negative pressure wound therapy have demonstrated improved healing in complex wounds through enhanced epithelial cell proliferation and angiogenesis and reduced MMP activity [44, 45].

MMPs control chemokine activity, the establishment of chemotactic gradients, and extravasation of leukocytes out of the blood into the injured tissue [46]. Epithelialization and keratinocyte cell migration is dependent on induction of MMP9 activity and [47] keratinocytes express MMP2 and 9 while fibroblasts express only MMP2 [48]. CXCL2 is resistant to MMP processing whereas CXCL5 is processed by multiple MMPs, including MM2 and MMP9, resulting in dramatic increase in chemokine activity [49]. Our findings conflict with a report demonstrating reduced expression and protein production of both MMP2 and MMP9 in diabetic excisional wounds [50], but are more supportive of clinical findings reporting elevated levels MMP2 and 9 in wound fluid obtained from diabetic patients [51, 52]. Interestingly, ESWT resulted in a modest increase in both MMP2 and MMP9 expression during normal wound healing whereas only a modest increase in MMP9 expression was noted during impaired wound healing. We speculate that the high and prolonged level of MMP9 proteases in the wound site leads to a disrupted and uncoordinated wound healing process by degrading matrix proteins and growth factors that are essential for normal healing.

Decreased cytokine and growth factor production has also been shown to play a role in impaired wound healing in diabetic wounds [4, 7]. In this study, we also identified 17 angiogenic-related cytokine gene transcripts that were not expressed or were downregulated during diabetic impaired wound healing when compared to normal acute healing, including *CDh5*, *FGF-1*, *FGF-2*, *FGFr3*, *FIGF*,

FZD5, *IL-1β*, *ITGAV*, *MMP2*, *NRP1*, *NRP2*, *PIGF*, *PTGS1*, *SERPINF1*, *STAB 1*, *TBX1*, and *TIMP1*. These gene targets could potentially be used as universal markers for impaired healing diagnosis. Interestingly, ESWT administration was unable to induce or augment expression of these mediators which play known pivotal roles in regulating normal wound healing processes by affecting the chemotactic attraction of inflammatory cells, proliferation of fibroblasts, keratinocytes, and endothelial cells as well as by neovascularization and extracellular matrix (ECM) synthesis [3].

Of these 17 transcripts, FGF-1 and FGF-2 (both which have angiogenic potency greater then VEGF and PDGF) are believed to be critical mediators of the early wound healing repair process, promotion of endothelial cell proliferation, new vessel and tube formation, and enhanced fibroblast migration. This mediation results in direct effects on structural cells involved in the synthesis of ECM, and affects fibroblast proliferation, granulation tissue formation and the differentiation of fibroblasts into myofibroblasts [53]. Healing of excisional wounds is delayed in mice lacking FGF-2, but not FGF-1 [54]. In addition, Tbx1, a transcription factor, has been linked to FGF signaling and involved in early blood vessel development [55–57]. The pronounced absence of mRNA transcripts for FGFs and their mediators in db^+/db^+ wounds ± ESWT treatment suggests a marked reduction in fibroblast infiltration and function which may contribute to the reported impaired granulation tissue formation and decreased angiogenesis in diabetic wounds [15].

Interestingly, Kuo et al. [23] reported ESWT increased skin fibroblast recruitment in ischemic skin-flaps. Moreover, Callaghan et al. [14, 29] showed accelerated wound healing under diabetic and normal wound healing conditions by upregulation of FGF-2 mediated angiogenesis. Furthermore, IL-1β stimulates fibroblast and keratinocyte proliferation and serves as potent chemoattractant for epidermal cells [1]. EGF supports fibroblast and keratinocyte proliferation, epithelialization, wound tensile strength and stimulates fibroblasts to secrete collagenase (MMP1) to degrade the matrix during the remodeling phase. Likewise, TIMP2 has been shown to accelerate keratinocyte migration in vivo [58]. Stabilin-1 (STAB1), a receptor found on activated $CD11b^+$, $F4/80^+$ tissue macrophages, mediates uptake of SPARC (osteonectin), a nonstructural component of ECM involved with angiogenesis, tissue remodeling, and wound healing. Clearance of SPARC from extracellular spaces promotes ECM synthesis and tissue remodeling [59]. High expression of Serpinf1/PEDF has been shown to stimulate endothelial cell migration, enhances vascular tube formation, and stimulates VEGF production from endothelial cells [60]. Cox-1 (PTGS1) induction of PGE_2 strongly contributes to keratinocyte proliferation and the selective inhibition of COX-1 [but not COX-2 (PTGS2)] has been shown to inhibit keratinocyte proliferation in healthy mice [61]. Evidence suggests that dysregulation of COX-1—coupled but not a COX-2—coupled PG biosynthesis pivotally contributes to strongly impaired repair conditions in diabetic mice [62]. This coincides with our observation that impaired wound healing was not associated with significant changes in gene expression for proangiogenic factors VEGF, PDGF, TGFα, TGFβ1, TGFβ2 TGFβ3. However, we can not rule out that angiogenesis and subsequent wound closure is delayed by increased local release of antiangiogenic factors such as thrombospondin and endostatin [13, 63, 64].

