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Chimeric Composite Skin Substitutes for Delivery of Autologous Keratinocytes to Promote Tissue Regeneration

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Abstract

Objective—We hypothesize that the pathogen-free human keratinocyte progenitor cell line, NIKS, cultured in a chimeric fashion with patient's primary keratinocytes would produce a fully-stratified engineered skin substitute tissue and serve to deliver autologous keratinocytes to a cutaneous wound.

Summary Background Data—Chimeric autologous / allogeneic bioengineered skin substitutes offer an innovative regenerative medicine approach for providing wound coverage and restoring cutaneous barrier function while delivering autologous keratinocytes to the wound site. NIKS keratinocytes are an attractive allogeneic cell source for this application.

Methods—Mixed populations of GFP-labeled NIKS and unlabeled primary keratinocytes were used to model the allogeneic and autologous components in chimeric monolayer and organotypic cultures.

Results—In monolayer co-culture, NIKS^{GFP} cells had no effect on the growth rate of primary keratinocytes and cell-cell junction formation between labeled and unlabeled keratinocytes was observed. In organotypic culture employing dermal and epidermal compartments, chimeric composite skin substitutes generated using up to 90% NIKS^{GFP} cells exhibited normal tissue architecture and possessed substantial regions attributable to the primary keratinocytes. Tissues expressed proteins essential for the structure and function of a contiguous, fully-stratified squamous epithelia and exhibited barrier function similar to that of native skin. Furthermore, chimeric human skin substitutes stably engrafted in an *in vivo* mouse model, with long-term retention of primary keratinocytes but loss of the NIKS^{GFP} cell population by 28 days after surgical application.

Conclusions—This study provides proof of concept for the use of NIKS keratinocytes as an allogeneic cell source for the formation of bioengineered chimeric skin substitute tissues, providing immediate formal wound coverage while simultaneously supplying autologous cells for tissue regeneration.

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Introduction

Skin serves an essential role in maintaining fluid homeostasis and acts as a barrier to infectious agents. Widespread loss of skin integrity, most often associated with major burns, results in morbidity or mortality due to the absence of these important functions. Current standards of care recommend resurfacing of the patient as quickly as possible to restore fluid homeostasis and cutaneous barrier function using autologous skin grafts. Unfortunately, patients with extensive burns may not have sufficient donor skin available for wound site coverage. One approach to treating such cases is to apply cadaver skin as a temporary dermal/epidermal cover, with subsequent application of autologous keratinocytes isolated from a biopsy of unwounded tissue and cultivated *ex vivo*. Epicel® (Genzyme Corporation), an example of such an autologous product, is supplied as a thin layer of keratinocytes lacking a dermal component. Although this product has proven to be a valuable life-saving treatment option, widespread clinical success has been hindered by technical challenges.¹ This product is extremely fragile and does not possess barrier function, a property which develops as the multilayered stratified squamous epithelium matures.² Significantly, Epicel® is only available for clinical application three to four weeks after the time of skin biopsy necessitating use of other surgical treatment modalities. Furthermore, bacterial contamination and other factors cause high failure rates of Epicel® that are not seen with traditional split-thickness skin grafts.³ As an alternative therapy, the use of chimeric cultures of autologous and allogeneic cells has been proposed as a solution for the complex clinical problem of severe skin loss.⁴⁻⁵ The use of both autologous and allogeneic cell sources could substantially decrease the time required for cell expansion, enabling earlier clinical application for permanent wound coverage. Importantly, application of fully-stratified chimeric skin substitutes would immediately restore full barrier function, aiding in re-establishing fluid homeostasis and preventing wound sepsis. The major obstacle to this approach has been the lack of a consistent source of pathogen-free allogeneic keratinocytes.

In the field of regenerative medicine, consistent human cell sourcing is a well recognized problem.⁶ The human keratinocyte progenitor cell line, NIKS, exhibits cell type-specific growth and differentiation characteristics, yet maintains an extended lifespan *in vitro*.⁷ The NIKS cells arose spontaneously from a culture of normal human primary keratinocytes established from neonatal foreskin tissue. NIKS keratinocytes are non-tumorigenic and free of adventitious agents such as viruses, bacteria, fungi, and mycoplasma.⁸ In organotypic culture NIKS keratinocytes form stratified squamous epithelia possessing tensile strength and barrier function similar to that of intact skin. Recently, composite skin substitutes containing a dermal component and an epidermis generated from NIKS keratinocytes (StrataGraft®) have been evaluated in a Phase I / Phase II clinical trial. In this study the clinical performance of StrataGraft® skin substitutes was found to be comparable to cadaver skin for the treatment of complex skin defects.⁸ No adverse events associated with the clinical application of skin substitute tissue were observed. Furthermore, *in vitro* evaluation and analysis of patient data indicated that skin substitutes generated from NIKS cells were well tolerated and did not induce strong allogeneic immune responses.⁹ The longevity, consistency, pathogen-free status, and clinical safety evaluation of the NIKS cell line makes it ideally suited for cell therapy and tissue engineering applications.

