Sustained Expression of Angiogenic Factors Stimulates Vascularized Granulation Tissue Depostion in Diabetic Mice

Allen R. Comer[1], Joely A. Straseski[2], Sara C. Pirnstill[1], Kristi K. Schaeve[1], Maggie L. Bach[1], Jennifer Murphy[2], B. Lynn Allen-Hoffmann[1,2]
[1]Stratatech Corporation, Madison, WI, USA and [2]University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI, USA

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Abstract

Introduction
Insufficient vascularization is a limiting factor in the healing of chronic wounds. Strategies to promote the formation of vascularized granulation tissue are promising approaches to promote wound healing. Topical application of individual angiogenic factors is limited by rapid degradation or clearance from the wound environment. This study describes the development of human skin substitutes designed to express sustained, elevated levels of angiogenic factors to promote deposition of highly-vascularized granulation tissue.

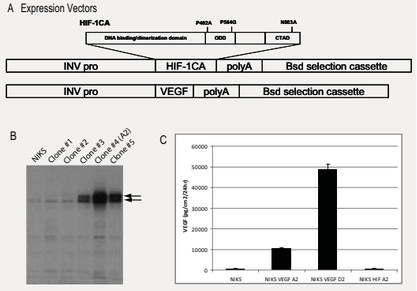
Methods
NIKS® keratinocytes were stably transfected with non-viral plasmid vectors encoding either a single angiogenic factor (VEGF) or a transcriptional regulator that induces multiple angiogenic factors (HIF-1α). Genetically-modified skin substitutes were evaluated for secretion of angiogenic factors and stimulation of endothelial cell proliferation, as well as the ability to promote deposition of vascularized granulation tissue after engraftment on nude or diabetic mice.

Results
Stably-transfected cells are genetically stable and non-tumorigenic. Skin substitutes modified to express VEGF secreted 100 fold more VEGF than unmodified tissue. Tissue modified to express HIF-1α secreted elevated levels of several pro-angiogenic chemokines. Conditioned medium from tissue expressing VEGF stimulated the proliferation of human microvascular endothelial cells compared to medium from unmodified skin substitutes. Tissue expressing either VEGF of HIF-1α promoted enhanced vascularization compared to unmodified skin substitutes after grafting on nude mice. Skin substitutes expressing VEGF also enhanced the deposition of vascularized granulation tissue in diabetic mice. Cell banks were produced to support GMP production.

Conclusions
Skin substitutes expressing elevated levels of angiogenic factors enhance vascularization in animal models of impaired wound healing. Uniform, non-viral genetic modification of NIKS® keratinocytes offers safety and consistency advantages compared to heterogeneous modification of primary keratinocytes with viral vectors. The ability of skin substitutes expressing elevated levels of angiogenic factors to promote vascularitoin in vivo suggests that these substitutes may accelerate the vascularization and healing of chronic wounds.

Results

Figure 1 Stably-Transfected NIKS® Cells Over-express Angiogenic Factors

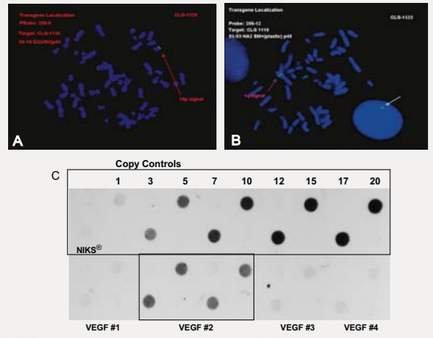


(A) NIKS® cells were transfected with non-viral expression vectors encoding a stabilized variant of HIF-1α (HIF-1^{CA}) or $VEGF_{165}$ and individual clones of stably-transfected cells were recovered. Point mutations resulting in the constitutively-active HIF-1^{CA} protein are shown.

(B) Western blot showing the elevated levels of HIF-1^{CA} (arrow) in different NIKS$^{HIF-1CA}$ clones. Data presented in the remaining figures is for the clone expressing the highest level of HIF-1^{CA} protein.

(C) Levels of VEGF secreted from ExpressGraft$_{Vascular}$ tissue or unmodified NIKS® tissue. VEGF levels were quantified by ELISA. Different clones stably transfected with the $VEGF_{165}$ vector express 10- to 100-fold higher levels of VEGF. Tissue expressing HIF-1^{CA} does not express elevated levels of VEGF.

