Published in final edited form as:

J Am Coll Surg. 2011 March ; 212(3): 340–348. doi:10.1016/j.jamcollsurg.2010.10.017.

A New Technique of ex vivo Gene Delivery of VEGF to Wounds Using Genetically Modified Skin Particles Promotes Wound Angiogenesis

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Abstract

Background—Transplantation of genetically modified keratinocytes has been shown to accelerate wound healing. However, this method is labor-intensive and time-consuming. We have developed a new technique of intraoperative gene-delivery to wounds that involves transplantation of transfected minced skin particles (MSPs) derived from harvested partial-thickness skin.

Study Design—MSPs measuring 0.8×0. ×0.35 mm were created from a split-thickness skin graft of a pig. In vitro transfection was carried out with adenoviral-LacZ (Ad-LacZ) for qualitative and adenoviral-VEGF (Ad-VEGF) for quantitative analysis. Transfected MSPs were transplanted to each of 2.5×2.5 cm full-thickness wounds on the dorsum of the pig. Non-transfected MSPs served as controls. Wound chambers were applied and injected with saline to create a wet environment.

Results—LacZ expression was detected in migrating cells originating from MSPs both in vitro and in vivo. VEGF expression in the wound fluid of Ad-VEGF-MSPs transplanted wounds on each of days 2–4 (mean±SEM (ng/ml): 6.74±1.89: day 2, 9.88±2.27: day 3, 9.87±1.28: day 4) was significantly higher (p<0.0001) compared to wounds transplanted with either untransfected MSPs, Ad-LacZ-MSPs or untransplanted controls. In vitro VEGF expression was significantly higher (p<0.0001) in Ad-VEGF 1×10¹⁰ transfected-MSPs compared to either Ad-VEGF 1×10⁹ transfected MSPs or untransfected MSPs. Wounds transplanted with Ad-VEGF-MSPs showed significantly higher (p<0.0001) numbers of newly formed blood vessels (12.6±0.9 vessels/HPF) compared to wounds transplanted either Ad-LacZ-MSPs (4.4±0.5) or untransfected MSPs (5.2±0.7). All MSP transplanted wounds (Ad-VEGF-MSPs, Untransfected-MSPs, Ad-LacZ-MSPs) showed significantly higher reepithelialization compared to untransplanted wounds on days 10 and 14 (p<0.0001).

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Disclosure Information: Nothing to disclose.

Abstract presented at the American College of Surgeons 95th Annual Clinical Congress, San Francisco, CA, October 2009, and was awarded as the "Scientific Exhibit of Exceptional Merit."

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Conclusions—We demonstrated successful transfection of MSPs that can be transplanted to wounds as a source of gene expressing cells. This technique may be used to deliver growth-modulating genes in wound healing.

INTRODUCTION

Gene therapy is a potentially attractive option in treatment of non-healing wounds. It aims at delivering the growth modulating genes to the cells in the wound microenvironment, enabling over or under-expression of specific molecules like cytokines and growth factors hence modifying the healing process. By providing a sustained delivery of these growth-modulating polypeptides, gene therapy has the advantage over direct application of recombinant factors, which are expensive and have a short half-life requiring frequent applications. *Ex vivo* gene delivery with transplantation of gene transfected cells to wounds is one of the methods of delivering specific genes to the wound environment. One advantage of this approach over direct application of genes, *in vivo* gene delivery, is adding an extra cell source to the regenerating wound: transplanted cells serve as both regenerative seeds and carriers for specific genes to maximize the healing potential. We have developed and validated a porcine wet wound healing model that utilizes polyurethane wound chambers tightly sealed to wound periphery and injected with saline [1–9]. Using this model, we have demonstrated that transplantation of keratinocytes with *ex vivo* gene delivery of hEGF can further enhance healing of full thickness wounds compared with wounds transplanted with non-hEGF-expressing keratinocytes [7]. We also demonstrated wound healing acceleration of diabetic pigs with transplantation of IGF-1 transfected keratinocytes [3]. Given the time and work required for cell culture and gene transfection to cells before transplantation, we have been developing a novel technique for *ex vivo* gene delivery to wounds that utilizes small pieces of skin (Minced Skin Particles or MSP) created mechanically in the operating room.