To date, there have been no reports on the generation of chimeric cultures of human autologous and allogeneic keratinocytes. We hypothesized that organotypic co-culture of NIKS and primary keratinocytes will generate a fully-stratified epidermal component of a composite skin substitute tissue and serve to deliver autologous keratinocytes to a cutaneous wound site. We genetically engineered NIKS cells to stably express green fluorescent protein (GFP) and generated monolayer and organotypic co-cultures with unlabeled primary keratinocytes. Organotypic culture of NIKS^{GFP} and primary human keratinocytes resulted in

a fully-stratified, chimeric skin substitute that exhibited normal tissue architecture and possessed barrier function comparable to native skin. When chimeric skin substitutes were transplanted onto mice, the NIKS^{GFP} keratinocytes were displaced and sloughed while the primary keratinocytes remained. These findings suggest that in a clinical setting, chimeric skin substitutes generated with NIKS keratinocytes would have the unique characteristics of providing immediate formal wound coverage while also supplying autologous cells for late stage wound closure.

Materials and Methods

Generation of Green Fluorescent Protein Expressing NIKS Keratinocytes (NIKSGFP)

NIKs keratinocytes⁷ stably expressing cytomegalovirus (CMV) promoter-driven GFP were generated by polycationic liposome-mediated transfection of pGreenLantern (Gibco-BRL, Rockville, MD) and pcDNA3neo (Invitrogen, Carlsbad, CA). NIKs cells cultivated on mitomycin C-treated Swiss mouse 3T3 fibroblast feeder layers¹⁰ were transfected using GeneFector (VennNova, Miami, FL) in Dulbecco's modified Eagle's medium. After 5 hours, cells were switched to standard keratinocyte culture medium⁷ for subsequent maintenance. Sterile fluorescence-activated cell sorting (FACS) was employed to isolate individual NIKS^{GFP} clones. The NIKS^{GFP} clone selected for use maintained a stable, high level of GFP expression for over 15 passages as determined by FACS analysis. GFP expression had no observable effects on differentiation of NIKS^{GFP} in monolayer or organotypic culture.¹¹

Monolayer Cell Culture Methods

Primary keratinocyte cultures were established from human neonatal foreskin tissue obtained after circumcision in accordance with Madison Meriter Hospital and the University of Wisconsin Human Subjects Committees and Institutional Review Boards. NIKS^{GFP} and primary human keratinocytes were cultivated on feeder layers in standard keratinocyte culture medium and maintained at 37°C in a humidified 5% CO₂ atmosphere.⁷ Cultures were passaged at weekly intervals at 3×10^5 cells per 100 mm tissue culture dish (approximately a 1:25 split).

Co-culture of NIKS^{GFP} and Primary Human Keratinocytes in Monolayer Culture

For monolayer studies, cells were seeded onto feeder layers in standard keratinocyte medium using 50%:50%, 10%:90%, or 1%:99% (NIKS^{GFP} : primary keratinocytes). Cultures consisting of 100% NIKS^{GFP} or 100% primary keratinocytes served as controls. For examination of cell-cell adhesion, eight well chamber slides (BD Biosciences, San Jose, CA) were plated at a density of 4×10^4 cells/well. At confluence, cultures were fixed for 10 minutes with 1% paraformaldehyde in phosphate buffered saline (PBS), with subsequent cold acetone fixation for 5 minutes. The presence of E-cadherin was detected by indirect immunofluorescence as described below. Samples were viewed with an Olympus IX-70 inverted microscope equipped with fluorescein and Texas Red band pass filters. Images were acquired with a DEI-750 camera (Optronics Engineering) and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

For studies comparing growth rates of chimeric co-cultures, triplicate 60 mm tissue culture dishes were plated at a total cell density of 10^5 cells/ chimeric culture using the previously stated percentages. Cultures were trypsinized after 4 days of growth and the total number of cells harvested for each chimeric or control culture was determined. To account for variances in plating efficiency between NIKS^{GFP} and primary keratinocytes, the expected cell recovery for each chimeric combination was calculated based on the cell recoveries of NIKS^{GFP} and primary keratinocyte control cultures. The cell recovery value for each

chimeric culture was calculated as a percent of the expected value. Statistical significance was determined using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-test ($p < 0.05$). A total of five experiments were conducted, using two independent primary keratinocyte sources. Results are reported as the mean $\pm$ % error.

Organotypic Cell Culture Methods

Composite human skin substitutes were generated with NIKS^{GFP} and/or primary keratinocytes using organotypic culturing methods, with culturing media and the dermal components provided by Stratatech Corporation (Madison, WI). Dermises consisting of human dermal fibroblasts embedded in type I collagen were contained in cell culture plate inserts (44 cm²). Keratinocytes were seeded onto the surface of the cellularized dermal matrix at a density of 5×10^6 cells/insert. For chimeric stratified squamous epithelia, seeding proportions of 90%:10% and 50%:50% (NIKs^{GFP} : primary keratinocytes) were used. Tissues generated from 100% NIKS^{GFP} or 100% primary keratinocytes served as controls. Cultures were kept submerged for 5 days, at which point they were raised to the air-interface and maintained for an additional 18 days forming fully-stratified squamous epithelia.