In summary, these studies indicate that diabetic wounds demonstrated delayed wound closure and tissue remodeling associated with decreased angiogenesis and increased inflammation. These effects were exacerbated throughout the entire wound healing process. Treatment with ESWT resulted in overexpression of key proangiogenic factors which neither enhanced nor interfered with wound tissue repair. The molecular mechanism of ESWT-mediated enhancement of angiogenesis remains to be completely established. ESWT has shown initial signs of making impaired wounds develop a more normal expression signature; however, ESWT treatment also nonspecifically augmented expression of 5 key factors that we identified were constitutively overexpressed in impaired wounds. Further studies are needed to reveal the importance of the differentially expressed transcripts, and their expression kinetics. Results from this study might have future applications, including the identification of markers for early diagnosis, targets for drug design, and indicators for treatment responsiveness and prognosis. Many of the limitations in the treatment of severe complex and difficult-to-heal wounds are based on lack of knowledge of the molecular mechanism(s) of wound healing. This is especially true of physical energy modalities such as ESWT, and is suggested by the inability to demonstrate a treatment effect in our model. This problem is magnified due to the fact that clinical treatments regimens do not translate to an effective small animal model, wherein serial ESWT treatments exacerbated wound healing. As the understanding of the key mediators of wound healing and treatment effects develop, we believe that molecular gene expression signatures early in the wound healing response will accurately predict risk stratification and correlate with different clinical outcomes. Future studies will also be undertaken in order to assess the functionality of the differentially-regulated transcripts with various protein assays. Therefore, understanding the mechanisms of wound healing and non-healing at a molecular level can lead to new treatment designs, prevention programs, and a better understanding of current treatments provided.