The concept of transplanting small particles of skin to accelerate wound healing is relatively old. The pinch-grafting technique was, for example, described by Reverdin in 1869 [10] and since then many other techniques have been developed for this purpose [11–13]. In 2002, we showed that transplanted minced skin particles in the wet wound environment survive and proliferate to enhance wound healing, in a fashion similar to transplanted cultured keratinocytes [14]. Combining the two approaches of transplantation of minced skin particles and *ex vivo* gene delivery, we evaluated the feasibility of transplantation of minced skin particles as efficient gene delivery vehicles to wounds. Specifically, we hypothesized that minced skin particles transfected either with the β -galactosidase gene or VEGF gene could express their encoded peptides in a wet wound environment. The β -galactosidase gene would be a marker for localization of expressed peptides in wounds. The VEGF gene would be a model for transfer of a possibly therapeutic gene into the wound.

METHODS

Animals

Four female Yorkshire pigs (Parson's Farm, Hadley, MA), weighing 40–60 kg at arrival, were allowed to acclimatize for 1 week before initiation of the experiment. All animal procedures were approved by the Harvard Medical Area Standing Committee on Animals and conformed to the regulations related to animal use and other federal statutes.

Animal procedure

Animals received induction anesthesia with zolazepam 10 mg/kg (Telazol; Fort Dodge Veterinaria, Vall de Bianya, Spain) and xylazine 2.5 mg/kg (Xyla-Ject; Phoenix, St Josephs,

MO) intramuscularly followed by general anesthesia with 2% isoflurane (Novapplus; Hospira, IL) and Oxygen (4L) via endotracheal tube. Intramuscular injection of buprenorphine (Buprenex; Bedford Laboratories, Bedford, OH) 5 µg/kg and fentanyl patch 25 µg/hr (Watson Laboratories, Corona, CA) for 72 hours were administered for post operative analgesia.

Adenovirus preparation

The VEGF-expressing adenoviral vector (Ad-VEGF) used for this study is a replication-defective adenoviral vector that encodes VEGF under the control of the hCMV major immediate-early promoter (GenVec, Gaithersburg, MD).

Harvesting skin graft and creating minced skin particles

Dorsum of the animal was waxed and shaved. The skin was thoroughly disinfected with 10% povidone iodine (Betadine; Purdue Products LP, Stamford, CT) and 70% isopropanol (Aaron Industries, Clinton, SC). Split thickness skin graft of 0.35 mm (0.014 inch) depth was harvested from the dorsal neck region with a pneumatic dermatome (Zimmer Inc, Warsaw, IN). The donor site was dressed with petrolatum gauze (Medline Industries, Mundelrin, IL) and sterile gauze (Kendall Healthcare, Mansfield, MA) under an adhesive Leukotape bandage (BSN medical Ltd, Brierfield, England). Skin graft was cut into 2.54 cm x 2.54 cm (645 mm²) square pieces with a scalpel. Immediately before transfection, each piece was minced separately with a manual mincing device to 1000 pieces of minced skin particles (MSPs) measuring 0.8 mm x 0.8 mm (0.64 mm²) with 0.35 mm thickness.

Preparation of genetically modified skin particles

For qualitative analysis, *in vitro* transfection of 1000 MSPs was carried out with 1×10^9 and 1×10^{10} particles of Ad-LacZ in 1ml of Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) containing 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Auckland, New Zealand) in the incubator at 37 °C for 2 hours. In addition, transfection of 1000 MSPs was carried out with adenoviral VEGF for quantitative analysis. After 2 hours-infection *in vitro*, viral vector-containing solution was removed and transfected MSPs were rinsed with DMEM containing 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin three times to remove unincorporated viral particles.

VEGF and LacZ expression in vitro

1000 pieces of Ad-VEGF transfected MSPs were placed in a well with 2 ml of DMEM with 10% FBS and antibiotics to evaluate VEGF expression *in vitro*. Untransfected MSPs and medium alone (with no MSPs) served as control. 500µl of medium was exchanged every day up to day 6 and VEGF expression in collected medium was evaluated by commercially available enzyme-linked immunosorbent assay for human VEGF (VEGF ELISA Kit, Human, EMD Biosciences, Inc, CA) according to manufacturer's protocol.