Composite skin substitute tissue samples were fixed for two hours in 1% paraformaldehyde in PBS. Each sample was divided evenly for cryopreservation and paraffin-embedding. After fixation, cryopreserved samples were equilibrated in 20% sucrose in PBS overnight at 4°C. Samples were then frozen in Tissue-Tek O.C.T. Compound (Sakura, Tokyo, Japan) with liquid nitrogen-chilled isopentane and stored at -80°C until sectioned. Paraffin-embedded sections (5 μ m) were stained with hematoxylin and eosin (H&E) to assess tissue morphology.

Barrier Function Analysis

The degree of skin surface hydration is proportional to the retention of electrical charge or capacitance.¹² A Nova DermaPhase Meter (DPM) 9003 equipped with a DPM 9107 sensor probe (NOVA Technology Corporation, Gloucester, MA) was used to measure the electrical impedance based capacitance of composite skin substitute tissues. For comparative analysis, impedance measurements were taken from native human skin as well as from skin where the barrier function had been disrupted by tape-stripping.^{13, 14} The probe was placed in direct contact with the skin surface and impedance measurements, ranging from 90 to 999 DPM units, were recorded at 0.5 second intervals for 10 seconds. Triplicate samples were tested and reported as the mean $\pm$ SEM.

Indirect Immunofluorescence

Indirect immunofluorescence analysis was employed to detect proteins associated with cell-cell adhesion or keratinocyte terminal differentiation. Cryopreserved sections (5 μ m) were fixed in cold acetone, washed, and blocked with 3% normal goat serum (NGS) in PBS. Samples were incubated for 1 hour at 37°C with mouse monoclonal antibodies against E-cadherin (1:80, BD Biosciences, San Jose, CA), keratin 1 (1:50, Novocastra Laboratories, Ltd., Newcastle upon Tyne, UK), keratinocyte-specific type I transglutaminase (1:100, Biomedical Technologies, Stoughton, MA), or filaggrin (1:100, Biomedical Technologies, Stoughton, MA) diluted with 3% NGS in PBS. Samples were incubated for 30 minutes at room temperature with goat anti-mouse IgG Alexa-594 secondary antibody (1:500, Invitrogen, Carlsbad, CA). Sections incubated with 3% NGS in PBS served as controls for non-specific staining. Nuclei were counterstained with 5 μ g/ml Hoechst 33258 in PBS.

Digital image capture of individual fluorescence signals was performed using the imaging system previously described or an Olympus IX-71 inverted fluorescent microscope equipped

with fluorescein, Texas Red, and Hoechst band pass filters, an Olympus DP70 digital camera and DP Controller software (Olympus, Center Valley, PA). Due to the intensity of GFP fluorescence in the squamous layer, image segmentation with separate analysis of GFP fluorescence in the basal, spinous, and granular layers was completed to assess tissue chimerism. Identical image manipulations were performed for the segmented regions of chimeric and control tissues. Dual- or tri-color images were created by overlay of single color images of the same field.

Grafting of Composite Human Skin Substitutes

All animal studies were performed in accredited facilities in accordance with the University of Wisconsin's Animal Research regulations and approved by the Animal Care and Use Committee. Immunocompromised athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) were anesthetized and cleansed with 4% chlorhexidine gluconate (Zeneca Pharmaceuticals, Wilmington DE). A full-thickness wound measuring 2×3 cm in size was excised from the dorsum of the animal. Composite skin substitutes were trimmed to the size of the wound, placed dermal side down onto the wound bed, and covered with non-adherent N-terface dressing (Winfield Laboratories, Richardson, TX) impregnated with bacitracin zinc (500 units) and polymyxin b sulfate (10,000 units) ointment (E. Fougera, Melville, NY). The grafts were further secured with Tegaderm transparent dressing and VetBond (3M, St. Paul, MN) to maintain a moist wound environment. Analgesia was administered in accordance with approved protocols. After 7 days all dressings were removed, digital photographs were taken of the wound sites, and biopsies spanning the wound edge were sampled from engrafted animals. Additional visual assessments were made and biopsies were taken at 14, 28, and 91 days after surgery. Biopsies of engrafted wound sites were fixed and processed for cryopreservation as previously described. H&E staining of sections allowed for evaluation of wound site and engrafted tissue morphology.