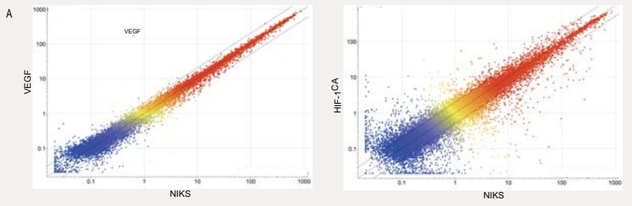
Figure 2 Identification of Transgene Integration Site and Copy Number



(A, B) Transgene integration site was determined by fluorescence in situ hybridization (FISH) using probes prepared from the vectors used to isolate the stably-transfected VEGF or HIF-1^{CA} clones. The VEGF clone contains a single detectable signal localized to the short arm of one copy of chromosome 16 **(A)**, while the HIF-1^{CA} clone **(B)** contains a single detectable signal on the long arm of one copy of chromosome 1. The intensity of the fluorescent signal suggests that the integration sites for both clones most likely contain multiple copies of the corresponding transgene.

(C) Transgene copy number for several VEGF clones was confirmed by DNA dot blot compared to a transgene-specific copy number dilution series (top two rows). The VEGF clone used for these studies (VEGF #2, in box on bottom rows) has 3-5 copies of the transgene. Transgene copy analysis of the HIF^{CA} clone is in progress.

Figure 3 Comparison of Gene Expression Profiles of ExpressGraft$_{Vascular}$ Tissue



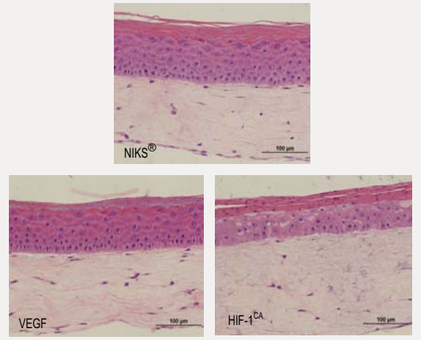
B **Fold Increase Over NIKS (ELISA)**

	NIKS^{VEGF}	NIKS$^{HIF-1CA}$
VEGF	**110.1**	1.5
CXCL1	0.9	**18.4**
CXCL5	0.8	**11.7**
CXCL6	0.6	**24.8**
CXCL8	1.1	**8.3**
Ang 1	1.2	0.8

(A) Gene expression profiles were compared for unmodified NIKS® tissue or tissue modified to express VEGF alone or HIF-1^{CA}. Scatter plots of the data show that the overall expression patterns of unmodified NIKS® tissue and tissue expressing VEGF are very similar, with the notable exception of much higher levels of VEGF expression in the stably-transfected tissue. The overall gene expression profile of tissue expressing HIF-1^{CA} differs more significantly from that of unmodified NIKS® tissue, with a larger number of differentially-expressed genes.

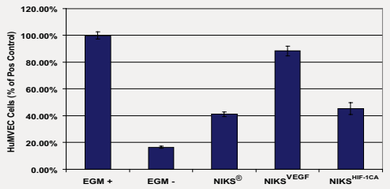
(B) Levels of several angiogenic cytokines that were identified as differentially expressed in the gene array analysis were quantified by ELISA in conditioned medium. Values presented are the relative expression (fold increase) compared to unmodified NIKS® tissue.

Figure 4 Epidermal Differentiation of ExpressGraft$_{Vascular}$ Tissue



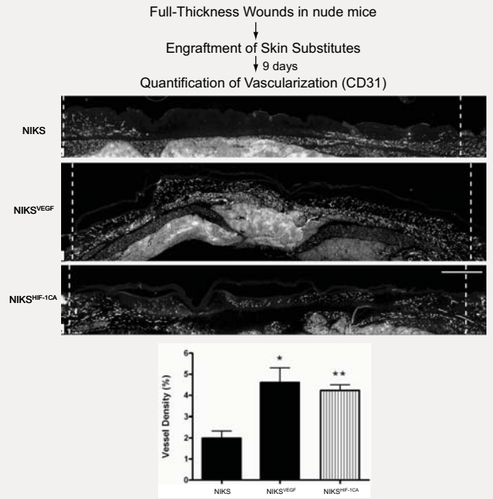
Sections of tissue produced by unmodified NIKS® cells or clones expressing VEGF or HIF-1^{CA} were stained with hematoxylin and eosin to visualize epidermal architecture. Tissue prepared from NIKS® cells and the VEGF clone exhibit normal epidermal differentiation, resulting in a stratified epidermal layer with well-defined basal, spinous, granular, and cornified layers. Tissue prepared from the HIF-1^{CA} clone exhibits altered (parakeratotic) epidermal differentiation, with a thinner epidermal layer and nucleated cells in the stratum corneum.