At the same time, 10 pieces of Ad-LacZ transfected MSPs were placed with the dermis down on a 30mm collagen-coated dish in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) containing added bovine pituitary hormone, epidermal growth factor, calcium-depleted fetal bovine serum, calcium dichloride, streptomycin, penicillin, gentamicin, and amphotericin (Gibco, Carlsbad, CA) to observe gene expression in migrating cells from transfected MSPs. LacZ expression was evaluated by X gal staining on day 4. Cells migrating from Ad-LacZ MSPs were washed twice with PBS, fixed for 30 min in 0.05% glutaraldehyde solution, washed twice with PBS, and stained for 3 hours with 0.5 mg/ml X-Gal substrate (Fisher Scientific, Worcester, MA).

Wounding procedure and gene delivery of VEGF and LacZ to wounds mediated by genetically modified MSPs

Squares measuring 2.54 cm x 2.54 cm (645 mm²) were outlined on the dorsum of the pig by tattoo machine (Special Electric Tattoo Marker; Spaulding Enterprises, Voorheesville, NY). After the skin was prepared with povidone iodine as described above, 14–18 full-thickness wounds measuring 2.54 cm x 2.54 cm and 0.8 cm deep were created along the tattoo lines on each of a total of 4 pigs and assigned to the study groups. Wounds were placed to ensure the even dispersal of treatment groups along the paraspinal area of the pig. Subsequently, 100 pieces (64 mm²) of Ad-VEGF transfected MSPs (transfection protocol is described above), Ad-LacZ transfected MSPs and untransfected MSPs were transplanted to each wound (645 mm²) in a randomized fashion with a 1:10 expansion ratio. No attention was paid to the orientation of the MSP's - dermal side up or down - on the bottom of the wound. Transplantation was performed after a 2 hour transfection period followed by thorough rinsing of the particles. Untransplanted wounds served as controls. Immediately after retransplantation, all wounds were sealed with flexible polyurethane chambers (Corium International, Grand Rapids, MI) and MSPs were allowed to adhere for 1 hour before we injected 2 ml of keratinocyte growth medium containing penicillin 100 units/ml and streptomycin 100 µg/ml to create a wet environment.

Daily maintenance and evaluation of VEGF expression

Wound fluid was collected and exchanged every day with 2 ml of sterile saline containing penicillin/ streptomycin. The samples were stored at approximately –80 °C for later analysis. VEGF expression in fluid was assessed by VEGF ELISA Kit according to manufacturer's protocol.

Histology and evaluation of LacZ expression, reepithelialization and angiogenesis

5 mm-wide wound biopsies were taken diagonally on days 6, 10 and 14. On day 14, pigs were euthanized by intravenous injection of Euthasol (Virbac AH, Fort Worth, TX). Wounds transplanted with Ad-LacZ transfected MSPs were cut to two samples. Half of them were flash-frozen in liquid nitrogen, sectioned (20 µm), and stained for the detection of β-galactosidase activity with X-Gal staining followed by eosin counterstaining. The other half of the samples of Ad-LacZ transfected MSPs and all samples of other groups were fixed in 10% formalin, embedded in paraffin, sectioned (6 µm), and stained with hematoxylin/ eosin (H&E) for reepithelialization measurements. For evaluation of angiogenesis, sections of formalin fixed tissue were used for immunoperoxidase analysis after baking at 60° C for 1 hour, deparaffinization and rehydration (100% xylene X4 for 3 minutes each, 100% ethanol X4 for 3 minutes each and running water for 5 minutes). The sections were blocked for peroxidase activity with 3% hydrogen peroxide in methanol for 10 minutes and washed under the running water for 5 minutes. The sections were digested with bacterial protease type XXIV (Sigma Chemical Co., St. Louis, MO) (0.01% in pre-warmed 0.005M Tris Buffer Saline, pH 7.6) at 37° C for 20 minutes. The sections were evaluated with immunostaining of vWF. Primary antibodies for vWF (DAKO, Glostrup, Denmark, Cat#A0082) were used for 40 minutes in room temperature. Rabbit-anti-mouse antibodies were used as secondary antibodies (DAKO Envision, Carpinteria, CA, Cat#K4011) for 30 minutes. The slides were rinsed with PBS between incubation. The sections were developed using 3, 3'-diaminobenzidine (DAB) (Sigma Chemical) as substrate and counter stained with Mayer's hematoxylin.