Detection of Human Tissue and NIKS^{GFP}-Labeled Cells using Fluorescence Microscopy

Cryopreserved sections were fixed in cold acetone, rinsed, and blocked for 30 minutes with 3% NGS in PBS. Sections were incubated for 1 hour at 37°C with Red-Phycoerythrin (RPE)-labeled anti-HLA-ABC (1:25, Accurate Chemical and Scientific Corp., Westbury, NY). Sections incubated with 3% NGS in PBS served as controls for non-specific staining. Nuclei were counterstained with Hoechst 33258. Samples were examined and digital images were captured using the imaging systems previously described. For each RPE or GFP image set, identical image manipulations were performed for chimeric and control tissues. Tri-color images were created by overlay of single color images of the same field.

Detection of NIKS^{GFP} Cells by Amplification of the Gene Encoding GFP

DNA encoding GFP expression from NIKS^{GFP} cells within tissues generated from 100% NIKS^{GFP} cells, 90%:10% (NIKS^{GFP} : primary keratinocytes), or 50%:50%, was detected using polymerase chain reaction (PCR) analysis. In addition to analysis of ungrafted tissues, biopsies from the wound site of chimeric tissues engrafted for 28 days were evaluated for retention of GFP-containing cells. Genomic DNA was isolated from tissue samples using a DNeasy Blood and Tissue kit according to the manufacturer's instructions (Qiagen, Valencia, CA). DNA was analyzed by PCR amplification using the following primer pairs: GFP (Fwd: AGGGCTATGTGCAGGAGAGA; Rev: GGGTGGACAGGTAATGGTTG) and Pv92 (Fwd: GGATCTCAGGGTGGGTGGCAATGCT; Rev: GAAAGGCAAGCTACCAGAAGCCCCAA). Amplification of GFP from ungrafted 100% NIKS^{GFP} tissue served as a positive control. Amplification of Pv92 served as an internal control for human specific DNA. Amplified products were visualized by agarose gel electrophoresis. To account for the presence of mouse genomic DNA in some samples, samples were run and loaded to achieve equivalent levels of detected human-specific DNA.

Positive control 100% NIKS^{GFP} tissue samples were loaded at a lower level due to the intensity of the GFP amplified product.

Results

Monolayer Co-culture of NIKS^{GFP} and Primary Keratinocytes Form Contiguous Epithelial Sheets

To evaluate if the NIKS keratinocyte cell line could serve as a “carrier” for autologous keratinocytes, the interaction of these two independent cell sources was examined both in monolayer and organotypic culture systems. To facilitate these studies, we exploited the ability of the NIKS cell line to be genetically engineered. NIKS keratinocytes stably transfected with plasmid DNA encoding GFP enabled individual cell tracking and provided a clear distinction between tissue derived from NIKS^{GFP} cells or unlabeled primary keratinocytes. Using a monolayer chimeric system we observed the interaction of both cell types to determine if co-culture with NIKS^{GFP} cells affected the growth of primary keratinocytes.

Indirect immunofluorescence for E-cadherin was performed to demonstrate the formation of cell-cell junctions between NIKS^{GFP} cells and primary keratinocytes. Relative amounts and localization of E-cadherin staining in co-cultures plated using proportions of 50%:50%, 10%:90%, or 1%:99% (NIKS^{GFP} : primary keratinocytes) were compared to 100% NIKS^{GFP} or 100% primary keratinocyte control cultures. In all cases, contiguous chimeric epithelial sheets were produced which exhibited mature cell-cell junctions between NIKS^{GFP} cells and unlabeled primary keratinocytes (Figure 1A). To determine if co-culturing affected the growth rate of either cell type, the cell recoveries after four days of growth were evaluated. No statistically significant differences in growth rate were detected, indicating that co-culturing of NIKS^{GFP} cells with primary keratinocytes did not affect the growth rate of either strain (Figure 1B). These studies not only demonstrate that cell-cell adhesion occurs between the putative carrier cell population and primary keratinocytes, but that in monolayer culture the NIKS^{GFP} cells do not alter the anticipated growth rate of the co-cultured primary keratinocytes.

Composite Skin Substitutes Generated From Two Cell Sources Form Chimeric Stratified Squamous Epithelia Possessing Cutaneous Barrier Function

To assess the development of stratified squamous epithelia generated from a mixed population of NIKS^{GFP} and unlabeled primary keratinocytes, composite skin substitutes were produced using organotypic culturing methods. Seeding proportions of 90%:10% and 50%:50% (NIKS^{GFP} : primary keratinocytes) were used to generate chimeric stratified squamous epithelia. Cultures seeded with 100% NIKS^{GFP} or 100% primary keratinocytes served as controls. Histological analysis confirmed that all tissues formed distinct basal, spinous, granular, and squamous layers characteristic of interfollicular epidermis (Figures 2A-D). The observed morphology of tissues composed of NIKS^{GFP} cells was comparable to that of unlabeled NIKS keratinocytes, indicating that genetic modification did not interfere with the formation of normal tissue architecture (data not shown).