Figure 5 Conditioned Medium From ExpressGraft$_{Vascular}$ Tissue Stimulates Endothelial Cell Growth



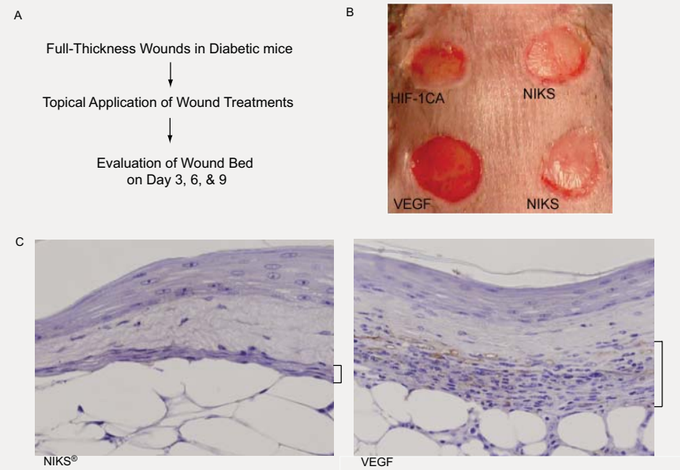
Conditioned medium from unmodified NIKS® skin substitutes or tissue made with clones expressing VEGF or HIF-1^{CA} was tested for the ability to promote the growth of human microvascular endothelial cells (HuMVEC). Positive control medium (EGM+) is fully-supplemented endothelial growth medium, while the negative control (EGM-) is unsupplemented endothelial basal medium. Conditioned medium from tissue was diluted 1:5 with unsupplemented EGM- medium and used to culture HuMVEC cells for 4 days, at which time cells were harvested and counted. Medium from unmodified NIKS® tissue stimulates HuMVEC growth compared to EGM- medium due to endogenous production of VEGF and other angiogenic factors. Medium from VEGF-expressing tissue stimulates HuMVEC growth twice as much as unmodified NIKS® tisssue and five times as much as the negative control (EGM-). This level of stimulation is comparable to that seen with fully-supplemented EGM+ medium. Medium from tissue expressing HIF-1^{CA} does not stimulate HuMVEC proliferation beyond that seen with unmodified NIKS® tissue.

Figure 6 Quantitative Analysis of Enhanced Vascularization of ExpressGraft$_{Vascular}$ Tissue *in vivo*



Experimental design is shown in **(A)**. Skin substitutes prepared from unmodified NIKS® cells or clones expressing VEGF or HIF-1^{CA} were grafted onto full-thickness wounds in athymic nude mice. After nine days, grafted tissue was harvested and vascular density was quantified by immunofluorescence detection of CD31. Representative photographs of CD31 staining are shown in **(B)**. Vessel density in the central region of the grafted tissue is greater in ExpressGraft$_{Vascular}$ tissue compared to tissue prepared from unmodified NIKS® cells. Quantification of the CD31 staining **(C)** shows that ExpressGraft$_{Vascular}$ tissue prepared from both the VEGF and HIF-1^{CA} clones results in statistically-significant increases in vascular density compared to unmodified NIKS® tissue. Fluorescently staining vessels were quantified using image intensity threshold analysis, with statistical analysis determined by one-way ANOVA and the Bonferroni test for multiple comparisons.

Figure 7 ExpressGraft$_{Vascular}$ Tissue Promotes Wound Vascularization in Diabetic Mice



Experimental design is shown in **(A)**. Unmodified NIKS® tissue or ExpressGraft$_{Vascular}$ tissue was applied to full-thickness wounds on diabetic (Lepr^{db}) mice. Three, six, and nine days later, skin substitutes were removed and the vascular appearance of the wound bed was assessed **(B)**. Day 6 analysis: Wounds treated with unmodified NIKS® tissues exhibit vascularization only at the wound periphery. Wounds treated with VEGF tissue show vascularization throughout the wound bed. Although not as pronounced, wounds treated with HIF-1^{CA} tissue exhibit a modest vascularization response, with increased vascularization located primarily at the wound margin. Tissues were sectioned and analyzed by immunohistochemistry with anti-CD31/PECAM (brown) **(C)**. Tissue expressing VEGF (right) promoted the formation of a thick layer of vascularized granulation tissue (brackets), compared to the relatively thin and poorly-vascularized tissue seen underneath unmodified NIKS® tissue (left).

Summary

Stably-transfected clones of the clinically-tested NIKS® epidermal progenitor cell line were isolated with non-viral vectors encoding VEGF or a constitutively-active variant of HIF-1α

Skin substitutes generated with stably-transfected NIKS® cells secrete multiple angiogenic factors at levels ~50-100 fold higher than unmodified skin substitutes

Skin substitutes generated with stably-transfected NIKS® cells stimulate endothelial cell proliferation *in vitro* and promote wound vascularization *in vivo*.

Conclusions

- The long-lived NIKS® epidermal progenitor cell line is an ideal source of cells for tissue engineering and is amendable to modification with non-viral vectors
- Second-generation skin substitutes prepared from genetically-modified NIKS® cells are promising candidates to enhance the vascularization and healing of chronic wounds

Acknowledgements

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