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References

1. Werner S, Grose R (2003) Regulation of wound healing by growth factors and cytokines. Physiol Rev 83(3):835–870
2. Singer AJ, Clark RA (1999) Cutaneous wound healing. N Engl J Med 341(10):738–746
3. Martin P (1997) Wound healing—aiming for perfect skin regeneration. Science 276(5309):75–81
4. Menke NB, Ward KR, Witten TM, Bonchev DG, Diegelmann RF (2007) Impaired wound healing. Clin Dermatol 25(1):19–25
5. Bauer SM, Bauer RJ, Velazquez OC (2005) Angiogenesis, vasculogenesis, and induction of healing in chronic wounds. Vasc Endovascular Surg 39(4):293–306
6. Ochoa O, Torres FM, Shireman PK (2007) Chemokines and diabetic wound healing. Vascular 15(6):350–355
7. Mast BA, Schultz GS (1996) Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. Wound Repair Regen 4(4):411–420
8. Broughton G II, Janis JE, Attinger CE (2006) Wound healing: an overview. Plast Reconstr Surg 117(7 Suppl):1e-S–32e-S
9. Mullner T, Mrkonjic L, Kwasny O, Vecsei V (1997) The use of negative pressure to promote the healing of tissue defects: a clinical trial using the vacuum sealing technique. Br J Plast Surg 50(3):194–199
10. Franz MG, Steed DL, Robson MC (2007) Optimizing healing of the acute wound by minimizing complications. Curr Probl Surg 44(11):691–763
11. Neal MS (2001) Angiogenesis: is it the key to controlling the healing process? J Wound Care 10(7):281–287
12. Romagnani P, Lasagni L, Annunziato F, Serio M, Romagnani S (2004) CXC chemokines: the regulatory link between inflammation and angiogenesis. Trends Immunol 25(4):201–209
13. Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O (2003) Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q J Nucl Med 47(3):149–161
14. Callaghan MJ, Chang EI, Seiser N et al (2008) Pulsed electromagnetic fields accelerate normal and diabetic wound healing by increasing endogenous FGF-2 release. Plast Reconstr Surg 121(1):130–141
15. Cianfarani F, Zambruno G, Brogelli L et al (2006) Placenta growth factor in diabetic wound healing: altered expression and therapeutic potential. Am J Pathol 169(4):1167–1182
16. Wang CJ (2003) An overview of shock wave therapy in musculoskeletal disorders. Chang Gung Med J 26(4):220–232
17. Yan X, Zeng B, Chai Y, Luo C, Li X (2008) Improvement of blood flow, expression of nitric oxide, and vascular endothelial growth factor by low-energy shockwave therapy in random-pattern skin flap model. Ann Plast Surg 61(6):646–653
18. Nishida T, Shimokawa H, Oi K et al (2004) Extracorporeal cardiac shock wave therapy markedly ameliorates ischemia-induced myocardial dysfunction in pigs in vivo. Circulation 110(19): 3055–3061
19. Stojadinovic A, Elster EA, Anam K et al (2008) Angiogenic response to extracorporeal shock wave treatment in murine skin isografts. Angiogenesis 11(4):369–380
20. Aicher A, Heeschen C, Sasaki K, Urbich C, Zeiher AM, Dimmeler S (2006) Low-energy shock wave for enhancing recruitment of endothelial progenitor cells: a new modality to increase efficacy of cell therapy in chronic hind limb ischemia. Circulation 114(25):2823–2830
21. Meirer R, Brunner A, Deibl M, Oehlbauer M, Piza-Katzer H, Kamelger FS (2007) Shock wave therapy reduces necrotic flap zones and induces VEGF expression in animal epigastric skin flap model. J Reconstr Microsurg 23(4):231–236
22. Davis TA, Stojadinovic A, Anam K et al (2009) Extracorporeal shock wave therapy suppresses the early proinflammatory immune response to a severe cutaneous burn injury. Int Wound J 6(1):11–21
23. Kuo YR, Wang CT, Wang FS, Yang KD, Chiang YC, Wang CJ (2009) Extracorporeal shock wave treatment modulates skin fibroblast recruitment and leukocyte infiltration for enhancing extended skin-flap survival. Wound Repair Regen 17(1): 80–87
24. Kuo YR, Wu WS, Hsieh YL et al (2007) Extracorporeal shock wave enhanced extended skin flap tissue survival via increase of topical blood perfusion and associated with suppression of tissue pro-inflammation. J Surg Res 143(2):385–392
25. Schaden W, Thiele R, Kolpl C et al (2007) Shock wave therapy for acute and chronic soft tissue wounds: a feasibility study. J Surg Res 143(1):1–12
26. Ennis WJ, Lee C, Meneses P (2007) A biochemical approach to wound healing through the use of modalities. Clin Dermatol 25(1):63–72
27. Ingber DE (2006) Cellular mechanotransduction: putting all the pieces together again. Faseb J 20(7):811–827
28. Kavros SJ, Liedl DA, Boon AJ, Miller JL, Hobbs JA, Andrews KL (2008) Expedited wound healing with noncontact, low-frequency ultrasound therapy in chronic wounds: a retrospective analysis. Adv Skin Wound Care 21(9):416–423
29. Walker NA, Denegar CR, Preische J (2007) Low-intensity pulsed ultrasound and pulsed electromagnetic field in the treatment of tibial fractures: a systematic review. J Athl Train 42(4):530–535
30. Kavros SJ, Miller JL, Hanna SW (2007) Treatment of ischemic wounds with noncontact, low-frequency ultrasound: the Mayo clinic experience, 2004–2006. Adv Skin Wound Care 20(4): 221–226
31. Fukumoto Y, Ito A, Uwatoku T et al (2006) Extracorporeal cardiac shock wave therapy ameliorates myocardial ischemia in patients with severe coronary artery disease. Coron Artery Dis 17(1):63–70
32. Rompe JD, Zoellner J, Nafe B (2001) Shock wave therapy versus conventional surgery in the treatment of calcifying tendinitis of the shoulder. Clin Orthop Relat Res 387:72–82
33. Kikuchi Y, Ito K, Ito Y et al (2010) Double-blind and placebo-controlled study of the effectiveness and safety of extracorporeal cardiac shock wave therapy for severe angina pectoris. Circ J 74(3):589–591
34. Schaden W, Fischer A, Sailler A (2001) Extracorporeal shock wave therapy of nonunion or delayed osseous union. Clin Orthop Relat Res 387:90–94
35. Belperio JA, Keane MP, Arenberg DA et al (2000) CXC chemokines in angiogenesis. J Leukoc Biol 68(1):1–8
36. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R (1986) Expression in rat fibroblasts of a human transforming growth factor-alpha cDNA results in transformation. Cell 46(2):301–309
37. Schreiber AB, Winkler ME, Derynck R (1986) Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor. Science 232(4755):1250–1253
38. Antoniades HN, Galanopoulos T, Neville-Golden J, Kiritsy CP, Lynch SE (1993) Expression of growth factor and receptor