Fluorescence microscopy was used to evaluate tissue chimerism by discriminating regions generated by NIKS^{GFP} cells from regions produced by unlabeled primary keratinocytes. GFP was detected throughout the epidermal compartment skin substitute composed of 100% NIKS^{GFP} keratinocytes (Figure 2E). Varying levels of GFP intensity were observed in the different layers, with the basal layer displaying the weakest fluorescence intensity and the squamous layer displaying the strongest. Contiguous regions of fluorescence were clearly discernable from non-fluorescing regions within chimeric skin substitutes (Figures 2F, 2G).

In stratified squamous epithelia generated by incorporating a small percentage of primary keratinocytes (90% NIKS^{GFP}:10% primary keratinocytes), non-fluorescing regions were found in a patterned array of cell stacks. This pattern was also observed in skin substitutes generated using equal proportions of NIKS^{GFP} and primary keratinocytes, although the expression of GFP was localized to the squamous layer. As anticipated, no GFP expression was observed in skin substitutes generated from primary keratinocytes (Figure 2H). These studies revealed that in chimeric skin substitutes, primary keratinocytes not only persist but generate substantial portions of the final tissue.

The degree of barrier function exhibited by chimeric and control tissues was measured by skin surface electrical impedance. Intact and tape-stripped human skin were used as positive and negative controls for barrier function, respectively. Although indirect, the magnitude of the impedance change during a defined measurement period correlates with the rate of transepidermal water loss. A large change in impedance corresponds to a higher rate of water flux, which is indicative of poorer barrier function. All composite skin substitute tissues were found to possess barrier function similar to that of native skin (Figure 3). In summary, at all cell ratios tested organotypic co-cultures of NIKS^{GFP} and primary keratinocytes developed stratified squamous epithelia possessing barrier function.

Chimeric Skin Substitutes Express Proteins Associated with Cell-Cell Adhesion and Keratinocyte Terminal Differentiation

To further evaluate the development of chimeric skin substitutes, indirect immunofluorescence was used to visualize markers of cell adhesion and terminal differentiation normally found within the interfollicular epidermis. As represented by tissue generated from 90%:10% (NIKS^{GFP} : primary keratinocytes), chimeric skin substitutes exhibited appropriate expression and localization for all proteins examined (Figure 4). The cellular adhesion protein E-cadherin was localized to the cell membrane and restricted to the basal, spinous, and granular layers of generated tissues (Figure 4A). Keratin 1, essential to the structural integrity of the developing tissue, was localized to the suprabasal layers of the epidermal compartment (Figure 4B). The intermediate-stage differentiation marker keratinocyte-specific type I transglutaminase was properly localized to the cell membrane of the spinous and granular layers (Figure 4C). This pattern corresponds to the function of transglutaminase which crosslinks proteins into the cornified envelope. Finally, expression of the intermediate-to-late stage differentiation protein, filaggrin, was appropriately localized to the granular layer of all tissues (Figure 4D). No differences in protein levels or localization were noted when chimeric tissues were compared to controls indicating that expression of these proteins was not adversely affected by tissue chimerism. As confirmed by the merged images (Figures 4E-H), tissue regions generated from each cell population contribute to the overall structure of the fully-stratified squamous epithelia. These results demonstrate that proteins critical to cell-cell adhesion and keratinocyte terminal differentiation are appropriately expressed and localized in the epidermal compartment of chimeric composite skin substitutes.

NIKSGFP and Chimeric Skin Substitute Tissues Engraft in an In Vivo Model

The ability of skin substitutes generated from chimeric co-culture, NIKS^{GFP} cells, or primary keratinocytes to stably engraft in an athymic nude mouse model was evaluated at 7, 14, 28, and 91 days after surgical application. At the time of grafting, the composite skin substitutes ranged in thickness from 75 to 100 μ m and were easily handled during surgical application to the excisional wound sites. All engrafted tissue exhibited a translucent pink coloration when evaluated 7 days after surgery and at all subsequent timepoints. Acquisition of this coloration suggests establishment of vascularization to the engrafted tissues. At all time points, no differences in wound site or graft appearance were observed between skin

substitutes. All engrafted human tissue was visually discernable through completion of the study, although the size of the wound site was reduced due to wound contraction associated with this *in vivo* rodent model.¹⁵ At each timepoint, biopsies were evaluated by histology and fluorescence microscopy to distinguish human from mouse tissue. The intersection of human and mouse epidermis at the wound edge was clearly evident based on tissue architecture and cellular traits such as size and granularity (arrow, Figure 5A). GFP fluorescence was used to identify engrafted epidermal tissue regions generated by NIKS^{GFP} cells (Figure 5B) and detection of the major histocompatibility complex (MHC) class I human leukocyte antigen complex (HLA-ABC) served to confirm the presence of human tissue. With few exceptions human nucleated cells, including keratinocytes, express MHC class I antigen on their cell surface.¹⁶ Staining for HLA-ABC was localized to the cell membrane in the basal and immediately suprabasal layers of the epidermis in all engrafted tissue (Figure 5C). Keratinocytes of the granular layer and the stratum corneum did not stain. Staining in the dermis was primarily localized to the region beneath the engrafted human tissue, identifying human dermal fibroblasts within the wound bed. HLA-ABC staining was specifically detected up to 91 days post-surgery (data not shown). These findings show that human tissue from chimeric skin substitutes stably engraft in this *in vivo* model and persist for up to 3 months.