By measuring the length of newly formed epithelium tongues from both sides of the wound edge as well as newly formed epithelium surrounding MSPs in H&E sections, the rate of reepithelialization of the wound was determined. Angiogenesis was evaluated by counting clusters of stained endothelial cells per field under x 40 magnification in vWF assay

sections. All observation and measurements were performed with Nikon ECLIPSE E400, Nikon DIGITAL SIGHT and Nikon DS-Fi1 with NIS-Elements D3.0.

Statistical analysis—In order to account for repeated measures taken on pigs for each group on multiple days and varying number of wounds, we used separate repeated measures mixed models in PROC MIXED which could accommodate these unbalanced designs for the outcomes: reepithelization, *in vitro* ELISA, and wound fluid ELISA, modeling the correlation with variance components. Fixed effects are estimated by Type III sums of squares. We used a generalized estimating equation (GEE) with Poisson log link for angiogenesis data in PROC GENMOD where the outcome was number of blood vessels per HPF, modeling the correlation between groups with repeated measures on wounds by compound symmetry for day 10 only with all groups and wounds taken on one pig. To obtain specific comparisons between groups for angiogenesis data and *in vitro* ELISA and between groups at different time points for all other models, we used the difference between least squares adjusted means obtained from the models, and used the Bonferroni-Sidak correction to adjust for multiple comparisons with an overall significance level of 0.05 for the models with group and day comparisons. All analyses were conducted in SAS Version 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Genetically modified MSPs express the LacZ transgene as well as VEGF *in vitro*

Split thickness skin grafts, harvested from dorsal neck of the pigs, were cut into small pieces of skin measuring $0.8 \times 0.8 \times 0.35$ mm using a manual mincing device. Immediately, 1000 MSPs were transfected with 1×10^9 and 1×10^{10} adenoviral-VEGF (Ad-VEGF) particles for 2 hours and rinsed before cultured in medium. These numbers were decided based on our previous *in vivo* gene delivery experiments with 1×10^{11} Ad-VEGF, which showed impaired wound healing due to viral cytotoxicity [8]. VEGF expression in culture medium was evaluated (Figure 1). We did not detect any difference between VEGF expression from Ad-VEGF (1×10^9 particles) MSPs and untransfected MSPs in the culture medium. However, VEGF expression was significantly different between 1×10^{10} and each other group ($p < 0.0001$ for each) as obtained from pairwise comparisons of adjusted means from a repeated measures analysis. We did not have a large enough sample size to compare groups between different days. On day 2 we found a VEGF expression of 105.7 ng/ml in the medium of Ad-VEGF (1×10^{10} particles) MSPs, 31.6 ng/ml in untransfected MSPs group and 14.5 ng/ml in the Ad-VEGF (1×10^9 particles) MSPs group. Cultured medium without any MSPs did not show any detectable VEGF. All MSPs cultured for this *in vitro* experiment were obtained from the same pig.

Similarly, 1000 MSPs were transfected with 1×10^{10} adenoviral-LacZ (Ad-LacZ) particles with the same protocol. Subsequently, 10 transfected MSPs were cultured on each collagen coated dish. X gal staining 48 hours after transfection demonstrated transgene expression in migrating fibroblasts (Figure 2a) and keratinocytes (Figure 2b) from MSPs *in vitro*.

Successful *ex vivo* gene delivery of VEGF and LacZ mediated by genetically modified MSPs

Ad-VEGF MSPs, Ad-LacZ MSPs and untransfected MSPs were transplanted into porcine full thickness wounds measuring 2.54×2.54 cm, with 1:10 expansion ratio. Untransplanted wounds served as control. VEGF expression in wound fluid collected from wound chambers was evaluated (Figure 3a). On day 1, we observed VEGF expression for Ad-LacZ MSPs (mean $\pm$ SEM: 1.1 ng/ml), untransfected MSPs ($1.49 \pm 0.2 \text{ ng/ml}$), and untransplanted

wounds (0.82 ± 0.4 ng/ml), secondary to intrinsic VEGF expression as a response to tissue injury.