mRNAs in skin epithelial cells following acute cutaneous injury. Am J Pathol 142(4):1099–1110
39. Luetteke NC, Qiu TH, Peiffer RL, Oliver P, Smithies O, Lee DC (1993) TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell 73(2):263–278
40. Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL, Dunn AR (1993) Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. Cell 73(2):249–261
41. DeLisser HM, Christofidou-Solomidou M, Strieter RM et al (1997) Involvement of endothelial PECAM-1/CD31 in angiogenesis. Am J Pathol 151(3):671–677
42. Fujiwara K (2006) Platelet endothelial cell adhesion molecule-1 and mechanotransduction in vascular endothelial cells. J Intern Med 259(4):373–380
43. Li S, Huang NF, Hsu S (2005) Mechanotransduction in endothelial cell migration. J Cell Biochem 96(6):1110–1126
44. Greene AK, Puder M, Roy R et al (2006) Microdeformational wound therapy: effects on angiogenesis and matrix metalloproteinases in chronic wounds of 3 debilitated patients. Ann Plast Surg 56(4):418–422
45. Saxena V, Hwang CW, Huang S, Eichbaum Q, Ingber D, Orgill DP (2004) Vacuum-assisted closure: microdeformations of wounds and cell proliferation. Plast Reconstr Surg 114(5):1086–1096 (discussion 1097–1088)
46. Parks WC, Wilson CL, Lopez-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4(8):617–629
47. McCawley LJ, O'Brien P, Hudson LG (1998) Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)- mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. J Cell Physiol 176(2):255–265
48. Sawicki G, Marcoux Y, Sarkhosh K, Tredget EE, Ghahary A (2005) Interaction of keratinocytes and fibroblasts modulates the expression of matrix metalloproteinases-2 and -9 and their inhibitors. Mol Cell Biochem 269(1–2):209–216
49. Van Den Steen PE, Wuyts A, Husson SJ, Proost P, Van Damme J, Opdenakker G (2003) Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. Eur J Biochem 270(18):3739–3749
50. Wall SJ, Bevan D, Thomas DW, Harding KG, Edwards DR, Murphy G (2002) Differential expression of matrix metalloproteinases during impaired wound healing of the diabetes mouse. J Invest Dermatol 119(1):91–98
51. Yager DR, Zhang LY, Liang HX, Diegelmann RF, Cohen IK (1996) Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids. J Invest Dermatol 107(5):743–748
52. Wysocki AB, Staiano-Coico L, Grinnell F (1993) Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. J Invest Dermatol 101(1):64–68
53. Cao R, Brakenhielm E, Pawliuk R et al (2003) Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nat Med 9(5):604–613
54. Miller DL, Ortega S, Bashayan O, Basch R, Basilico C (2000) Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. Mol Cell Biol 20(6):2260–2268
55. Aggarwal VS, Liao J, Bondarev A et al (2006) Dissection of Tbx1 and Fgf interactions in mouse models of 22q11DS suggests functional redundancy. Hum Mol Genet 15(21):3219–3228
56. Mitsiadis TA, Tucker AS, De Bari C, Cobourne MT, Rice DP (2008) A regulatory relationship between Tbx1 and FGF signaling during tooth morphogenesis and ameloblast lineage determination. Dev Biol 320(1):39–48
57. Lania G, Zhang Z, Huynh T et al (2009) Early thyroid development requires a Tbx1-Fgf8 pathway. Dev Biol 328(1):109–117
58. Terasaki K, Kanzaki T, Aoki T, Iwata K, Saiki I (2003) Effects of recombinant human tissue inhibitor of metalloproteinases-2 (rh-TIMP-2) on migration of epidermal keratinocytes in vitro and wound healing in vivo. J Dermatol 30(3):165–172
59. Kzhyshkowska J, Workman G, Cardo-Vila M et al (2006) Novel function of alternatively activated macrophages: stabilin-1-mediated clearance of SPARC. J Immunol 176(10):5825–5832
60. Apte RS, Barreiro RA, Duh E, Volpert O, Ferguson TA (2004) Stimulation of neovascularization by the anti-angiogenic factor PEDF. Invest Ophthalmol Vis Sci 45(12):4491–4497
61. Kampfer H, Brautigam L, Geisslinger G, Pfeilschifter J, Frank S (2003) Cyclooxygenase-1-coupled prostaglandin biosynthesis constitutes an essential prerequisite for skin repair. J Invest Dermatol 120(5):880–890
62. Kampfer H, Schmidt R, Geisslinger G, Pfeilschifter J, Frank S (2005) Wound inflammation in diabetic ob/ob mice: functional coupling of prostaglandin biosynthesis to cyclooxygenase-1 activity in diabetes-impaired wound healing. Diabetes 54(5):1543–1551
63. Streit M, Velasco P, Riccardi L et al (2000) Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. EMBO J 19(13):3272–3282
64. Italiano JE Jr, Richardson JL, Patel-Hett S et al (2008) Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood 111(3):1227–1233