The pattern of GFP expression in engrafted chimeric and control skin substitute tissues was also examined. Expression of GFP localized to the epidermal region, and was especially evident in the stratum corneum, in engrafted 100% NIKS^{GFP} tissue at 7, 14, and 28 days post-surgery (Figures 6A-C). In chimeric skin substitute tissue generated from 90%:10% (NIKs^{GFP} : primary keratinocytes), GFP fluorescence was detected 7 days after surgical application with organization of the labeled cells in widening cellular stacks (Figure 6D), although at more infrequent intervals than observed prior to grafting. By 14 days post-surgery, GFP-labeled cells were detected in the most suprabasal layers only, at sporadic intervals along the length of the tissue section (Figure 6E). In chimeric tissues generated from 50%:50% (NIKs^{GFP} : primary keratinocytes), GFP-labeled cells were rarely detected at either 7 or 14 days post-application (Figures 6G, H). By 28 days, no GFP-labeled cells were observed in chimeric tissues (Figures 6 F, I). GFP fluorescence was not detected in any tissue 91 days after surgical application (data not shown). As anticipated, GFP fluorescence was not observed at any time in engrafted composite skin substitute tissue generated from 100% primary keratinocytes (data not shown). At each timepoint GFP-labeled cells were detected in a dose-dependent manner in a pattern suggesting loss of the NIKS^{GFP} cell population through desquamation. To complement assessment of GFP fluorescence, genomic DNA was analyzed for the presence of the gene encoding GFP. Although a dose-dependent reduction in GFP DNA was found in chimeric tissues prior to surgical application, no GFP DNA was detectable by PCR analysis in the corresponding engrafted chimeric tissues 28 days post-application (Figure 7). The detection of human-specific Pv92 DNA confirmed that lack of GFP signal was not attributable to the absence of human DNA. Our findings demonstrate that primary keratinocytes are retained and NIKS^{GFP} cells are lost in chimeric human skin substitutes stably-engrafted *in vivo*.

Discussion

It has been over a decade since Suzuki *et al.* initially proposed the use of chimeric cultures as a treatment option for severe skin loss.^{4, 5} The lack of a consistent source of pathogen-free allogeneic keratinocytes has hindered further development of this therapeutic approach. Here we show that chimeric skin substitutes generated with NIKS^{GFP} cells possess normal tissue architecture, accurately express proteins essential for the structure and function of stratified squamous epithelial tissue, exhibit normal barrier function, and stably engraft in an *in vivo* mouse model, with long-term retention of primary keratinocytes and loss of

NIKS^{GFP} keratinocytes. These findings support the use of fully-stratified chimeric skin substitutes generated from NIKS keratinocytes interspersed with autologous cells as a treatment option for severe cutaneous trauma such as catastrophic burns.

The feasibility of co-cultured skin substitutes has been previously demonstrated in several animal models. Co-cultured keratinocytes from different strains of mice (BALB/c and C3H/He) grafted back onto either experimental mouse strain resulted in long-term survival of syngeneic keratinocytes and rejection of the allogeneic keratinocytes.⁴ The long-term survival of syngeneic keratinocytes has been demonstrated using chimeric keratinocyte cultures containing up to 98% allogeneic and as few as 2% syngeneic cells.¹⁷ Additionally, chimeric syngeneic-xenogeneic (mouse-human) cultured keratinocytes grafted onto mice resulted in complete resurfacing of the wound with syngeneic cells due to the elimination of the xenogeneic cells.⁵ However, translation of this approach for human therapeutic application has not been reported. The major impediment to the development of a human chimeric system results from a dearth of appropriate allogeneic cell sources to serve as “carriers” for the autologous cells. The NIKS human keratinocyte progenitor cell line may have the potential to circumvent this impediment, in that these non-tumorigenic, pathogen-free cells exhibit typical keratinocyte-specific growth and differentiation while maintaining an extended lifespan *in vitro*. Moreover, allogeneic skin substitutes generated with NIKS keratinocytes have been evaluated in a Phase I / Phase II clinical trial focused on the treatment of complex skin defects. To facilitate identification of the carrier cell population, we took advantage of the unique properties of the NIKS cell line to be genetically engineered and generated a NIKS clone stably expressing GFP.

In monolayer culture, co-culturing NIKS^{GFP} cells and unlabeled primary keratinocytes resulted in the production of contiguous chimeric epithelial sheets. E-cadherin protein was properly localized to the cell membrane revealing that mature cell-cell junctions had been established between both cell types. Furthermore the growth rate in monolayer culture remained consistent, indicating that co-culture with NIKS^{GFP} cells did not inhibit or enhance the growth of primary keratinocytes.