VEGF expression was slightly higher in the Ad-LacZ MSPs and untransfected MSPs in comparison with untransplanted wounds, presumably due to some contribution to VEGF expression by the cells in the transplanted MSPs. However, wounds transplanted with Ad-VEGF MSPs showed significantly higher VEGF expression (3.04 ± 0.52 ng/ml on day 1, 6.74 ± 1.89 ng/ml on day 2, 9.88 ± 2.27 ng/ml on day 3, 9.87 ± 1.28 ng/ml on day 4) compared to each of the other three groups separately for each day ($p < 0.0001$) and always significantly different from untransplanted wounds for days 2–6 ($p < 0.0001$). Each of these results were obtained from pairwise comparisons of adjusted means obtained from repeated measures analysis between each group at each day of interest. Qualitative evaluation of gene expression with X gal staining on sections of wounds with Ad-LacZ MSPs showed transgene expression around transplanted MSPs (Figure 3b) as well as migrating cells (Figure 3c).

MSPs mediated gene delivery of VEGF does not enhance wound reepithelialization

Re-epithelialization of wounds was evaluated using H&E sections of wound biopsies (Figure 4). All three groups transplanted with MSPs (Ad-VEGF MSPs, Untransfected MSPs, Ad-LacZ MSPs) showed significantly higher reepithelialization rates compared to untransplanted wounds on day 10 and 14 ($p < 0.0001$), as determined from pairwise comparisons of adjusted means at each day obtained from repeated measures analysis. By day 14, all three groups of transplanted wounds were fully epithelialized, whereas reepithelialization of untransplanted wounds was only $58.9 \pm 4.8\%$. There was no significant difference in reepithelialization between wounds transplanted with Ad-VEGF MSPs, Ad-LacZ MSPs and untransfected MSPs, indicating that adenoviral constructs did not alter reepithelialization of the wounds. Neo-epidermis of Ad-VEGF MSPs and Ad-LacZ MSPs were as thick as untransfected MSPs and showed well developed rete ridges. The adenoviral-vector did not impair the regeneration of new epidermis, and the VEGF transgene did not enhance the speed of reepithelialization (Figure 5). We did not observe any differences in local inflammation between the adenoviral-minced skin particle groups and untransfected-minced skin group.

MSPs mediated gene delivery of VEGF enhances angiogenesis in wounds

Angiogenesis in wounds was evaluated with vWF staining. The number of newly formed blood vessels in biopsies from on 10 was counted in each group using vWF antibody stained slides. All wounds transplanted with MSPs (Ad-VEGF MSPs, Untransfected MSPs, Ad-LacZ MSPs) each showed significantly higher number of vessels compared to untransplanted wounds (2.0 ± 0.3 , $P < 0.0001$ for each comparison) (Figure 6). However, Ad-VEGF MSP transplanted wounds contained a well developed subepidermal plexus of blood vessels (Figure 7) and significantly higher number of blood vessels (12.6 ± 0.9 , $P < 0.0001$) compared to wounds transplanted with untransfected MSPs and Ad-LacZ MSPs. These results were from pairwise comparisons of adjusted means between groups obtained from GEE analysis of angiogenesis data.

DISCUSSION

The healing wound is a site of intense metabolism and complex molecular interactions. Growth factors are key players in mediating cell-cell interactions, cell migration, proliferation, and differentiation during the process of wound repair [15]. Upon wounding, expression of growth factors and their receptors is temporarily elevated and subsides once the wound healed. Gene therapy for wound healing aims at delivering these growth

promoting genes to the site of wound repair and is a potentially attractive option in treatment of non-healing wounds. Gene therapy has been used in clinical trials of wound treatment as well as treatment of conditions like Leber's amaurosis, malignant gliomas, Parkinson's disease, X-linked adrenoleukodystrophy and various cancers [16,17]. *Ex vivo* gene delivery to wounds commonly involves transfer of growth modulating genes to isolated and culture expanded skin cells and subsequent transplantation of the cells into the wound environment. This approach has some advantages over *in vivo* gene delivery techniques. It adds an extra cell source to the regenerating wound: transplanted cells serve as both regenerative seeds and carriers for specific genes to maximize the healing potential. In addition, the amount of gene expression can be standardized by *ex vivo* gene delivery [16]. Morgan et al. showed the first successful gene delivery to keratinocytes using retro-viral hGH in 1987 [18]. Subsequently in 1990, they demonstrated *in vivo* hGH expression after transplantation of the transfected keratinocytes in nude mice, as first *ex vivo* gene delivery to wounds [19]. The potential application of this approach to promote wound healing has been explored by various researchers [20–24]. In particular, we have demonstrated that *ex vivo* delivery of hEGF can significantly improve the healing of porcine full-thickness wounds [4,7]. We have also showed wound healing acceleration in diabetic porcine full thickness wounds with *ex vivo* gene delivery of IGF-1 [3]. A limitation of this approach is the relatively long preparation time for creating the genetically modified cells, which takes many days and a special laboratory facility to isolate and culture skin cells.