The lack of a fully-stratified tissue and a corresponding lack of barrier function in monolayer cultures of autologous keratinocytes prompted us to evaluate the capacity of a chimeric system to form functional stratified squamous epithelia. Examination of the barrier properties of these stratified squamous epithelial tissues provides the first evidence that NIKS^{GFP} and chimeric skin substitutes possess cutaneous barrier function similar to that of native skin. Furthermore, in this initial report the expression and appropriate localization of proteins essential to the structure and function of the epidermis has been established for skin substitute tissue generated from both NIKS^{GFP} and mixed NIKS^{GFP} / primary keratinocyte populations. Histological analysis of chimeric skin substitutes confirmed the formation of typical epidermal architecture which incorporated discrete regions derived from either NIKS^{GFP} or primary keratinocytes as verified by fluorescence microscopy. Fluorescing and non-fluorescing regions were found in a pattern of widening cell stacks suggesting the formation of discrete units of terminally differentiating cells resulting from labeled or unlabeled proliferative keratinocytes. Using an *in vivo* rodent model, Clayton *et al.* have recently demonstrated that 84% of the keratinocyte progenitors within the basal layer divide asymmetrically to generate one proliferative and one differentiating keratinocyte.¹⁸ The remaining 16% undergo symmetric division generating two proliferative or two differentiating cells. The fate of an individual dividing cell is stochastic.¹⁹ Our observation of discrete differentiating tissue regions specifically labeled with GFP is consistent with the findings of Clayton *et al.* GFP intensity was also found to vary with the different epidermal layers. This variance may result in part from the nature of the differing functions of each layer within the epidermis. The proliferative basal layer may exhibit weaker fluorescence

due to dilution of GFP resulting from cell division and the high nuclear to cytoplasmic ratio. In contrast, the squamous layer displays strong fluorescence resulting from the collapsed structure of the enucleated squamous cells which amplifies the GFP signal. This intensity variation did not preclude observation of contiguous fluorescing and non-fluorescing regions. In both ratios of chimerism examined, 90%:10% and 50%:50%, it was discovered that the fluorescing tissue regions were being displaced by non-fluorescing tissue. Although this effect was consistent with the proportion of primary cells originally seeded into the chimeric culture, we propose that this effect is dynamic in nature, changing as the tissue matures *in vitro*. Importantly, our results indicate that primary keratinocytes have the capacity to persist and may ultimately replace the proportion of tissue composed of NIKS^{GFP} cells, the carrier population.

An athymic nude mouse model was employed to evaluate the *in vivo* performance of chimeric skin substitute tissue. In contrast to the challenges posed by the physical properties of products such as Epicel®, including fragility and difficulty in handling,¹ surgical application of chimeric skin substitutes was facilitated by the handling characteristics of the fully-stratified composite tissues. In all cases, tissues engrafted into the excisional wound site and displayed a consistent appearance in both coloration and contraction properties throughout the study. Histological analysis revealed a seamless transition from human to mouse epidermis. No involution of either human or mouse epidermis was observed. This boundary was confirmed by localization of GFP and by direct immunofluorescent detection of human MHC class I HLA-ABC. Furthermore, specific detection of human cells indicated that tissue from chimeric skin substitutes stably engrafted and persisted for up to 3 months in this *in vivo* model. Complementing the detection of human tissue, direct visualization of GFP fluorescence identified engrafted epidermal tissue generated by NIKS^{GFP} cells in chimeric and control skin substitute tissues. Temporal and dose-dependent responses were observed suggesting loss of the labeled cell population through desquamation with retention of unlabeled primary keratinocytes. GFP expression was driven by the CMV promoter which is susceptible to transcriptional inactivation due to DNA methylation and histone deacetylation.²⁰ Analysis for genomic DNA encoding GFP in biopsies taken from chimeric tissues engrafted for 28 days confirmed that the loss of GFP fluorescence resulted from depletion of labeled cells and not from down-regulation of GFP expression, indicating that terminally differentiating NIKS^{GFP} cells are being displaced by primary keratinocyte progenitors. Eventual loss of the NIKS^{GFP} carrier population was predicted by both the extent of chimerism found prior to grafting and the frequency of GFP signal localization to the enucleated squames that would be shed once introduced into an *in vivo* environment. The lowest seeding percentage of primary keratinocytes examined in our study was 10%, however additional optimization may demonstrate similar results using an even lower percentage. The need for relatively few autologous keratinocytes is clinically relevant because it translates to a reduction in the time required for clonal expansion prior to organotypic culture, reducing the time to patient application of a fully-stratified skin substitute. Moreover, application of tissue possessing cutaneous barrier function is a significant improvement over the use of autologous cell products lacking this important characteristic.

Unlike skin grafts, organotypic cultures do not contain highly antigenic elements such as Langerhans cells and endothelial cells. Similar to other allogeneic skin substitute products²¹⁻²³, it is probable that tissue containing NIKS keratinocytes will ultimately be replaced when applied onto humans, further supporting the utility of NIKS cells as a temporary allogeneic cell source for chimeric skin substitutes. As evidenced by the successful generation and utilization of NIKS^{GFP} cells, NIKS cells are readily amenable to genetic manipulation. As suggested by Sheridan *et al.*, composite skin substitutes composed of autologous keratinocytes and genetically modified allogeneic cells may enhance tissue

engraftment.²⁴ The exogenous expression of factors which promote wound healing has been explored using virally transduced keratinocytes, however the use of these keratinocytes have not yet been examined in the context of a chimeric allogeneic / autologous skin substitute.^{25,}
²⁶ We have genetically engineered NIKS keratinocytes to express biologically active molecules such as host defense peptides^{27, 28} and pro-angiogenic factors using non-viral vectors²⁹. The use of these genetically modified cells as allogeneic carrier populations for chimeric skin substitutes will be investigated. Our study of chimeric cultures composed of NIKS^{GFP} and primary keratinocytes supports the hypothesis that the NIKS cell line can function as an allogeneic carrier in the format of an engineered chimeric skin substitute tissue. The delivery of autologous patient cells in a fully-stratified, biologically active tissue substitute represents the new generation of personalized regenerative medicine therapies.

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Figure 1.

(A) Phase contrast, GFP localization (green), and E-cadherin (red) detection in a confluent co-culture of NIKS^{GFP} and primary keratinocytes plated in equal proportions. Arrows indicate junction between individual NIKS^{GFP} and primary keratinocytes. Scale bar: 50 μ m. (B) Growth rate of NIKS^{GFP} and primary keratinocytes plated separately and at specific ratios of 50%:50%, 10%:90%, or 1%:99% (NIKS^{GFP} : primary keratinocytes). Results are shown as percent of expected value correcting for differences in plating efficiency between the two strains of keratinocytes.

Figure 2.

Assessment of stratified squamous epithelia generated from 100% NIKS^{GFP} cells (A,E), 90%:10% (NIK^{GFP} : primary keratinocytes) (B,F), 50%:50% (C,G) and 100% primary keratinocytes (D,H). H&E staining confirmed formation of distinct basal, spinous, granular, and squamous layers (A-D). Fluorescence microscopy was employed to discriminate tissue regions generated by NIKS^{GFP} cells (green) from tissue produced by unlabeled primary keratinocytes (EH). Sections were counterstained with Hoechst 33258 (blue) to visualize nuclei. A white dashed line in E-H denotes the dermal / epidermal boundary. Scale bar represents 100 μ m.

Figure 3.

Barrier function properties of composite skin substitute tissues as measured by skin surface electrical impedance (DPM units). The values obtained for native skin, and skin where the barrier had been disrupted by tape-stripping, are also presented. Data represents the mean from triplicate samples. Error bars represent the standard error of the mean.

Figure 4. 90%:10% (NIKS^{GFP} : primary keratinocytes) chimeric skin substitute evaluated for expression of E-cadherin (A, E) keratin 1 (B, F) transglutaminase-1 (C, G) and filaggrin (D, H). GFP fluorescence (green) permitted visualization of tissue chimerism, whereas Hoechst 33258 (blue) identified nuclei localization. A white dashed line denotes the dermal / epidermal boundary. Scale bar represents 100 μ m.

Figure 5. Detection of engrafted NIKS^{GFP} tissue. A representative section spanning the human / mouse interface of the wound edge from NIKS^{GFP} tissues 28 days after surgery was stained with H&E (A). Fluorescence microscopy was employed to visualize tissue generated by NIKS^{GFP} cells (green) in the adjacent section (B). Expression of human MHC Class I (red) in the epidermal region co-localized with NIKS^{GFP} tissue (C). Hoechst 33258 (blue) permitted visualization of nuclei. Scale bar represents 200 μ m.

Figure 6.

Detection of GFP fluorescence in engrafted skin substitute tissues generated from either 100% NIKS^{GFP} cells (A-C), 90%:10% (NIKS^{GFP} : primary keratinocytes) (D-F) or 50%:50% (G-I). At post-operative days 7 (A, D, G) and 14 (B, E, H), GFP-labeled cells were detected in all tissues in a dose-dependent manner. Although GFP fluorescence was detected in 100% NIKS^{GFP} tissues, no labeled cells were observed in chimeric tissues 28 days (C, F, I) after surgical application. Hoechst 33258 (blue) permitted visualization of nuclei. A white dashed line in D-I denotes the dermal / epidermal boundary. Scale bar represents 100 μ m.

Figure 7.

Detection of NIKSGFP cells by genomic DNA analysis in chimeric tissues prior to and 28 days after surgical application. Whereas GFP-containing cells were detected in the positive control sample and chimeric tissues, no amplification of DNA encoding GFP was detected by PCR analysis in engrafted chimeric tissue 28 days after surgical application. Detection of Pv92 confirmed the presence of human DNA in tissues both prior to and after engraftment.