In this study, we attempted at *ex vivo* gene delivery to porcine full thickness wounds using minced skin particles (MSPs) instead of cultured cells. The similarities between pig and human skin, in terms of the structure of dermis and epidermis and the cell turnover time make it a very useful tool for evaluation of wound healing [25,26]. In 2002, we showed that transplanted minced skin particles in the wet wound environment survive and proliferate to enhance wound healing, in a fashion similar to transplanted cultured keratinocytes [14]. We used a manual mincing device that produces small pieces of skin measuring 0.8×0.8 mm out of harvested skin graft in the operating room. This way the lengthy and laborious process of enzymatic isolation and subsequent cultivation of skin cells is eliminated. As the marker genes for qualitative and quantitative assessment of gene expression, VEGF and LacZ were delivered to MSPs by adenoviral vectors. In nature, VEGF is produced by endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, platelets, neutrophils and macrophages [27–31]. It effects on multiple components of the wound healing cascade, including angiogenesis [32], epithelialization and collagen deposition [33]. VEGF was used as the model gene for modulating wound healing as well as a quantitative marker gene. LacZ served as a qualitative assessment tool. Gene expression was assessed both *in vitro* and *in vivo* following transplantation of transfected MSPs to wounds.

We confirmed LacZ gene expression in migrating and proliferating cells from transfected MSPs *in vitro* as well as *in vivo*. Our *in vitro* analysis revealed successful delivery of VEGF to MSPs using 1×10^{10} adenoviral particles, evidenced by significant VEGF overexpression for at least 5 days. MSPs transfected with lower number of viral particles (1×10^9) and untransfected MSPs also showed a baseline and minimal expression, presumably secondary to VEGF released from keratinocytes and fibroblasts in the MSPs. Following transplantation, the wounds transplanted with Ad-VEGF MSPs had a significantly higher VEGF expression compared to Ad-LacZ MSPs, untransfected MSPs and untransplanted wounds. This difference remained significant until day 5. The level of *in vivo* VEGF expression in this study was similar to our previous data using the same amount of Ad-VEGF as *in vivo* gene delivery with microseeding [8]. Therefore, gene transfer efficiency of our new method seems to be comparable to the efficiency of *in vivo* gene delivery. As a result of VEGF overexpression, wound angiogenesis increased significantly in Ad-VEGF MSPs compared to Ad-LacZ MSPs, untransfected MSPs and untransplanted wounds.

Despite enhancement of angiogenesis in wounds, we observed no significant improvement in reepithelialization between the VEGF transfected, LacZ transfected and untransfected MSP groups. This is in contrast to a recently reported study by Dickens et al., which showed acceleration of both reepithelialization and angiogenesis in porcine full thickness wounds following transplantation of VEGF transfected keratinocytes with the similar level and duration of VEGF overexpression and the same size wounds [24]. Perhaps, one explanation for this difference can be a greater regenerative capacity of transplanted small pieces of skin compared with keratinocytes alone used by Dickens. In other words, the wounds transplanted with MSPs have already reached their maximal healing potential and the additional benefit of VEGF overexpression and angiogenesis acceleration is too small to detect. It should be emphasized that using 1×10^{10} adenoviral particles for successful gene transfer to wounds, we did not observe any cytotoxic effect in relation to the viral vector and the healing potential, as the rate of wound reepithelialization in the transfected and untransfected groups were similar. This study was done as a proof of principle to assess the feasibility of gene delivery to minced skin particles. We demonstrated successful transfection of MSPs that can be transplanted to wounds as a source of gene-expressing cells. Additionally, we demonstrated angiogenesis acceleration in wounds using *ex vivo* gene delivery of VEGF with this new technique. This is the first report of successful delivery of genes to wounds by means of genetically modified skin particles. Utilizing this method, therapeutic genes of interest can be transplanted to wounds intraoperatively shortly after harvesting skin. This technique can be used to deliver growth modulating genes to treat non-healing wounds.

Acknowledgments

This work was supported by Public Health Grants R01 GM51449 from the National Institutes of Health.

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Figure 1.

VEGF expression in vitro. In vitro VEGF expression increased in Ad-VEGF 1×10^{10} MSPs 2 days after transfection and was maintained on day 5. Ad-VEGF 1×10^9 showed no statistically significant difference in VEGF expression compared to untransfected MSPs. Culture medium did not contain VEGF as determined from pairwise comparisons of adjusted means at each day of interest from repeated measures analysis.

Figure 2.
X-gal staining 48 hours after transfection of MSPs. Fibroblasts (a) and keratinocytes (b) expressing the LacZ transgene can be seen migrating from the MSPs.

Figure 3.

In vivo gene expression of VEGF and LacZ. **(a)** In vivo VEGF expression in wound fluid. Wounds with transplanted Ad-VEGF MSPs showed a significantly higher VEGF expression from day 2 to 4 (* $p < 0.0001$ as determined from pairwise comparisons of adjusted means at each day of interest from repeated measures analysis). VEGF expression was also detected in other transplanted and non-transplanted wounds as well. Ad-VEGF, $n=3$ per time point; untransfected, $n=3$ per time point; Ad-LacZ, $n=1$ per time point; untransplanted, $n=2$ per time point. The mean $\pm$ SEM is reported. **(b)** and **(c)** X gal staining of wounds with Ad-LacZ MSPs 6 days after transplantation demonstrated LacZ transgene expression around transplanted Ad-LacZ MSPs **(b)** and migrating cells in wound tissue **(c)**. Scale bar = 50 μm .

Figure 4.

Reepithelialization of wounds. On day 6 there was no significant differences among the groups in rate of re-epithelialization. On day 10 and 14, reepithelialization of all wounds in the transplanted groups (Adeno-LacZ-MSP, Ad-VEGF-MSP, untransfected MSP) were each significantly higher than untransplanted wounds (* $p < 0.0001$), but no significant difference was detected between wounds in the transplanted groups, as determined from pairwise comparisons of adjusted means at day 10 and 14 from repeated measures analysis). The mean $\pm$ SEM is reported. Day 6: $n=2$ per transplanted group; untransplanted $n=4$. Day 10: Ad-VEGF MSPs $n=5$, untransfected MSPs $n=5$, Ad-LacZ MSPs $n=4$, untransplanted $n=4$. Day 14: Ad-VEGF MSPs $n=3$, untransfected MSPs $n=4$, Ad-LacZ MSPs $n=3$, untransplanted $n=4$.

Figure 5. Cross section of 4 different groups of wounds with newly formed epithelium. All transplanted wounds were fully epithelialized and revealed a well-formed and mature epithelium, dermo-epidermal junction and rete ridges. Scale bar = 1 mm

Figure 6.

The number of vessels per HPF in wounds on day 10. The mean $\pm$ SEM is shown in the figure. Immunostaining with vWF antibody in Ad-VEGF MSP wounds showed significantly higher number of newly formed blood vessels per field compared to each of the other three groups. $n=5$ per group. $*p<0.0001$ compared to Ad-LacZ MSPs, untransfected MSPs or untransplanted; $**p<0.0001$ for untransplanted wounds compared to any of the wounds in the transplanted groups (Adeno-LacZ-MSP, Ad-VEGF-MSP, untransfected MSP). Results were obtained from pairwise comparisons of adjusted means between groups that come from GEE analysis of angiogenesis data.

Figure 7.

Angiogenesis in wounds. Immunohistochemical staining for angiogenesis using vWF **(a)** Ad-VEGF MSP wounds on day 10 showed well developed subepidermal plexus of new blood vessels compared to untransfected MSP wounds on day 10. **(b)** Enlarged image of the section marked with the square. Scale bar = 200 μ m.